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Effect of Probiotic Administration Route and Dietary Nutrient Density on Growth Performance, Gut Health, and some Hematological Variables in Healthy or *Eimeria* Infected Broiler Chickens

Research Article

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ABSTRACT

This experiment aimed to evaluate the effects of probiotic administration route (PAR), dietary nutrient density (DND) and *Eimeria* challenge (ECH) on performance, gut morphology, and hematological variables in broiler chickens. Eggs carrying 17.5-day-old broiler embryos were injected with 0.1 mL distilled water (vehicle control) or 0.1 mL distilled water containing probiotic. A group of 72 intact eggs was also included in the experimental design representing un-injected control. Hatchlings from intact and probiotic injected eggs were further evaluated in a 42-d floor-pen trial along with 465 additional hatchmate chicks. Chicks were placed in two environmentally controlled houses, each having 30 floor pens randomly assigned to the following six treatments: conventional chicks receiving a standard diet with (group 1) or without (group 2) probiotic; conventional chicks receiving a 5% diluted diet with (group 3) or without (group 4) probiotic; and *in ovo* probiotic-administered chicks receiving standard diet (group 5) or 5% diluted diet (group 6). All birds in one of the houses were inoculated with a pathogenic dose of an attenuated live *Eimeria* vaccine at 28 d. Chicks receiving *in ovo* probiotic showed reductions in hatch weight and yolk sac weight compared to control ($P<0.05$); the same group, however, had a significantly higher weight gain (WG) during the first 10 days post-hatch but this improvement disappeared with age. Groups receiving standard diet exhibited better growth performance than those fed the 5% diluted diet. *Eimeria* challenge caused significant adverse effects on performance traits, intestinal morphology, and hematological variables. It is concluded that neither PAR nor DND could alleviate *Eimeria* induced deteriorations in productivity and health of broiler chickens.

KEY WORDS broiler, coccidiosis, *in ovo* injection, nutrient density, probiotic.

INTRODUCTION

Coccidiosis is a worldwide poultry health and welfare problem that imposes substantial economic losses on the industry. The situation is conventionally inhibited or treated by using anticoccidial drugs. The development of drug-resistant *Eimeria* species (Conway and McKenzie, 2007) and the presence of residues of coccidiostats in poultry products (Piątkowska *et al.* 2012), however, triggered a variety of efforts to recognize and introduce efficient and safe alternatives for the drugs with less possibility of resistance development. Probiotics are defined as microorganisms that when administered to human or animal diets in adequate amounts, induce health benefit for the host (Fuller, 1989). These organisms could enhance immune system function in animals with different health status (Pourakbari *et al.* 2016; Wang *et al.* 2018a; Wang *et al.* 2018b). The first few days post-hatch are extremely critical

for newborn chicks since opportunistic pathogens may colonize and invade their intestinal lumen, leading to poorer health and reduced post-hatch productivity. Early access to probiotic bacteria may be an effective way to competitively excluding the harmful organisms. Unfortunately, under practical conditions, an important part of the critical period is spent in the hatchery without any access to exogenous feed and water (Uni, 1999); dietary administration of probiotics, therefore, is not possible immediately after hatch. Different methods have been developed to fill the time gap for early delivery of probiotic bacteria to baby chicks including spray method, vent lip method (Seifi *et al.* 2017) and *in ovo* injection technique (De Oliveira *et al.* 2014). *In ovo* injection provides an opportunity to introduce probiotics to the bird prior to hatch; this may bring advantages for the chick by accelerating its gut microbiome establishment and decreasing the risk of post-hatch colonization by harmful microorganisms. There is some evidence indicating that post-hatch performance, immunity, and disease-resistance may be improved by *in ovo* probiotic supplementation (Pender *et al.* 2016; Majidi-Mosleh *et al.* 2017). Pender *et al.* (2016) reported that intra-egg probiotic administration reduced *Eimeria* lesion severity and post-hatch mortality in broiler chicks. The authors also found that *in ovo* probiotic delivery improved early performance and provided protection against a mixed *Eimeria* infection.

Dietary concentrations of certain nutrients have also been shown to influence the bird resistance and immunity against *Eimeria*. According to the literature, dietary vitamin E and arginine (Perez-Carbajal *et al.* 2010), vitamin A (Erasmus *et al.* 1960), zinc (Southern and Baker, 1983), betaine (Amerah and Ravindran, 2015), amino acids (Rochell *et al.* 2016), protein (Britton *et al.* 1964), and omega-3 fatty acids (Korver *et al.* 1997) may boost the bird resistance to coccidial infections. On the other hand, coccidiosis has been shown to deteriorate nutrient digestion and absorption due to the resulting intestinal destructions, decreased secretion and activity of digestive enzymes and reduced expression of nutrient transporters (Su *et al.* 2014; Su *et al.* 2015; Yin *et al.* 2015). There is evidence that low dietary nutrient density may improve broiler chickens immunity (Guo *et al.* 2010). In another research, feeding broilers with dense diets (103.75 and 107.5% *vs.* 100% of recommended nutrient levels) led to higher counts of beneficial bacteria (*Lactobacillus* and *Bifidobacteria*) in caeca (Nabizadeh *et al.* 2017). Accordingly, unknown interactions may be exploited between dietary nutrient density and probiotic administration route on health and performance of broilers exposed to enteric infections such as coccidiosis.

The current study aimed at evaluating the effect of probiotic administration route (*in ovo* or in-feed), dietary nutrient density and *Eimeria* challenge on growth performance, gut morphology, and some hematological variables in broiler chickens.

MATERIALS AND METHODS

The procedures used in this study were approved by the Animal Care and Use Committee of the Ferdowsi University of Mashhad, Iran.

Probiotics, eggs and injections

Lactobacillus salivarius (strain: HBUAS53102; Accession number: MH393176.1; 4C) and *L. plantarum* (strain ZLS006; Accession number: CP020858.1; 10ceca), previously isolated from broiler chickens gut in our lab (Majidzadeh-Heravi *et al.* 2011), were dissolved in sterile-distilled water so that each 0.1 mL of the final solution contained 10^9 cfu of each bacterium.

Four hundred thirty-two broiler breeder eggs carrying 17.5 d-old alive embryos were divided into 24 replicate sets of 18 eggs and treated as follows: 1) a group of 4 sets of 18 eggs remained intact and served as an un-injected control; 2) another group of 4 sets of 18 eggs was injected with 0.1 mL of sterile distilled water (vehicle control) and 3) the remaining 16 sets of 18 eggs were injected with 0.1 mL of the previously prepared probiotic-containing solution. All eggs were candled prior to the injections to define and mark their air cell center points and also to make sure the embryos were alive. Then, the marked points were disinfected using ethanol-impregnated cotton bullets. A hole was made at the point using an 18-gauge needle taking care to maintain inner shell membrane integrity. Then, injections were performed using 1 ml insulin syringes with 5 mm (length) 25-gauge disposable needles. After the injections, the holes were resealed using a glue gun. Eighteen eggs of each replicate were put in one partition of four hatchery trays, each having 8 separate partitions, in a way that each tray accommodated one replicate of the un-injected control along with one replicate of vehicle control and 4 replicates of the probiotic injected group. The trays were then transferred into a hatchery room (temperature: 37.1 ˚C; relative humidity: 75%). The incubation and injection procedures of this study were implemented in a commercial hatchery complex (Fariman broiler breeder company, Fariman, Mashhad, Iran).

Diets and housing conditions

Hatchlings from intact and probiotic injected eggs were further evaluated in a 42-d floor-pen trial. Moreover, 465 additional one-day-old chicks from the same parent stock (Ross 308, 47 week-old) and incubation batch were also used in the study. The chicks were transferred to two separate environmentally controlled houses, each having 30

floor-pens randomly assigned to the following six treatment groups: conventional chicks fed standard starter, grower and finisher diets without (group 1) or with (group 2) supplemental probiotic (10^9 cfu of each *L. salivarius* and *L. plantarum* per kg of diet); conventional chicks fed 5% diluted starter, grower and finisher diets without (group 3) or with (group 4) supplemental probiotic; *in ovo* probiotic-receiving chicks fed the standard diets (group 5); and *in ovo* probiotic-receiving chicks fed the diluted diets (group 6). Each replicate pen contained 7 female and 6 male chicks. Nutrient compositions of feed ingredients used in this study were adapted from NRC (1994). Diets were formulated to meet the minimum nutrient requirements of Ross 308 broilers (Aviagen, 2014a; Table 1). Management practices recommended for Ross 308 broilers (Aviagen, 2014b) were implemented during the rearing period.

Coccidial challenge

On day 28, all birds of one of the houses were crop-intubated with a clinically pathogenic dose of an attenuated anticoccidial vaccine, Livacox-T®. Each preventative dose of the vaccine (0.01 mL) contained 300 to 500 sporulated oocysts from each *Eimeria acervulina*, *E. maxima* and *E. tenella*. The infective dose used in the present study was 50 times higher than the preventive dose (Mohiti-Asli and Ghanaatparast-Rashti, 2015). The experiment, therefore, had twelve treatment groups made by the combination of the following 3 main factors: 1) administration route of the probiotic which had three levels including no probiotic administration, *in ovo* probiotic administration, and in-feed probiotic administration; 2) dietary nutrient density which had two levels including standard nutrient density and low nutrient density (with 5% dilution rate); and 3) challenge induction which had two levels including no challenge and Emeria challenge.

Evaluated variables

Hatching measurements

At the end of the 21st day of incubation, hatchlings from each replicate set of 18 eggs were counted and weighed. Hatchability rate and hatch weight were calculated according to the following two formulas:

Hatchability (%)= (number of hatched eggs in a replicate/number of eggs assigned to the replicate (18 eggs)) × 100

Hatch weight (g/chick)= total weight of hatchlings in a replicate (g) / number of hatchlings in the replicate

Fifteen newly hatched chicks (8 female and 7 male chicks) per *in ovo* treatment group were dissected to measure yolk sac weight and yolk sac-free body weight.

Post-hatch measurements

Performance

Group weight and feed intake were measured at 10, 24, 28, and 42 days of age. Average weight gain of a replicate group during a specific period was calculated by subtracting the initial average weight of the group from its final average weight.

Feed conversion ratio (FCR) was calculated as the amount of feed consumed per unit of body weight and was corrected for mortality. The mortality rate in each replicate was obtained by dividing the number of dead birds by the initial number of birds in the group and then multiplying the quotient by 100.

Jejunal histomorphology

On the 35th day (seventh day post-challenge coinciding with the peak of the severity of the disease clinical symptoms), one female bird from each replicate was selected, weighed and sacrificed by cervical dislocation and about 1 cm of the middle part of jejunum was removed, washed and fixed in a 10% formalin solution. The formalin solution was renewed after 24 h. Formalin-fixed samples were kept at room temperature until further morphological evaluations according to standard protocols. In brief, samples were dehydrated by serially exposing them to ascending grades of ethyl alcohol. Then, clearing and removal of ethylic alcohol were done using xylene followed by paraffinizing. Subsequently, paraffin-embedded tissues were cut with a thickness of 5-6 µm using a microtome. Three sections per sample were provided and unwrinkled by spreading them on a 40 ˚C water bath and mounting them on a glass slide by putting the slides under the water and lifting up to pick up the sections. Then slides with sections were put on a warmed plate (45 ˚C) to remove water and paraffin. After deparaffinization by xylene and rehydration by descending grades of ethyl alcohol, slide-mounted sections were stained by hematoxylin-eosin and evaluated under a light microscope for villus height (VH), villus width (VW), crypt depth (CD), and lamina properia thickness (LPT). Villus surface area (VSA) was calculated using the formula (Khodambashi Emami *et al.* 2017):

$$VSA\ (mm^2) = 2\pi \times (VW/2) \times VH$$

Each variable was measured 5 times per replicate, and finally, an average of the 5 readings was used for the respective replicate.

Differential white blood cell (WBC) counts

At 35 d of age, one female bird from each pen was bled and blood smears were provided. The smears were then fixed using methanol and stained with Giemsa.

Table 1 Ingredients and nutrient composition of experimental diets

Ingredients (%)	Starter (1 to 10 d of age)		Grower (11 to 24 d of age)		Finisher (25 to 42 d of age)	
	Standard	5% Diluted	Standard	5% Diluted	Standard	5% Diluted
Corn	49.22	57.30	52.50	60.41	57.78	65.43
Soybean meal (44% CP)	41.59	37.40	37.89	33.88	32.34	28.61
Vegetable oil	4.52	0.80	5.41	1.65	5.96	2.17
Limestone	1.09	1.06	1.01	0.98	0.93	0.91
Dicalcium phosphate	1.88	1.77	1.66	1.56	1.49	1.40
Common salt	0.37	0.35	0.37	0.35	0.37	0.35
Mineral premix[1]	0.30	0.30	0.30	0.30	0.30	0.30
Vitamin premix[2]	0.30	0.30	0.30	0.30	0.30	0.30
DL-methionine	0.37	0.34	0.32	0.29	0.29	0.27
L-lysine HCl	0.24	0.27	0.17	0.20	0.18	0.20
L-threonine	0.11	0.11	0.08	0.08	0.06	0.06
Sum	100	100	100	100	100	100
Nutrients composition						
Apparent metabolizable energy (AME) (kcal/kg)	3000	2850	3100	2945	3200	3040
Crude protein (CP) (%)[3]	23.00	21.86	21.53	20.45	19.51	18.54
Calcium (%)	0.96	0.91	0.87	0.83	0.79	0.75
Available phosphorus (%)	0.48	0.46	0.44	0.41	0.40	0.38
Sodium (%)	0.16	0.15	0.16	0.15	0.16	0.15
Met (%)	0.72	0.68	0.65	0.61	0.59	0.56
Met + Cys (%)	1.08	1.03	0.99	0.94	0.91	0.86
Lysine (%)	1.44	1.37	1.29	1.23	1.16	1.10
Threonine (%)	0.97	0.92	0.88	0.84	0.78	0.74
DCAD (mEq/kg)[4]	247.77	233.80	236.63	223.22	213.97	201.69

[1] Provided the followings per kilogram of diet: Mn: 120 mg; Fe: 60 mg; Zn: 101.64 mg; Cu: 12 mg and Se: 0.24.
[2] Provided per kg of diet: vitamin A: 10800 IU; vitamin D_3: 2400; vitamin E: 21.6 IU; vitamin K_3: 2.4 mg; Thiamin: 2.16 mg; vitamin niacin: 12 mg; Pantothenic acid: 36 mg; Pyridoxine: 3.6 mg; Folic acid: 1.2 mg; vitamin B_{12}: 0.018; Biotin: 0.18 and Choline chloride: 360 mg.
[3] Analyzed crude protein contents of starting, growing, finishing diets were 23.53%, 21.93%, 19.71% and 22.19%, 20.65%, 18.54% for standard and low-density diets, respectively.
[4] Dietary cation anion difference.

The processed smear slides were evaluated under a light microscope for leukocyte differential counts (Samour, 2006). Heterophil to lymphocyte ratio was also determined as a measure of stress.

Statistical analysis

All raw data were subjected to Minitab's outlier and normality tests. After detecting and excluding outliers and ensuring the normal distribution of data, further data analysis was performed using SAS (2004) general linear method (GLM) procedure. Hatchery data were analyzed in a completely randomized design (CRD) because calculated relative efficiencies of randomized complete block design (RCBD) to CRD were less than 100. Data collected prior to Emeria challenge (1 to 28 d of age) were analyzed in a randomized complete block design with a 2 (DND levels: standard or 5% diluted) × 3 (PAR levels: no supplementation, *in ovo* supplementation or in feed supplementation) factorial arrangement with houses considered as blocks. Data collected after the challenge (29 to 42 d of age) were analyzed in a 2 (DND levels) × 2 (ECH levels: challenged or unchallenged) × 3 (PAR levels) split-plot factorial design for which challenge was the main plot while DND and PAR were sub-plots.

RESULTS AND DISCUSSION

Hatching measurements

Data on the effect of *in ovo* administration of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium per egg) on hatchability, hatch weight (HW), absolute yolk sac weight (AYSW), relative yolk sac weight (RYSW), absolute yolk-free body weight (AYFBW) and relative yolk-free body weight (RYFBW) of newly hatched broiler chicks are shown in Tables 2 and 3. Hatchability was not significantly affected by the *in ovo* treatments.

Interestingly, hatchlings from probiotic-injected eggs had significantly lower HW, AYSW, RYSW, and AYFBW while having higher RYFBW than those from the intact or vehicle control eggs ($P<0.05$). De Oliveira *et al.* (2014) did not find any alteration in RYSW and RYFBW of 19- or 21-d-old broiler embryos following *in ovo* injection of different probiotic preparations at day 17.5 of incubation; however, they found that *in ovo* injection of certain bacteria caused higher chick body lengths. It has been shown that there is a positive correlation between chick body length and utilization of egg nutrients by the embryo (Willemsen *et al.* 2008).

Table 2 The Effect of *in ovo* injection of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium per egg) on hatchability rate and hatch weight of broiler chicks

Treatment	Hatchability (%)	Hatch body weight (g/chick)
Control[1]	98.61	45.45[a]
Distilled water[1]	98.61	45.80[a]
Probiotic[2]	97.20	44.20[b]
SEM	1.179	0.346
P-value	0.6275	0.0099

[1] Each mean represents 4 replicates of 18 eggs each.
[2] Each mean represents 16 replicates of 18 eggs each.
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 3 The Effect of in ovo injection of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium per egg) on absolute and relative yolk sac weight and yolk free body weight of newly hatched broiler chicks

Treatment	LBW (g)	AYSW (g)	RYSP (%)	AYFBW (g)	RYFBP (%)
Control[1]	45.66[a]	2.85[a]	6.25[a]	42.80[a]	93.75[b]
Distilled water[1]	45.81[a]	2.92[a]	6.38[a]	42.89[a]	93.62[b]
Probiotic[2]	43.46[b]	2.57[b]	5.90[b]	41.02[b]	94.10[a]
SEM	0.259	0.051	0.114	0.260	0.114
P-value	< 0.0001	< 0.0001	0.0150	< 0.0001	0.0150

[1] Each mean represents 15 observations.
[2] Each mean represents 13 observations except for the mean of LBW which was obtained from 15 observations.
LBW: live body weight; AYSW: absolute yolk sac weight; RYSW: relative yolk sac weight; AYFBW: absolute yolk-free body weight and RYFBW: relative yolk-free body weight.
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.

There is evidence that short-chain fatty acids, naturally produced by gut microbes, may stimulate host gut motility and blood flow (Kamath *et al.* 1988). Also, it has been reported that some bacterial species can decarboxylate amino acid tryptophan to tryptamine which in turn induces the release of serotonin from enterochromaffin cells and serotonin promotes gut motility (Takaki *et al.* 1985). Thus, reductions in HW, AYSW, RYSW, and AYFBW in hatchlings receiving probiotic *in ovo* could to a large extent be attributed to the accelerated yolk sac absorption due to the increased intestinal motility and blood flow in these chicks. The absorbed yolk materials are deposited in body tissues or used to meet embryonic maintenance requirements and a part of them is also excreted into an extra-embryonic structure called allantois. Therefore, higher yolk absorption would be accompanied by a higher amount of waste excreted out of the body resulting in a reduced hatch weight compared to hatchlings with slower yolk sac absorption. Given the benefits of rapid yolk sac absorption in improving chick's post-hatch health and performance, higher hatch weight does not always mean higher chick quality. New opportunities may be offered to hatcheries to improve their chick's quality by *in ovo* injection of certain probiotic bacteria.

Post-hatch measurements

Pre-challenge growth performance

The effect of dietary nutrient density and administration route of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium per injected egg or kg of diet) on growth performance of broiler chickens in different time periods from 1 to 28 days of age is shown in Tables 4 and 5. Birds fed the standard diet had significantly higher body weight (BW) and weight gain (WG) but lower feed intake (FI) and feed to gain ratio (F:G) as compared to those fed 5% diluted diet. Chickens are able to regulate their feed intake based on the feed energy density, so that birds on a low-energy diet eat more feed than those on a high-energy diet, although energy intake is not maintained, leading to a reduced performance (Leeson *et al.* 1996).

Notably, birds hatched from the probiotic-injected eggs, which have had the lowest hatch weight, gained more weight during the first 10 days post-hatch, confirming the positive effects of the faster yolk sac absorption on post-hatch performance; however, the effect disappeared with age.

During 1 to 28 d of age, birds receiving probiotic in their feed had higher F: G compared to unsupplemented birds as well as to those which received probiotic *in ovo*. This may be due to the dietary dose of the probiotic. In other words, if it was added in doses lower than 10^9 cfu per kg of diet, the probiotic might show positive effects on performance traits.

Mountzouris *et al.* (2010) reported that PoultryStar ME, a commercial probiotic, showed better efficacy on performance traits of broiler chickens when used at a low inclusion rate (10^8 cfu per kg of diet), whereas its effectiveness was significantly decreased at higher inclusion levels (10^9 and 10^{10} cfu per kg of diet).

Table 4 The effect of administration route of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium per egg or kg of diet) and dietary nutrient density on average body weight and weight gain of broiler chickens (pre-challenge)

Effects	Average body weight (g per bird)		Weight gain (g per bird)		
	10 d of age	28 d of age	1 to 10 d of age	11 to 28 d of age	1 to 28 d of age
Dietary nutrient density (DND)[1]					
Standard	239.4[a]	1346.6[a]	194.8[a]	1107.2[a]	1301.96[a]
5% diluted	225.8[b]	1239.5[b]	180.8[b]	1013.8[b]	1194.52[b]
SEM	1.74	7.31	1.74	6.97	7.29
Probiotic administration route (PAR)[2]					
No probiotic administration	231.3	1305.6	185.9[b]	1074.2	1260.2
In ovo	235.9	1296.9	192.4[a]	1061.0	1253.4
In feed	230.5	1276.8	185.0[b]	1046.2	1231.2
SEM	2.13	8.95	2.13	8.54	8.93
			P-values		
DND	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
PAR	0.1694	0.0749	0.0340	0.0773	0.0773
DND × PAR	0.6066	0.1258	0.6353	0.0862	0.0862

[1] Each mean represents 30 observations.
[2] Each mean represents 20 observations.
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 The effect of administration route of probiotic bacteria (10^9 cfu of each of *L. plantarum* and *L. salivarius* per egg or kg of diet) and dietary nutrient density on feed intake and feed to gain ratio of broiler chickens (pre-challenge)

Effects	Feed intake (g per bird)			FCR		
	1 to 10 d of age	11 to 28 d of age	1 to 28 d of age	1 to 10 d of age	11 to 28 d of age	1 to 28 d of age
Dietary nutrient density (DND)[1]						
Standard	237.0[b]	1800.5[b]	2037.4[b]	1.217[a]	1.628[a]	1.566[a]
5% diluted	241.1[a]	1819.8[a]	2060.9[a]	1.337[b]	1.797[b]	1.727[b]
SEM	1.37	4.18	4.75	0.0100	0.0102	0.0083
Probiotic administration route (PAR)[2]						
No probiotic administration	239.0	1817.9	2056.9	1.290	1.696	1.636[b]
In ovo	240.6	1805.2	2045.8	1.255	1.706	1.637[b]
In feed	237.5	1807.3	2044.8	1.287	1.735	1.668[a]
SEM	1.681	5.12	5.81	0.0123	0.0125	0.0102
			P-values			
DND	0.0383	0.0019	0.0010	< 0.0001	< 0.0001	< 0.0001
PAR	0.4378	0.1825	0.2722	0.0883	0.0830	0.0494
DND × PAR	0.7769	0.3182	0.4196	0.9258	0.1178	0.1033

[1] Each mean represents 30 observations.
[2] Each mean represents 20 observations.
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.

Post-challenge growth performance

The effects of administration route of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium per egg or kg of diet; PAR) and dietary nutrient density (DND) on growth performance of healthy or *Eimeria* challenged (ECH) broiler chickens are shown in Tables 6 and 7. The exposure of broiler chickens to the pathogenic dose of the trivalent *Eimeria* vaccine led to significantly worsened BW, WG, feed intake and F:G ratio. Birds receiving the low nutrient density diet, as expected, showed a higher FI and poorer WG and F:G ratio. There was no effect of PAR on performance traits during the post-challenge life of the birds. A significant ECH × DND interaction was observed for feed intake during 29 to 35 d, 29 to 42 d and d 1 to 42 d. Unchallenged birds fed diluted diet had the highest FI followed by unchallenged birds fed standard diet, differing significantly from each other as well as from those challenged and fed diluted or standard diets, (P<0.05) whereas the latter two groups had similar FI.

Eimeria infection seemed to reduce the ability of birds to adjust their feed intake in response to dietary metabolizable energy content during the first week post-challenge. In addition to the physical damages to the gut structure, *Eimeria* infections cause reductions in expression of intestinal nutrient transporters and digestive enzymes (Su *et al.* 2014; Su *et al.* 2015; Yin *et al.* 2015) and thereby digestibility of nutrients may be affected; this, in turn, would affect the neural pathways that control feed intake since some nutrients, amino acids in particular, play important roles in neurotransmission (D'Mello, 2003).

Table 6 The effect of administration route of probiotic bacteria (10^9 cfu of each of *L. plantarum* and *L. salivarius* per egg or kg of diet), dietary nutrient density and *Eimeria* challenge on average body weight and weight gain of broiler chickens (Note: *Eimeria* challenge was induced at 29 d of age)

Effects	Average body weight (g per bird)		Average gain (g per bird)			
	35 d of age	42 d of age	29 to 35 d	36 to 42 d	29 to 42 d	1 to 42 d
***Eimeria* challenge (ECH)[1]**						
Unchallenged	1843.99[a]	2474.11[a]	554.08[a]	630.12[a]	1184.20[a]	2429.43[a]
Challenged	1658.85[b]	2155.67[b]	362.61[b]	496.83[b]	859.44[b]	2110.69[b]
SEM	10.635	15.736	10.965	21.67	15.709	15.562
Nutrient density (DND)[1]						
Standard	1809.95[a]	2391.39[a]	463.32	581.44[a]	1044.76[a]	2346.72[a]
5% diluted	1692.89[b]	2238.39[b]	453.37	545.50[b]	998.87[b]	2193.39[b]
SEM	12.105	16.519	9.042	12.025	13.804	16.490
Probiotic administration route (PAR)[2]						
No probiotic administration	1765.25	2318.11	459.67	552.86	1012.53	2272.68
In ovo	1753.91	2315.83	457.06	561.92	1018.98	2272.35
In feed	1735.09	2310.73	458.31	575.64	1033.95	2265.15
SEM	14.825	20.231	11.075	14.727	16.907	20.196
			P-values			
ECH	0.0003	0.0001	0.0002	0.0122	0.0001	0.0001
DND	< 0.0001	< 0.0001	0.4412	0.0409	0.0238	< 0.0001
PAR	0.3572	0.9657	0.9862	0.5502	0.6583	0.9566
ECH × DND	0.7820	0.8684	0.4516	0.6132	0.9571	0.8692
ECH × PAR	0.8357	0.9184	0.8288	0.5649	0.7800	0.9207
DND × PAR	0.6941	0.8160	0.7539	0.8088	0.8911	0.8165
ECH × DND × PAR	0.8105	0.9849	0.5145	0.9060	0.9326	0.9864

[1] Each mean represents 30 observations.
[2] Each mean represents 20 observations.
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 7 The effect of administration route of probiotic bacteria (10^9 cfu of each of *L. plantarum* and *L. salivarius* per egg or kg of diet), dietary nutrient density and *Eimeria* challenge on feed intake and feed to gain ratio of broiler chickens (Note: *Eimeria* challenge was induced at 29 d of age)

Effects	Feed intake (g per chick)				FCR			
	29 to 35 d	36 to 42 d	29 to 42 d	1 to 42 d	29 to 35 d	36 to 42 d	29 to 42 d	1 to 42 d
***Eimeria* Challenge (ECH)[1]**								
Unchallenged	1200.43[a]	1418.40[a]	2618.83[a]	4674.29[a]	2.184[a]	2.28	2.216[b]	1.927[a]
Challenged	949.33[b]	1187.83[b]	2137.17[b]	4180.04[b]	2.640[b]	2.41	2.502[a]	1.985[b]
SEM	14.894	15.005	21.313	22.770	0.0366	0.058	0.0246	0.0062
Dietary nutrient density (DND)[1]								
Standard	1048.27[b]	1292.70	2340.97[b]	4378.40[b]	2.33[a]	2.247[a]	2.27[a]	1.869[a]
5% diluted	1101.50[a]	1313.53	2415.03[a]	4475.93[a]	2.49[b]	2.445[b]	2.45[b]	2.044[b]
SEM	10.294	11.850	16.258	16.694	0.041	0.0387	0.027	0.0114
Probiotic administration route (PAR)[2]								
No probiotic administration	1064.60	1292.50	2357.10	4413.10	2.39	2.37	2.363	1.948
In ovo	1077.40	1298.85	2376.25	4422.05	2.42	2.34	2.365	1.952
In feed	1082.65	1318.00	2400.65	4445.45	2.42	2.33	2.350	1.969
SEM	12.607	14.513	19.912	20.446	0.051	0.047	0.0324	0.0139
				P-values				
ECH	0.0003	0.0004	< 0.0001	0.0001	0.0009	0.1799	0.0012	0.0027
DND	0.0007	0.2211	0.0025	0.0002	0.0077	0.0008	< 0.0001	< 0.0001
PAR	0.5856	0.4406	0.3113	0.5334	0.8981	0.7838	0.9363	0.5327
ECH × DND	0.0046	0.8573	0.0485	0.0481	0.8473	0.6809	0.7501	0.4124
ECH × PAR	0.6860	0.5114	0.9126	0.9386	0.8423	0.7731	0.7744	0.9847
DND × PAR	0.9919	0.7264	0.8385	0.7633	0.8107	0.9219	0.9309	0.7499
ECH × DND × PAR	0.8683	0.8933	0.9687	0.9116	0.8331	0.9542	0.9358	0.9483

[1] Each mean represents 30 observations.
[2] Each mean represents 20 observations.
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.

Also, coccidiosis increases digesta transit time probably due to the impaired muscular activity (Aylott *et al*. 1968) and this stasis, in turn, may reduce feed intake.

The growth depression and body weight loss during *Eimeria* infection observed in the present study may be attributed to decreased feed intake along with impaired digestion, absorption and utilization of nutrients due to the damaged, shortened and flattened intestinal villi (Fernando and McCraw, 1973). Furthermore, it has been shown that coccidiosis may elevate circulating levels of corticosterone (Duckworth *et al*. 2001). Corticosterone accelerates muscle protein degradation without affecting protein synthesis in these tissues (Hayashi *et al*. 1994). Therefore, *Eimeria*-induced growth suppression in chickens may be due in part to increased blood corticosterone levels and muscle protein degradation.

Intestinal morphology

The effects of administration route of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium per egg or kg of diet), dietary nutrient density (DND), and *Eimeria* challenge (ECH) on jejunum histomorphology of broiler chickens are presented in Table 8. Birds exposed to coccidial infection had shorter and wider villi, deeper crypts and decreased villus surface area (VSA) as compared with their uninfected counterparts (P<0.05). In addition, challenged birds had lower jejunal villus height (VH) to crypt depth (CD) ratio compared with unchallenged healthy birds. The VH was also significantly affected by the route of probiotic administration, in a way that *in ovo* probiotic-administered birds had the longest villi and the unsupplemented birds had the shortest. Significant interactions were observed between ECH and DND on VH. Unchallenged birds fed the standard diet had the highest VH followed by unchallenged birds fed the diluted diet, differing significantly from each other and from those challenged and fed standard or diluted diets; whereas no difference was observed between the latter two groups.

Eimeria spp. affect gut physical integrity by penetration of epithelial cells of the intestinal mucosa (Yun *et al*. 2000). Gut epithelial cells serve as barriers against pathogens. Intestinal morphometric variables could be considered as integrity and health indices of the organ during enteric infections. Indeed, destructive actions of enteric pathogens on enterocytes are manifested as reduced villus height and increased crypt depth (Danforth *et al*. 1989). Destruction of affected enterocytes may occur as apoptosis (program; led death) or necrosis (abnormal cellular death with pathologic reasons). Apoptosis is a defense mechanism through which the host destructs its *Eimeria* infected enterocytes, breaking the life cycle of the parasite. However, in initial stages of their development, some *Eimeria* species (such as *E. tenella* and *E. necatrix*) are able to inhibit apoptosis of parasitized enterocytes and thereby, provide a stable nutritious environment for themselves allowing them to mature and scape the surveillance mechanisms of the host immune system. After maturation, the parasite induces apoptosis of the enterocyte, thus providing conditions for its release into the lumen (Del Cacho *et al*. 2004). This interactions lead to an increased regeneration of enterocytes and may ultimately cause reductions in VH and VH:CD ratio along with increases in the CD.

Differential white blood cell (WBC) counts

The effects of administration route of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium per egg or kg of diet), dietary nutrient density (DND) and *Eimeria* challenge (ECH) on differential count of white blood cells in peripheral blood of broiler chickens are given in Table 9. The exposure to the *Eimeria* infection led to marked rises in percentages of blood monocytes (M), eosinophils (E), heterophils (H) as well as in heterophile to lymphocyte ratio while population size of lymphocytes (L) was adversely affected by the infection (P<0.05). Birds receiving probiotic by *in ovo* injection had a higher monocyte count than un-supplemented birds. Birds fed probiotic-containing diet showed a higher eosinophil count compared with those hatched from intact eggs and reared on the probiotic-free diet. There was no significant effect of dietary nutrient density on proportional sizes of different WBC subpopulations. Significant ECH × PAR interactions occurred for monocyte and eosinophil counts (Figures 3 and 4; P<0.05) so that probiotic administration by either *in ovo* injection or dietary supplementation caused marked increments in blood eosinophil and monocyte counts in *Eimeria* challenged birds whereas these parameters were not influenced by PAR in healthy birds.

There is evidence that enteric infections such as coccidiosis induce physiological stress leading to increased circulatory levels of corticosterone (Duckworth *et al*. 2001) and this may cause some hematological alterations including increased H population, decreased L population and increased H to L ratio (Shini *et al*. 2009). Pro-inflammatory cytokines, released by activated macrophages during the infection, are the main mediators of the heterophil population increment. These cytokines mediate changes in blood glucocorticoids including corticosterone (Krams *et al*. 2012). The changes in blood corticosterone concentrations may affect endocrine system functions (e.g. conversion of noradrenaline to adrenaline and production of thyroid hormones), leading to a wide variety of events including immune suppression. The H:L ratio is used as a quantitative indicator for the hypothalamic-pituitary-adrenal axis activity and increases by the stress intensity.

Table 8 The effect of administration route of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium per egg or kg of diet), dietary nutrient density and *Eimeria* challenge on jejunum morphology of broiler chickens at 35 d of age (seven days after inoculation with *Eimeria* oocysts; *Eimeria* challenge was induced at 29 d of age)

Effects	Villus height (VH)	Villus wide	Villus surface area	Crypt deapth (CD)	VH:CD
	µm		mm^2	µm	-
***Eimeria* challenge (ECH)[1]**					
Unchallenged	1007.2[a]	179.6[b]	0.563[a]	286.4[a]	3.69[a]
Challenged	627.3[b]	216.5[a]	0.426[b]	359.5[b]	1.82[b]
SEM	46.816	7.902	0.0288	13.71	0.157
Dietari nutrient density (DND)[1]					
Standard	838.5	197.9	0.500	309.7	2.95
5% diluted	796.0	198.3	0.489	336.1	2.57
SEM	24.97	6.302	0.0231	12.25	0.155
Probiotic administration route (PAR)[2]					
No supplementation	755.4[b]	204.6	0.479	316.3	2.66
In ovo	864.6[a]	193.8	0.507	327.8	2.86
In feed	831.9[ab]	195.9	0.500	324.7	2.75
SEM	30.59	7.719	0.0283	15.01	0.190
			P-value		
ECH	0.0046	0.0299	0.0286	0.0199	0.0011
DND	0.2380	0.9615	0.7389	0.1371	0.0935
PAR	0.0487	0.5790	0.7738	0.8531	0.7436
ECH × DND	0.0110	0.6641	0.1229	0.2698	0.3376
ECH × PAR	0.7249	0.3670	0.3715	0.7311	0.8947
DND × PAR	0.0690	0.2199	0.3576	0.6249	0.1900
ECH × DND × PAR	0.6328	0.2517	0.9693	0.1288	0.2688

[1] Each mean represents 30 observations.
[2] Each mean represents 20 observations.
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 9 The effect of administration route of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium per egg or kg of diet), dietary nutrient density and *Eimeria* challenge on differential white blood cell (WBC) counts in broiler chickens (Note: *Eimeria* challenge was induced at 29 d of age)

Effects	Monocyte	Eosinophil	Lymphocyte (L)	Heterophil (H)	H:L
	% of total WBC count				-
***Eimeria* challenge (ECH)[1]**					
Unchallenged	1.80[b]	1.13[b]	67.80[a]	29.27[b]	0.439[b]
Challenged	5.16[a]	3.04[a]	48.87[b]	42.93[a]	0.964[a]
SEM	0.592	0.342	2.187	2.372	0.0816
Dietary nutrient density (DND)[1]					
Standard	3.22	2.38	57.81	36.59	0.737
Diluted	3.75	1.78	58.86	35.61	0.667
SEM	0.329	0.212	1.342	1.224	0.0597
Probiotic administration route (PAR)[2]					
Un-supplemented	2.82[b]	1.58[b]	60.44	35.16	0.613
In ovo	3.47[ab]	2.63[a]	57.06	36.84	0.732
In feed	4.15[a]	2.05[ab]	57.50	36.30	0.761
SEM	0.403	0.259	1.644	1.500	0.073
		P-value			
ECH	0.0169	0.0181	0.0039	0.0162	0.0111
DND	0.2711	0.0581	0.5921	0.5821	0.4278
PAR	0.0890	0.0324	0.3351	0.7465	0.3606
ECH × DND	0.4075	0.1368	0.8977	0.9174	0.5411
ECH × PAR	0.0629	0.0314	0.1559	0.4197	0.2969
DND × PAR	0.2916	0.7794	0.7695	0.5560	0.2711
ECH × DND × PAR	0.9018	0.5149	0.6606	0.6537	0.2815

[1] Each mean represents 30 observations.
[2] Each mean represents 20 observations.
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.

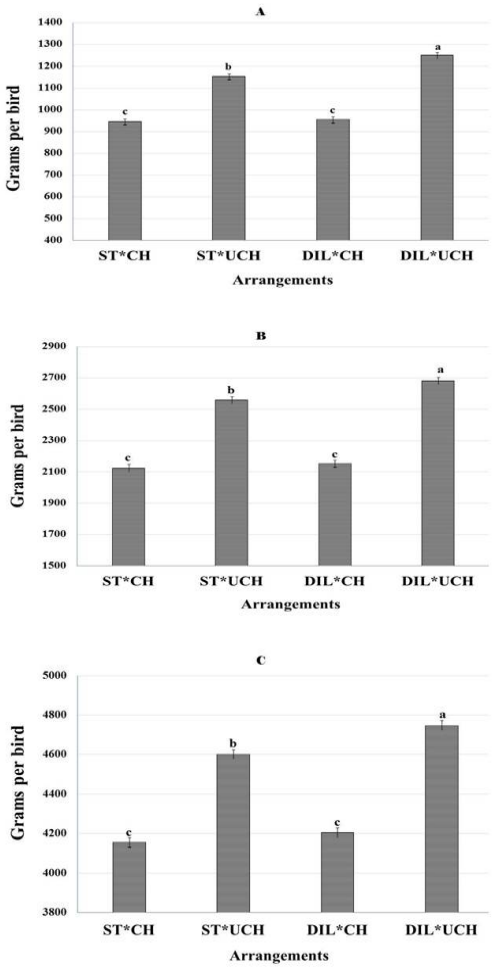

Figure 1 Interactions between *Eimeria* challenge and dietary nutrient density (standard diet or 5% diluted diet) on feed intake of broiler chickens from 29 to 35 d of age (seven days after inoculation with *Eimeria* oocysts; Chart A), 29 to 42 d of age (two weeks after inoculation with *Eimeria* oocysts; chart B) and throughout the rearing period (chart C)

ST: standard diet; DIL: diluted diet; CH: challenged and UCH: unchallenged

[a, b] Arrangements with no common superscript letter differ significantly (P<0.05)

Error bars represent standard error of the mean (SEM)

The ratio has been known as one of the most sensitive indicators of stress in the chicken (Shini *et al*. 2009). Ola *et al*. (2017) reported that broilers exposed to a mixed *Eimeria* infection (*E. acervulina*, *E. maxima*, *E. necatrix*, and *E. tenella*) showed a higher total count of WBC and increased relative counts of heterophils, eosinophils and monocytes while the relative count of lymphocytes was significantly decreased.

Monocytes can exhibit quick motile responses to infections, release cytokines and differentiate into macrophages or dendritic cells (Geissmann *et al*. 2010). Eosinophils have a substantial role in the control of parasitic infections (Ramirez *et al*. 2018).

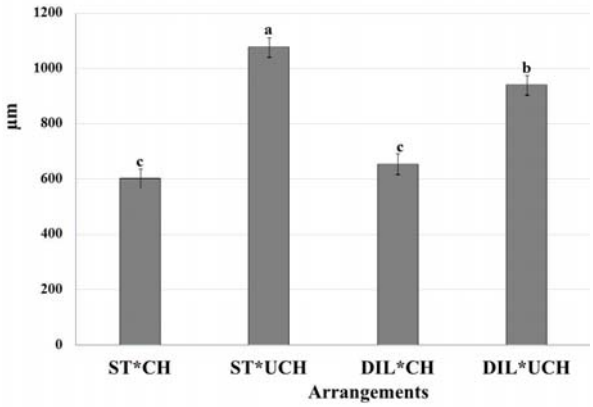

Figure 2 Interactions between *Eimeria* challenge and dietary nutrient density (standard diet or 5% diluted diet) on jejunum villus height in broiler chickens at 35 d of age (seven days after inoculation with *Eimeria* oocysts)

CH: challenge; UCH: unchallenged and PFree: probiotic-free diet

[a, b] Arrangements with no common superscript letter differ significantly (P<0.05)

Error bars represent standard error of the mean (SEM)

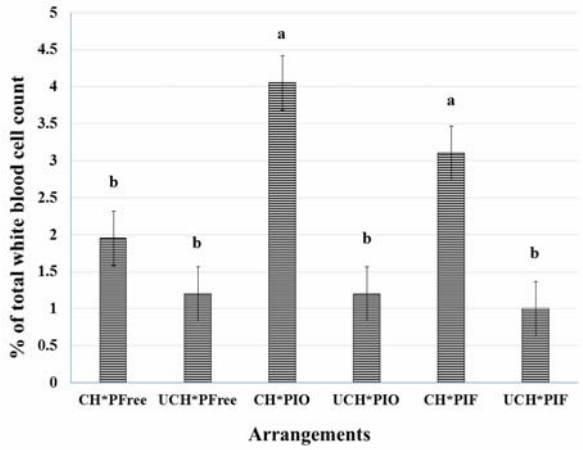

Figure 3 Interactions between *Eimeria* challenge and administration route of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium per egg or kg of diet) on eosinophil count at 35 d (seven days after inoculation with *Eimeria* oocysts)

CH: challenge; UCH: unchallenged; PFree: probiotic-free diet; PIO: probiotic *in ovo* and PIF: probiotic in feed

[a, b] Arrangements with no common superscript letter differ significantly (P<0.05)

Error bars represent standard error of the mean (SEM)

In the current study, probiotic administration, either via *in ovo* injection or via feed, augmented eosinophil and monocyte populations in peripheral blood of broilers affected by the coccidian infection and this could be translated as magnification of innate immune response by probiotic bacteria in infected birds; however, owing to the higher mortality rate observed in dietary probiotic-administered *Eimeria* infected birds (data not shown), it seems that the

probiotic used in our study overstimulated the immune system, worsening the situation.

CONCLUSION

In summary, *in ovo* injection of probiotic bacteria (a combination of *L. plantarum* and *L. salivarius*; 10^9 cfu of each bacterium) led to a decreased hatch weight, probably by accelerating yolk sac absorption, followed by an improved growth performance during 1 to 10 days of age. Neither dietary nutrient density (standard diet or low-density diet) nor probiotic administration route (no supplementation, *in ovo* injection or dietary supplementation) could alleviate clinical symptoms of coccidiosis. However, further investigations should be conducted to evaluate the effect of *in ovo* injection of probiotics on yolk sac absorption and chick quality. *In ovo* probiotic supplementation also caused a long-lasting benefit on jejunum morphology in terms of villus length. This suggests that the continued dietary inclusion of probiotics may not be necessary to elicit their advantages. In other words, introducing small amounts of the organisms to the chick prior to hatch or early post hatch may be as effective as the continuous administration. Determination of optimum doses and supplementation routes of probiotic bacteria in broilers exposed to the main enteric pathogens also seems to bring some good practical findings.

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