Effects of riboflavin and *Bacillus subtilis* on internal organ development and intestinal health of Ross 708 male broilers with or without coccidial challenge

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ABSTRACT In a companion study, we found that inclusion of different doses of riboflavin affected growth performance of Ross 708 male broilers' responses to coccidial challenge (by 5 *Eimeria* spp on day 14 of age) and dietary *Bacillus subtilis* (***B. subtilis***) supplementation. The current study was conducted to further test whether supplementation of *B. subtilis* and riboflavin will reduce negative impact and inflammation caused by *Eimeria* spp proliferation and help proper function of internal organs. A total of 1,248 Ross × Ross 708 male broiler chicks were randomly placed in 96 floor pens (8 blocks, 12 treatments). Treatments were arranged in a 3 (riboflavin) × 2 (*B. subtilis)* × 2 (Coccidial challenge) factorial arrangement in a randomized complete block design. Coccidial challenge reduced the weight of sampled birds on day 27 and day 36 and increased the relative weights of the internal organs of proventriculus, duodenum, jejunum, ileum, and spleen to BW on day 27, which may be because of inflammation caused by proliferation of *Eimeria* spp. The increased relative weights of duodenum, jejunum, ileum, and spleen on coccidial challenged birds were lost on day 36. Correlation analysis also indicated that the jejunum weight was positively related to villus height, *Eimeria acervulina,* and *Eimeria maxima* on day 27 but was not on day 36. The loss of the positive relationships may be because of recovery of the birds from coccidiosis on day 36. Even though the coccidial challenge and riboflavin interactively affected feed conversion ratio and BW gain and supplementation of dietary *B. subtilis* reduced mortality from day 35 to 42 in the companion study, the same response of internal organs was not observed in the current study. Coccidial challenge compromised development of internal organs of Ross 708 male broilers at an early age, but the negative effects subsided with age of birds rather than supplementation of riboflavin and *B. subtilis* at current tested levels under our experimental set up.

Key words: coccidiosis, riboflavin, *Bacillus subtilis*, internal organ development

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INTRODUCTION

Coccidiosis is the leading cause of enteric disease in poultry production. It is caused by protozoan parasites of the genus *Eimeria,* which proliferate in the epithelial cells of intestinal tissue and provoke intestinal inflammation (McDougald and Fitz-Coy, 2013). *Eimeria* spp are host specific (Conway and McKenzie, 2007). In poultry, 7 different species are known to induce coccidiosis, but most infections are caused by *Eimeria acervulina, Eimeria maxima, Eimeria necatrix,* and *Eimeria tenella* (Lillehoj and Trout, 1996; McDougald and Fitz-Coy, 2013). Each *Eimeria* spp has a specific region for proliferation in the host intestine (Lillehoj et al., 2004). The intracellular proliferation of the *Eimeria* spp in the host intestine involves production of several types of inflammatory cytokines (Oh et al., 2019), which may impair the normal structure and development of the intestine as well as other internal organs of digestion and immunity. Increased production of inflammatory cytokines reduces immune status of birds (Srinivasan et al., 2017). Immunosuppression caused by coccidiosis may be either transient or permanent (Muller, 2002) and make the birds vulnerable to other diseases (McDougald and Fitz-Coy, 2013). *Eimeria* spp multiply exclusively in the intestine, which causes morphological changes in the intestine, leading to reduction in nutrient

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absorption (McDougald and Fitz-Coy, 2013). Previously, coccidiostats have been used to control coccidiosis and enteric diseases, but because of possible risk for the development of antibiotic resistance, use of coccidiostat has been banned in the European Union (Cogliani et al., 2011) and voluntarily withdrawn in the USA (FDA, 2013). Enteric diseases are the greatest challenge to antibiotic-free broiler production (Van Immerseel et al., 2009; Timbermont et al., 2011). Coccidiosis alone can cause an economic loss of £ 3 billion annually to UK poultry industry (Williams, 1999). Approximately $127 million/yr was spent on anticoccidials in the United States to prevent coccidiosis (Chapman, 2009).

To produce antibiotic-free broilers, it is very important to enhance intestinal integrity, intestinal health, and acquired immunity of birds so that diseases such as coccidiosis will impose minimal negative impact on nutrient digestion, absorption, and growth performance of birds. To enhance intestinal integrity, antibiotic alternatives including probiotics such as *Bacillus subtilis* can be added to feed. *Bacillus* is a group of Gram positive, rod-shaped, spore forming, aerobic, or facultative anaerobic bacteria. It has been reported that *B. subtilis* can tolerate the pelleting heat and pressure associated with feed manufacturing and can germinate in the intestine after they are fed through the feed within a short period of the time (Latorre et al., 2014). *B. subtilis* can enhance growth rate and reduce feed conversion ratio (Molnar et al., 2011), nutrient digestibility (Mountzouris et al., 2010), immunomodulation (Park et al., 2020), and reduce the number of pathogenic bacteria while still maintaining beneficial microflora in the gut. Furthermore, it can enhance intestinal morphology by improving villus height (**VH**) and reducing crypt depth (**CD**) (Jayaraman et al., 2013). Therefore, *B. subtilis* may be used as an antibiotic alternative to enhance gut health during challenged conditions such as coccidiosis or other enteric diseases. However, supplementation of *B. subtilis* during the coccidial challenged condition did not produce consistent improvement in BWG and feed conversion ratio day 0 to 54 (Wang et al., 2018). Similarly, supplementation of *B. subtilis* reduced BWG day 0 to 21 (Harrington et al., 2016) and no effects on day 0 to 42 (Harrington et al., 2016; Reis et al., 2017; Upadhaya et al., 2019). The different results on growth performance while using probiotics may be associated with production of bile salt hydrolase (**BSH**) by probiotics as well as other intestinal microflora, which could reduce host lipid metabolism and growth performance (Lin, 2014; Geng and Lin, 2016). Exhaustive worldwide human gut microbiome analysis done by Song et al. (2019) showed that *Bacillus* has a higher number of strains with BSH paralogs.

Riboflavin (also known as Vitamin B2) is an essential water-soluble vitamin that acts as the coenzyme in metabolic pathways for carbohydrate and protein metabolism. Along with this primary function, riboflavin has 2 other functions that is BSH inhibitor and antioxidant. It can inhibit the BSH enzymes produced by various intestinal bacterial organism including those used as probiotics, consequently enhancing feed conversion efficiency and improving growth performance of chickens (Lin et al., 2014; Smith et al., 2014; Geng et al., 2020) and providing protection to body tissue as an antioxidant (Ashoori and Saedisomeolia, 2014). Riboflavin may optimize the functions of probiotics by reducing BSH activity (Rani et al., 2017). However, limited research is available where extra riboflavin is supplemented along with *B. subtilis* in broiler diets.

Healthy and well-developed internal organs play a crucial role in achieving sustained growth performance. We hypothesized that supplementation of riboflavin and *B. subtilis* in the feed would help broilers defend against induced subclinical coccidiosis by enhancing the intestinal integrity and reducing the damage caused by *Eimeria* spp in the intestine, which could improve immune organs development. Therefore, the objectives of this study were to determine effects of dietary riboflavin, *B. subtilis,* and subclinical coccidial challenge on internal organ development and intestinal morphology of Ross 708 male broilers.

MATERIALS AND METHODS

Experimental Design

This experiment was conducted in a broiler house at the Poultry Research Unit of Mississippi State University (**MSU**). Animal care and husbandry protocol was approved by the Institutional Animal Care and Use Committee of MSU. The experiment follows a $3 \times 2 \times 2$ factorial arrangement of treatments. This results in a total of 12 treatments, containing 3 different levels of riboflavin (0.75, 6.6 [commercially recommended], and 20 ppm/kg supplemented on basal diet [Table 1]), with (calculated 1.1×10^8 CFU/kg of finished feed, actual plate counting verified viable 4.1×10^7-1.5×10^8/kg in the finished feed) or without probiotic *B. subtilis PB6* (CLOSTAT^R Dry, Kemin Industries, Iowa) and with or without coccidial challenge. A 20 × dose of commercial coccidial vaccine (Coccivac^R-B52, Merck Animal Health, Omaha, NE) consisting of 5 different strains of *E. acervulina, E. maxima, E. maxima MFP, Eimeria mivati, and E. tenella* were orally gavaged individually to all challenged birds with 1 mL sterile water, and nonchallenged birds were gavaged with 1 mL of sterile water on day 14 (Wang et al., 2019). Each treatment had 8 replicate pens arranged in a randomized complete block design with each replicate in each block.

Experimental Diet Preparation

Corn and soybean meal–based diets without supplementation of riboflavin were made to meet or exceed Ross 708 nutritional recommendations (Aviagen, 2014) other than dietary riboflavin. The proximate analysis, digestible amino acids, and metabolizable energy values of corn and soybean were obtained from near-infrared spectroscopy (NIR system, model: XDS-XM-1100 series,

Table 1. Feed ingredient composition and calculated nutrient contents of a basal diet for periods of starter (day 0–14), grower (day 14–28), and finisher (day 28–41) feeding phases.

Ingredients[1]%	Starter day 0–14	Grower day 14–28	Finisher day 28–41
Yellow Corn	60.50	62.61	68.24
Soybean Meal	32.13	29.50	23.70
Choline Chloride	0.01	0.01	0.01
Dicalcium Phosphate	2.29	2.08	1.83
Limestone	1.27	1.14	1.06
Salt	0.33	0.33	0.33
Premix[2]	0.25	0.25	0.25
L-Lysine HCl	0.43	0.35	0.35
DL-Methionine	0.40	0.35	0.32
L-Threonine	0.17	0.12	0.10
Sodium Bicarbonate	0.002	0.002	0.002
Soybean Oil	2.21	3.26	3.80
Sand[3]	-	-	-
Calculated composition[4]			
CP%	20.30	19.12	16.92
Ca%	0.96	0.87	0.78
M.E.(kcal/kg)	3,000	3,099	3,196
Dig. Lysine%	1.28	1.15	1.02
Dig. Methionine%	0.71	0.64	0.59
Dig. TSAA%	0.95	0.87	0.80
Dig. Threonine%	0.86	0.77	0.68
Riboflavin ppm	1.477	1.433	1.344
Choline chloride ppm	771	726	680
P available%	0.48	0.44	0.39
Sodium%	0.16	0.16	0.16
Potassium%	0.80	0.76	0.67
Chloride%	0.20	0.20	0.20

[1] Ingredient nutrient compositions were analyzed before formulating the diet.

[2] Premix provided the following per kilogram of finished diet: retinal acetate, 2.654 μg; cholecalciferol, 110 μg; DL-α-tocopherol acetate, 9.9 mg; menadione, 0.9 mg; vitamin B12, 0.01 mg; folic acid, 0.6 μg; choline, 379 mg; D-pantothenic acid, 8.8 mg; niacin, 33 mg; thiamine, 1.0 mg; D-biotin, 0.1 mg; pyridoxine, 0.9 mg; ethoxyquin, 28 mg; manganese, 55 mg; zinc, 50 mg; iron, 28 mg; copper, 4 mg; iodine, 0.5 mg; selenium, 0.1 mg.

[3] Experimental additives: commercial probiotics *Bacillus subtilis* PB6 1.1×10^{8}, CFU/kg of finished feed, and riboflavin at 0.00075 g/kg, 0.0066 g/kg, 0.020 g/kg were added and replaced sand in diet without these additives.

[4] Nutrient contents were calculated on a dry matter basis.

FOSS, Sweden) and a commercial database (Precise Nutrition Evaluation, Adisseo, Alpharetta, GA) before formulating the diets. Least-cost software from Creative Formulation Concepts, Educational version LLC (Pierz, MN) was used to formulate the feed. A special vitamin premix without riboflavin was used. Riboflavin was added to diets separately to produce diets of different levels of riboflavin. A commercially available riboflavin consisting of 80% pure riboflavin was used (Lutavit^{R} Riboflavin SG 80, BASF, Germany). Standard basal mash diet was prepared at the MSU Poultry Research Unit feed mill for starter, grower, and finisher diets. Each basal batch was mixed in a vertical screw mixer (907 kg capacity, Jacobson) for 5 min dry, followed by 10 min of mixing after addition of soy oil.

The dietary treatments were prepared at the USDA Poultry Research Unit (Starkville, MS). For each dietary treatment, riboflavin and *B. subtilis* were mixed in a small horizontal mixer (11 kg capacity) before being added to the allotted basal diet. Then, diets were mixed in a horizontal ribbon mixer (907 kg capacity) to ensure a homogenous mixture before pelleting.

Diets consisting of 3 different levels of riboflavin and with or without *B. subtilis* were pelleted. Feed samples were collected after cooling for each treatment. Starter diet was crumbled after pelleting, which were fed to birds before day 14. Grower and finisher diets were fed as pellets to birds after day 14 and day 28, respectively.

Bird Management

Ross 708 broiler chicks were obtained from a commercial hatchery and feather sexed after arrival on day 0. Thirteen male chicks were placed in each of 96 pens measuring 0.91 × 1.2 m with an area of 0.084 m^2 per bird. Each pen contained a commercial type tube feeder and 3 nipple drinkers. To make a similar condition to commercial setting, used litter obtained from MSU commercial broiler houses was used. Fresh pine shavings were top-dressed over the used litter obtained. Temperature and lighting program followed commercial practices recommended by Ross 708 commercial management guidelines. House temperature was maintained at 32°C for the first week, then target temperature drop was approximately 4°C until fourth week. After the fourth week, the temperature was maintained at 18.3°C. Twenty-four hours of light was provided for first week, after that a dark period was maintained for 4 h each day.

Birds were reared in a house equipped with an evaporative cooling system and negative air pressure fans. Temperature and humidity were maintained according to the requirements of Ross 708. Observation of the bird's health condition, availability of feed, water, and environmental housing conditions were recorded twice a day.

Sampling Procedure

One bird per pen was randomly selected for gastrointestinal sampling on day 13, 27, and 36. Selected birds were tagged, live weight of birds were taken before they were humanely euthanized using CO_2 asphyxiation. Euthanized birds were then necropsied for the following sampling.

Measurement of Digestive Organs

The weight of empty gizzard and proventriculus was taken. The length and weight of empty small intestine, that is, duodenum, jejunum, and ileum, were recorded. The length of the jejunum was defined as the length from the terminal end of the duodenum loop to the Meckel's diverticulum. The length of ileum is from Meckel's diverticulum to ileo-ceca junction. The absolute (g), relative weight [g/g BW (%)], absolute length (cm), and absolute weight to length (g/cm) were measured and calculated for each organ from each sampled bird.

Measurement of Immune Organs

The weights of the immune organs, spleen and bursa were taken out, and weight of these organs were recorded. These organs are related to synthesis of the B-lymphocytes and T-lymphocytes. The relative weights [g/g BW (%)] were calculated for each organ from each sampled bird.

Coccidial Lesion Score

On day 27 and day 36, one bird from each replicate was euthanized, and coccidiosis lesion scoring in all 3 sections of small intestine and ceca was done according to methods described by Conway and McKenzie (2007). Lesions were scored on the scale of 0 to 3, in which 0 (no gross lesion), 1 (0–4 petechiae on serosa per cm), 2 (4–10 petechiae on serosa per cm), and 3 (10- numerous petechiae on serosa per cm). *Eimeria* spp involved in formation of lesion in intestinal segments were identified based on lesion color, shape, and location of lesion in intestine (Conway and McKenzie, 2007). White color patched lesions present in duodenum and extended to jejunum during severe infection were considered to be produced by *E. acervulina*. Red pinpoint lesions colonized mainly in jejunum and extended to duodenum and ileum during severe infection were considered to be *E. maxima* (Conway and McKenzie, 2007).

Intestinal Tissue Sampling

Birds used for measurement of internal organ development were also used for collection of intestinal sections. Two centimeter of each section of the small intestine, that is, duodenum, jejunum, and ileum, were collected from the middle part of each section. The section was then stored in a 20 mL screw-capped vial containing 10 mL of 10% buffered formalin. The section which was fixed in the formalin was cut into smaller sections then sent to College of Veterinary Medicine Laboratory of Mississippi State University for preparation of histological slides. The slides were stained with hematoxylin and eosin. Histological slides were observed in a compound microscope (Laxco SeBa, Bothell, WA), and images were captured. Captured images were measured using the ImageJ software (National Institutes of Health, Bethesda, MD). The VH, villus width (**VW**), and CD were measured. For each of the samples, 5 different intact villi were selected and measured, and the average length was taken. For the VW, the width of the selected villus was taken in 3 different locations (top, middle, and bottom) section of the villus and averaged to make the VW of the single villus. The ratio of VH to CD was calculated by dividing the VH to CD for each of the sections of the intestine.

Results obtained from the intestinal measurements suggest that most of the changes or the effects were because of either the inclusion of *Bacillus* in the diet or because of coccidial challenge. Therefore, histological samples from only birds fed the recommended level of riboflavin were measured. This resulted in a total of 4 treatments arranged in the 2 (*Bacillus*) × 2 (coccidial challenge) factorial arrangement with or without *Bacillus* in the diet and with or without coccidial challenge to the birds. On day 27, intestinal segments were collected from all 3 sections of small intestine, and on day 36, only jejunum section was collected for histology.

Statistical Analysis

Variables measured before coccidial challenge on day 14 were analyzed using 2-way ANOVA within a randomized complete block design. In this case, doses of riboflavin and *Bacillus* supplementation were considered as fixed effects. Variables measured after coccidial challenge were analyzed using 3-way ANOVA. The doses of riboflavin, *Bacillus* supplementation in the diet, and coccidial challenge were considered fixed effects. In both cases, block was used as the random effect as the blocking was done according to location within the house, and each treatment was replicated once in each block. Pen was considered the experimental unit. Data were analyzed using the Proc GLM procedure of the SAS 9.4 (SAS version 9.4, SAS Institute, 2013). The level of significance was determined at $P \leq 0.05$. Fisher's protected LSD was used to determine multiple comparisons between the treatments. Pearson partial correlation analysis was used to analyze the correlation between intestinal histomorphology with intestinal measurements. Spearman partial correlation was used to analyze the correlation between the intestinal histomorphology and intestinal measurements with intestinal lesion score.

RESULTS

Internal organ absolute and relative weights and small intestine absolute and relative weights and lengths are reported in Table 2, Table 3, and Table 4 (day 13); Table 5, Table 6, and Table 7 (day 27); and Table 8, Table 9, and Table 10 (day 36). Villus depth and CD measurements are reported in Table 11. Lesions caused by *E. acervulina* and *E. maxima* are presented in Table 12 and Table 13 (day 27) and Table 14 and Table 15 (day 36), respectively. In addition, relationship between intestinal histomorphology and other intestinal measurements are reported in Table 16 and Table 17.

Internal Organ and Intestine Measurements on Day 13

Absolute Weights of Internal Organs on Day 13 Even though BW was not affected, birds fed 20 ppm of riboflavin had higher pancreas weights than birds fed 6.6 ppm (commercially recommended level) of riboflavin on day 13 ($P = 0.013$; Table 2).

Relative Weights to Body Weight on Day 13 Relative weight of gizzard and pancreas were interactively affected by riboflavin and *B. subtilis* on day 13 ($P = 0.025$ and $P = 0.036$, respectively). When riboflavin was fed at 0.75 ppm, diet with *B. subtilis* lowered relative gizzard weight. In birds fed diet without *B. subtilis*, relative gizzard weight was higher in birds fed riboflavin at 0.75 ppm than birds fed riboflavin at 6.6 ppm and 20 ppm ($P = 0.025$). Feeding riboflavin at 6.6 ppm, supplementation of *B. subtilis* in diet increased relative pancreas weight. When birds were not supplemented with *B. subtilis,* riboflavin at 6.6 ppm lowered relative pancreas weights compared with that of birds fed riboflavin at 0.75 ppm and 20 ppm ($P = 0.036$; Table 3).

Intestine on Day 13 Riboflavin and *B. subtilis* interactively affected duodenum length. Duodenum length was longer in birds fed 20 ppm riboflavin than those fed 0.75 ppm riboflavin, when dietary *B. subtilis* was not supplemented ($P = 0.048$). *B. subtilis* supplementation increased jejunum length ($P = 0.037$; Table 4)

Internal Organ and Intestine Measurements on Day 27

Absolute Weights of Internal Organs on Day 27 Coccidial challenge on day 14 decreased subsequent BW on day 27 ($P = 0.014$). When birds were fed 6.6 ppm riboflavin, *B. subtilis* supplementation lowered gizzard weight ($P = 0.012$). When *B. subtilis* was supplemented in the diets, gizzard weight was highest in birds fed riboflavin at 0.75 ppm. Riboflavin at 20 ppm lowered gizzard weight compared with riboflavin at 6.6 ppm, when *B. subtilis* was not supplemented ($P = 0.012$). When *B. subtilis* was not supplemented, pancreases were heavier in birds fed 6.6 ppm riboflavin than birds fed 0.75 ppm riboflavin, and birds fed riboflavin at 6.6 ppm along with *B. subtilis* had lower weight than birds fed the same level of riboflavin without *B. subtilis* ($P = 0.008$). Liver weights were reduced when birds were fed diet with *B. subtilis* ($P = 0.036$). Coccidial challenge reduced the weight of bursa ($P = 0.026$; Table 5).

Relative Weights to Body Weight on Day 27 The relative gizzard weight was reduced when supplementing riboflavin at 20 ppm compared with 0.75 ppm ($P = 0.037$). On nonchallenged birds, *B. subtilis* supplementation lowered relative weight of gizzard compared with birds without supplementation of *B. subtilis* ($P = 0.043$). The relative proventriculus ($P = 0.001$) and spleen ($P = 0.001$) weight was increased by coccidial challenge (Table 6).

Intestine on Day 27 The coccidial challenge increased the relative and absolute weights of the duodenum, jejunum, and ileum ($P < 0.001$), along with an increase in the weights of the intestine. Coccidial challenge increased the length of duodenum ($P = 0.0002$) and jejunum ($P < 0.0001$). Coccidial challenge also increased

Table 2. The live body weight (g) and internal organ weight (g) of Ross 708 male broilers fed riboflavin and *Bacillus subtilis* diet on day 13.

Riboflavin	Bacillus	BW	Proventriculus	Gizzard	Spleen	Pancreas	Liver	Bursa	Heart
0.75		326	2.24	11.37	0.245	$1.58^{a,b}$	11.97	0.553	2.29
6.6		335	2.31	10.90	0.256	1.49^{b}	12.24	0.532	2.44
20		329	2.38	11.10	0.254	1.66^{a}	12.05	0.541	2.32
SEM		6.8	0.049	0.233	0.0100	0.0402	0.325	0.0275	0.059
	No	329	2.31	11.10	0.255	1.55	12.16	0.523	2.32
	Yes	331	2.31	11.14	0.248	1.61	12.02	0.561	2.38
	SEM	5.5	0.040	0.190	0.0082	0.033	0.265	0.0224	0.048
P-value									
Riboflavin		0.623	0.163	0.356	0.737	0.013	0.829	0.868	0.158
Bacillus		0.767	0.931	0.871	0.505	0.202	0.698	0.235	0.310
Riboflavin × Bacillus[1]		0.241	0.229	0.866	0.900	0.055	0.498	0.262	0.959

$^{a\text{-}c}$Means in a column not sharing a common superscript were different ($P < 0.05$).

[1] Means of nonsignificant interaction is not listed.

Table 3. The relative internal organ weight to BW (%) of Ross 708 male broilers fed riboflavin and *Bacillus subtilis* diet on day 13.

		Relative weight/BW %						
Riboflavin	Bacillus	Proventriculus	Gizzard	Spleen	Pancreas	Liver	Bursa	Heart
0.75		0.692	3.50	0.075	0.487	3.65	0.169	0.698
6.6		0.694	3.27	0.077	0.450	3.68	0.158	0.733
20		0.725	3.39	0.077	0.506	3.67	0.162	0.708
SEM		0.0124	0.052	0.0026	0.0121	0.071	0.0070	0.0185
	No	0.706	3.50	0.078	0.472	3.67	0.157	0.705
	Yes	0.702	3.27	0.075	0.490	3.66	0.169	0.722
	SEM	0.0101	3.39	0.0021	0.0099	0.058	0.0057	0.0151
Riboflavin × Bacillus								
0.75	No	0.707	3.62^{a}	0.077	0.499^{a}	3.65	0.160	0.700
0.75	Yes	0.677	3.38^{b}	0.073	0.475^{a}	3.64	0.179	0.697
6.6	No	0.682	3.26^{b}	0.078	0.417^{b}	3.70	0.151	0.730
6.6	Yes	0.707	3.28^{b}	0.075	0.483^{a}	3.66	0.166	0.736
20	No	0.729	3.31^{b}	0.078	0.501^{a}	3.67	0.162	0.685
20	Yes	0.721	3.47^{a,b}	0.077	0.512^{a}	3.67	0.161	0.732
SEM		0.0176	3.386	0.0037	0.0171	0.100	0.0099	0.0262
P-value								
Riboflavin		0.114	0.009	0.788	0.006	0.954	0.513	0.393
Bacillus		0.748	0.735	0.379	0.210	0.844	0.171	0.433
Riboflavin × Bacillus		0.300	0.025	0.917	0.036	0.972	0.567	0.601

$^{a\text{-}c}$Means in a column not sharing a common superscript were different ($P < 0.05$).

relative weight by the length of the ileum (P = 0.045; Table 7).

Internal Organ and Intestine Measurements on Day 36

Absolute Weights of Internal Organs on Day 36 Similar to day 27, coccidial challenge on day 14 decreased subsequent BW on day 36 (P = 0.013). The interaction of riboflavin and coccidial challenge affected the proventriculus and liver weight. For birds fed riboflavin at 6.6 ppm, coccidial challenge increased proventriculus weight compared with nonchallenged birds. Nonchallenged birds fed riboflavin at 6.6 ppm had lowered proventriculus weight compared with birds fed riboflavin at 20 ppm (P = 0.035). Similarly, coccidial challenge reduced liver weight when the birds were fed riboflavin at 20 ppm. On nonchallenged birds, riboflavin supplementation at 20 ppm increased liver weights in comparison to birds supplemented with riboflavin at 6.6 ppm (P = 0.038). The increased bursa weight due to the coccidial challenge on day 27 had disappeared on day 36 (P = 0.454; Table 8).

Relative Weights to Body Weight on Day 36 The increase of spleen weight due to coccidial challenge on day 27 was lost on day 36. The interaction of riboflavin and coccidial challenge affected the relative proventriculus and liver weight (P = 0.030 and P = 0.037). Coccidial

Table 4. The small intestine absolute weight, relative weight to BW, length, and ratio of weight to length because of dietary supplementation of different doses of riboflavin and *Bacillus subtilis* on day 13 of Ross 708 male broilers.

		Weight (g)			Relative weight to BW (%)			Length (cm)			Relative weight (g/cm)		
Riboflavin	Bacillus	Duo	Jej	Ile	Duo	Jej	Ile	Duo	Jej	Ile	Duo	Jej	Ile
0.75		5.92	8.80	5.20	1.80	2.71	1.58	21.52	42.40	40.98	0.274	0.211	0.129
6.6		5.88	8.84	5.35	1.75	2.67	1.62	21.50	42.22	40.51	0.274	0.208	0.134
20		5.90	8.96	5.49	1.80	2.74	1.69	21.69	43.43	41.30	0.273	0.210	0.134
SEM		0.130	0.234	0.120	0.038	0.072	0.049	0.288	0.797	0.577	0.0054	0.0065	0.0042
	No	5.88	8.76	5.23	1.77	2.68	1.58	21.53	41.71^{b}	40.42	0.275	0.209	0.131
	Yes	5.92	8.98	5.47	1.80	2.73	1.67	21.61	43.66^{a}	41.45	0.273	0.210	0.133
	SEM	0.106	0.191	0.098	0.031	0.059	0.040	0.235	0.651	0.471	0.0044	0.0053	0.0034
Riboflavin × Bacillus													
0.75	No	5.80	8.59	5.05	1.80	2.71	1.55	21.03^{b}	41.99	40.56	0.268	0.207	0.131
0.75	Yes	6.04	9.02	5.36	1.81	2.70	1.61	22.01^{a,b}	42.81	41.41	0.279	0.214	0.127
6.6	No	5.90	8.67	5.33	1.75	2.63	1.62	21.34^{a,b}	41.11	39.78	0.280	0.208	0.133
6.6	Yes	5.86	9.01	5.37	1.75	2.70	1.61	21.66^{a,b}	43.34	41.24	0.268	0.208	0.135
20	No	5.94	9.01	5.30	1.77	2.69	1.57	22.22^{a}	42.01	40.91	0.276	0.211	0.130
20	Yes	5.86	8.91	5.68	1.83	2.78	1.80	21.16^{a,b}	44.84	41.70	0.271	0.208	0.138
SEM		0.184	0.330	0.169	0.053	0.102	0.069	0.407	1.126	0.815	0.0077	0.0092	0.0059
P-value													
Riboflavin		0.978	0.884	0.263	0.513	0.783	0.320	0.878	0.529	0.622	0.993	0.952	0.650
Bacillus		0.781	0.413	0.085	0.541	0.530	0.114	0.806	0.037	0.126	0.725	0.898	0.721
Riboflavin × Bacillus		0.636	0.697	0.559	0.868	0.885	0.184	0.048	0.657	0.905	0.322	0.851	0.600

$^{a\text{-}c}$Means in a column not sharing a common superscript were different ($P < 0.05$).

Abbreviations: Duo, duodenum; Ile, ileum; Jej, jejunum.

Table 5. The live body weight (g) and internal organ weight (g) on day 27 of Ross 708 male broilers fed riboflavin and *Bacillus subtilis* diet and challenged with coccidiosis.

Riboflavin	Bacillus	Cocci.	BW	Proventriculus	Gizzard	Spleen	Pancreas	Liver	Bursa	Heart
0.75			1,274	5.73	26.38	1.29	3.76	39.71	2.22	7.39
6.6			1,276	5.69	25.01	1.23	3.87	40.32	2.19	7.44
20			1,264	5.72	24.26	1.28	3.90	38.58	2.23	7.24
SEM			22.0	0.129	0.451	0.059	0.088	0.994	0.114	0.188
	No		1,293	5.80	25.94	1.30	3.88	40.76^{a}	2.22	7.55
	Yes		1,250	5.63	24.49	1.24	3.81	38.31^{b}	2.21	7.17
	SEM		18.0	0.105	0.368	0.048	0.071	0.811	0.092	0.153
		Noncha	1,305^{a}	5.65	25.66	1.20	3.86	39.74	2.36^{a}	7.51
		Cha	1,238^{b}	5.78	24.77	1.33	3.82	39.33	2.07^{b}	7.21
		SEM	18.0	0.105	0.368	0.048	0.071	0.812	0.092	0.153
Riboflavin × Bacillus										
0.75	No		1,270	5.69	26.18^{a,b}	1.35	3.61^{b}	40.56	2.24	7.44
0.75	Yes		1,278	5.76	26.57^{a,b}	1.23	3.90^{a,b}	38.85	2.21	7.34
6.6	No		1,336	5.83	26.77^{a}	1.24	4.12^{a}	42.19	2.21	7.76
6.6	Yes		1,217	5.55	23.25^{c}	1.22	3.63^{b}	38.44	2.17	7.12
20	No		1,273	5.89	24.86^{b,c}	1.29	3.89^{a,b}	39.52	2.21	7.44
20	Yes		1,256	5.56	23.65^{c}	1.27	3.91^{a,b}	37.64	2.25	7.04
SEM			32.0	0.18	0.638	0.083	0.123	1.405	0.159	0.266
P-value										
Riboflavin			0.927	0.979	0.005	0.746	0.466	0.450	0.960	0.734
Bacillus			0.110	0.235	0.007	0.401	0.521	0.036	0.920	0.085
Cocci.			0.014	0.386	0.094	0.051	0.693	0.723	0.026	0.170
Riboflavin × Bacillus			0.122	0.492	0.012	0.766	0.008	0.722	0.951	0.598
Riboflavin × Cocci.[1]			0.673	0.028[2]	0.610	0.624	0.538	0.257	0.849	0.214
Bacillus × Cocci.[1]			0.419	0.527	0.136	0.516	0.515	0.717	0.115	0.246
Riboflavin × Bacillus × Cocci.[1]			0.563	0.171	0.446	0.286	0.381	0.410	0.963	0.943

$^{a\text{-}c}$Means in a column not sharing a common superscript were different ($P < 0.05$).

Abbreviations: Cha, Challenged with cocci; Noncha, Nonchallenged.

[1] Means of nonsignificant interactions are not listed.

[2] Fisher's LSD test was not able to separate means of combination of riboflavin and coccidial challenge effects on proventriculus weight.

Table 6. The relative internal organ weight to BW (%) on day 27 of Ross 708 male broilers fed riboflavin and *Bacillus subtilis* diet and challenged with coccidiosis.

Riboflavin	Bacillus	Cocci.	Proventriculus	Gizzard	Spleen	Pancreas	Liver	Bursa	Heart
0.75			0.449	2.06^{a}	0.101	0.296	3.11	0.174	0.575
6.6			0.450	1.97^{a,b}	0.096	0.306	3.16	0.171	0.588
20			0.454	1.93^{b}	0.102	0.311	3.05	0.177	0.576
SEM			0.0088	0.037	0.0040	0.0073	0.060	0.0080	0.0133
	No		0.452	2.01	0.100	0.301	3.14	0.172	0.587
	Yes		0.450	1.96	0.100	0.308	3.08	0.176	0.572
	SEM		0.0070	0.015	0.0030	0.0060	0.049	0.0066	0.0108
		Noncha	0.433^{b}	1.98	0.091^{b}	0.298	3.06	0.181	0.580
		Cha	0.468^{a}	2.00	0.108^{a}	0.310	3.16	0.167	0.580
		SEM	0.00725	0.030	0.0035	0.0060	0.049	0.0066	0.0108
Bacillus × Cocci.									
	No	Noncha	0.434	2.048^{a}	0.091	0.295	3.12	0.172	0.582
	No	Cha	0.469	1.98^{a,b}	0.109	0.306	3.18	0.172	0.593
	Yes	Noncha	0.433	1.91^{b}	0.092	0.302	3.01	0.190	0.578
	Yes	Cha	0.467	2.01^{a,b}	0.107	0.314	3.14	0.162	0.566
	SEM		0.0102	0.042	0.0050	0.0084	0.069	0.0093	0.0153
P-value									
Riboflavin			0.924	0.037	0.611	0.345	0.434	0.897	0.733
Bacillus			0.879	0.215	0.948	0.372	0.337	0.634	0.320
Cocci.			0.001	0.650	0.001	0.169	0.149	0.146	0.998
Riboflavin × Bacillus[1]			0.232	0.619	0.447	0.401	0.694	0.775	0.775
Riboflavin × Cocci.[1]			0.082	0.923	0.678	0.792	0.092	0.696	0.409
Bacillus × Cocci.			0.915	0.043	0.828	0.950	0.663	0.141	0.467
Riboflavin × Bacillus × Cocci.[1]			0.515	0.427	0.506	0.906	0.484	0.881	0.603

$^{a\text{-}c}$Means in a column not sharing a common superscript were different ($P < 0.05$).

Abbreviations: Cha, Challenged with cocci.; Noncha, Nonchallenged.

[1] Means of nonsignificant interactions are not listed.

Table 7. Small intestine absolute weight, relative weight to BW, length, and ratio of weight to length on day 27 because of dietary supplementation of different doses of riboflavin and *Bacillus subtilis* on Ross 708 male broilers challenged with coccidiosis.

			Weight (g)			Relative weight to BW (%)			Length (cm)			Relative weight (g/cm)		
Riboflavin	Bacillus	Cocci.	Duo	Jej	Ile	Duo	Jej	Ile	Duo	Jej	Ile	Duo	Jej	Ile
0.75			12.03	22.29	13.38	0.946	1.75	1.06	26.80	63.22	63.23	0.450	0.358	0.215
6.6			11.95	21.60	13.86	0.947	1.70	1.10	27.21	62.08	59.77	0.442	0.351	0.233
20			11.18	20.91	13.11	0.893	1.67	1.04	25.99	61.74	60.35	0.428	0.341	0.219
SEM			0.305	0.446	0.290	0.0250	0.039	0.024	0.439	1.301	1.342	0.0114	0.3490	0.0064
	No		11.97	21.86	13.72	0.932	1.70	1.07	26.80	62.85	61.87	0.447	0.350	0.224
	Yes		11.47	21.35	13.17	0.926	1.71	1.07	26.54	61.84	60.36	0.433	0.351	0.221
	SEM		0.250	0.369	0.240	0.0204	0.032	0.020	0.359	1.061	1.060	0.0093	0.0084	0.0052
		Noncha	10.98^{b}	20.09^{b}	12.58^{b}	0.845^{b}	1.54^{b}	0.97^{b}	25.68^{b}	58.30^{b}	59.67	0.430	0.351	0.214^{b}
		Cha	12.46^{a}	23.12^{a}	14.32^{a}	1.012^{a}	1.87^{a}	1.16^{a}	27.65^{a}	66.39^{a}	62.56	0.450	0.350	0.229^{a}
		SEM	0.369	0.369	0.240	0.020	0.032	0.020	0.359	1.061	1.096	0.0090	0.0084	0.0099
Bacillus × Cocci.														
No		Noncha	10.98	20.25	12.86	0.837	1.55	0.98	25.85	60.28	61.33	0.424	0.341	0.212
No		Cha	12.96	23.46	14.59	1.026	1.84	1.16	27.74	65.42	62.40	0.469	0.359	0.236
Yes		Noncha	10.99	19.92	12.30	0.853	1.53	0.96	25.50	56.32	58.00	0.435	0.360	0.217
Yes		Cha	11.96	22.77	14.05	1.000	1.90	1.17	27.57	67.37	62.73	0.431	0.341	0.224
SEM			0.354	0.552	0.339	0.0289	0.0452	0.028	0.5071	1.502	1.550	0.0132	0.0118	0.0074
P-value														
Riboflavin			0.097	0.104	0.180	0.217	0.366	0.239	0.149	0.703	0.157	0.396	0.475	0.134
Bacillus			0.166	0.330	0.108	0.854	0.692	0.937	0.608	0.504	0.335	0.309	0.942	0.668
Cocci.			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	0.065	0.125	0.942	0.045
Riboflavin × Bacillus[1]			0.633	0.348	0.965	0.792	0.273	0.442	0.123	0.091	0.062	0.755	0.068	0.334
Riboflavin × Cocci.[1]			0.244	0.984	0.454	0.571	0.843	0.345	0.191	0.185	0.256	0.616	0.394	0.482
Bacillus × Cocci.			0.162	0.732	0.987	0.470	0.444	0.528	0.865	0.052	0.241	0.073	0.124	0.266
Riboflavin × Bacillus × Cocci.[1]			0.668	0.812	0.949	0.167	0.859	0.557	0.341	0.37	0.520	0.933	0.909	0.785

[a-c] Means in a column not sharing a common superscript were different ($P < 0.05$).

Abbreviations: Cha, Challenged with cocci; Duo, duodenum; Ile, ileum; Jej, jejunum; Noncha, Nonchallenged.

[1] Means of nonsignificant interaction is not listed.

Table 8. Live body weight (g) and internal organ weight (g) on day 36 of Ross 708 male broilers fed riboflavin and *Bacillus subtilis* diet and challenged with coccidiosis on day 36.

Riboflavin	Bacillus	Cocci.	BW	Proventriculus	Gizzard	Spleen	Pancreas	Liver	Bursa	Heart
0.75			2,200	7.88	34.76	2.24	5.18	60.66	3.37	12.75
6.6			2,183	7.54	34.55	2.46	5.23	57.76	3.25	12.74
20			2,289	7.86	34.45	2.42	5.21	60.90	3.31	12.70
SEM			38.0	0.165	0.989	0.096	0.135	1.130	0.157	0.265
	No		2,245	7.73	35.20	2.38	5.32	59.51	3.31	12.84
	Yes		2,203	7.79	33.97	2.38	5.09	60.03	3.31	12.62
	SEM		31.0	0.134	0.808	0.078	0.110	0.923	0.129	0.217
		Noncha	$2,281^{a}$	7.69	34.81	2.33	5.21	59.78	3.24	12.95
		Cha	$2,160^{b}$	7.82	34.36	2.42	5.20	59.76	3.38	12.51
		SEM	31.0	0.134	0.808	0.078	0.110	0.923	0.129	0.216
Riboflavin × Cocci										
0.75		Noncha	2,251	$7.81^{a,b}$	34.14	2.24	5.20	$59.40^{a,b,c}$	3.31	12.81
0.75		Cha	2,148	7.94^{a}	35.38	2.24	5.16	$61.91^{a,b}$	3.43	12.69
6.6		Noncha	2,241	7.17^{b}	34.49	2.27	5.13	56.64^{c}	3.27	13.03
6.6		Cha	2,124	7.91^{a}	34.60	2.66	5.34	$58.87^{a,b,c}$	3.22	12.45
20		Noncha	2,349	8.11^{a}	35.80	2.48	5.31	63.30^{a}	3.14	12.68
20		Cha	2,230	$7.61^{a,b}$	33.11	2.36	5.10	$58.50^{b,c}$	3.48	12.73
SEM			54.3	0.233	1.399	0.135	0.191	1.599	0.223	0.375
P-value										
Riboflavin			0.115	0.267	0.976	0.214	0.963	0.107	0.873	0.992
Bacillus			0.354	0.750	0.288	0.994	0.148	0.690	0.977	0.472
Cocci.			0.013	0.503	0.697	0.420	0.926	0.986	0.454	0.153
Riboflavin × Bacillus[1]			0.542	0.932	0.642	0.117	0.754	0.266	0.462	0.683
Riboflavin × Cocci.			0.988	0.035	0.356	0.148	0.549	0.038	0.682	0.296
Bacillus × Cocci.[1]			0.620	0.451	0.222	0.923	0.501	0.968	0.988	0.561
Riboflavin × Bacillus × Cocci.[1]			0.999	0.537	0.876	0.241	0.835	0.797	0.835	0.670

[a-c] Means in a column not sharing a common superscript were different ($P < 0.05$).
Abbreviations: Cha, Challenged with cocci.; Noncha = Nonchallenged.
[1] Means of nonsignificant interaction is not listed.

Table 9. The relative internal organ weight to BW (%) on day 36 of Ross 708 male broilers fed riboflavin and *Bacillus subtilis* diet and challenged with coccidiosis.

Riboflavin	Bacillus	Cocci.	Proventriculus	Gizzard	Spleen	Pancreas	Liver	Bursa	Heart
0.75			0.359	1.60	0.103	0.236	2.77	0.154	0.582
6.6			0.347	1.59	0.114	0.242	2.67	0.147	0.589
20			0.345	1.51	0.106	0.230	2.67	0.146	0.556
SEM			0.0072	0.041	0.0044	0.0066	0.044	0.0068	0.0114
	No		0.346	1.58	0.106	0.238	2.67	0.147	0.577
	Yes		0.355	1.55	0.109	0.234	2.73	0.151	0.575
	SEM		0.0058	0.034	0.0036	0.0054	0.036	0.0055	0.0093
		Noncha	0.338	1.54	0.103	0.232	2.64	0.142	0.573
		Cha	0.362	1.59	0.112	0.240	2.76	0.155	0.579
		SEM	0.0059	0.034	0.0037	0.0054	0.036	0.0055	0.0093
Riboflavin × Cocci.									
0.75		Noncha	$0.348^{a,b,c}$	1.54	0.101	0.232	$2.65^{a,b}$	0.147	0.568
0.75		Cha	$0.370^{a,b}$	1.66	0.105	0.241	2.88^{a}	0.161	0.596
6.6		Noncha	0.321^{c}	1.55	0.103	0.233	2.58^{b}	0.145	0.585
6.6		Cha	0.373^{a}	1.63	0.126	0.252	$2.76^{a,b}$	0.149	0.594
20		Noncha	$0.346^{a,b,c}$	1.52	0.106	0.232	$2.70^{a,b}$	0.135	0.566
20		Cha	$0.342^{b,c}$	1.49	0.106	0.228	2.63^{b}	0.156	0.547
SEM			0.0102	0.059	0.0063	0.0093	0.062	0.0096	0.0161
P-value									
Riboflavin			0.301	0.251	0.174	0.445	0.183	0.652	0.102
Bacillus			0.259	0.532	0.642	0.626	0.233	0.560	0.895
Cocci.			0.006	0.255	0.090	0.295	0.027	0.101	0.656
Riboflavin × Bacillus[1]			0.625	0.866	0.208	0.941	0.130	0.575	0.927
Riboflavin × Cocci.			0.030	0.396	0.149	0.468	0.037	0.655	0.329
Bacillus × Cocci.[1]			0.217	0.097	0.693	0.917	0.679	0.768	0.833
Riboflavin × Bacillus × Cocci.[1]			0.527	0.822	0.211	0.898	0.809	0.890	0.457

[a-c] Means in a column not sharing a common superscript were different ($P < 0.05$).
Abbreviations: Cha, Challenged with cocci.; Noncha, Nonchallenged.
[1] Means of nonsignificant interactions are not listed.

Table 10. Small intestine absolute weight, relative weight to BW, length, and ratio of weight to length on day 36 because of dietary supplementation of different doses of riboflavin and *Bacillus subtilis* on Ross 708 male broilers challenged with coccidiosis.

			Weight (g)			Relative weight to BW (%)			Length (cm)			Relative weight (g/cm)		
Riboflavin	Bacillus	Cocci.	Duo	Jej	Ile	Duo	Jej	Ile	Duo	Jej	Ile	Duo	Jej	Ile
0.75			17.76	30.39	20.25	0.811	1.39	0.921^{a}	34.72^{a}	80.88	90.30	0.513	0.378	0.225
6.6			17.07	29.72	19.64	0.785	1.37	0.904^{a}	$34.33^{a,b}$	79.21	87.76	0.497	0.381	0.224
20			16.79	30.05	18.78	0.738	1.32	0.825^{b}	32.94^{b}	80.86	87.41	0.516	0.376	0.215
SEM			0.445	0.694	0.531	0.0197	0.034	0.0250	0.520	1.454	1.188	0.0122	0.0102	0.0057
	No		16.96	30.43	19.61	0.758	1.36	0.877	33.85	81.78	89.05	0.503	0.376	0.221
	Yes		17.45	29.68	19.51	0.798	1.36	0.890	34.14	78.85	87.93	0.514	0.381	0.222
	SEM		0.363	0.567	0.434	0.0161	0.028	0.0204	0.425	1.188	0.970	0.0099	0.0083	0.0047
		Noncha	17.12	31.20	21.13^{a}	0.756	1.38	0.935^{a}	33.73	80.08	89.78	0.510	0.393^{a}	0.236^{a}
		Cha	17.30	28.90	17.99^{b}	0.800	1.34	0.832^{b}	34.26	80.54	87.19	0.507	0.363^{b}	0.206^{b}
		SEM	0.363	0.567	0.434	0.0161	0.028	0.0204	0.425	1.188	0.970	0.0099	0.0083	0.0047
Riboflavin × Cocci.														
0.75		Noncha	17.12	$30.45^{a,b}$	21.62	0.765	1.37	0.962	34.22	79.94	92.23	0.500	0.384	0.235
0.75		Cha	18.41	$30.33^{a,b}$	18.89	0.858	1.41	0.881	35.22	81.81	88.38	0.526	0.373	0.214
6.6		Noncha	17.17	$30.47^{a,b}$	20.93	0.771	1.38	0.947	33.75	77.03	87.66	0.509	0.397	0.240
6.6		Cha	16.96	$28.97^{b,c}$	18.35	0.799	1.37	0.862	34.91	81.38	87.86	0.485	0.364	0.208
20		Noncha	17.06	32.70^{a}	20.83	0.732	1.41	0.897	33.22	83.28	89.47	0.522	0.399	0.233
20		Cha	16.53	27.41^{c}	16.73	0.744	1.24	0.753	32.66	78.44	85.34	0.510	0.353	0.197
SEM			0.629	0.982	0.751	0.0278	0.048	0.0354	0.735	2.057	1.681	0.0172	0.0145	0.0081
Bacillus × Cocci.														
No		Noncha	16.98	31.23	21.09	0.737	1.36	0.919	33.58	80.63	$88.75^{a,b}$	0.508	0.391	0.239
No		Cha	16.95	29.63	18.13	0.780	1.36	0.835	34.13	82.94	89.34^{a}	0.498	0.360	0.203
Yes		Noncha	17.26	31.17	21.16	0.775	1.41	0.952	33.88	79.54	90.81^{a}	0.513	0.396	0.233
Yes		Cha	17.64	28.18	17.85	0.821	1.32	0.829	34.40	78.15	85.04^{b}	0.515	0.366	0.210
	SEM		0.513	0.802	0.614	0.0227	0.039	0.0289	0.600	1.679	1.372	0.0141	0.0118	0.0066
0.75	No	Noncha	$17.57^{a,b}$	31.53	22.73	$0.769^{b.c.d}$	1.40	0.996	34.06	84.44^{a}	94.00	0.516	0.379	0.244
0.75	No	Cha	$17.32^{a.b}$	29.77	18.69	$0.801^{b,c}$	1.38	0.866	34.69	$82.25^{a,b}$	89.13	0.503	0.364	0.21
0.75	Yes	Noncha	16.65^{b}	29.38	20.51	$0.760^{b,c,d}$	1.34	0.928	34.38	75.44^{b}	90.44	0.485	0.39	0.226
0.75	Yes	Cha	19.50^{a}	30.89	19.08	0.9140^{a}	1.44	0.896	35.75	$81.376^{a,b}$	87.63	0.549	0.381	0.219
6.6	No	Noncha	$17.20^{a,b}$	30.89	21.41	$0.755^{b,c,d}$	1.36	0.944	34.00	75.50^{b}	84.94	0.51	0.411	0.254
6.6	No	Cha	16.17^{b}	30.11	18.86	$0.751^{b,c,d}$	1.39	0.875	34.00	84.31^{a}	90.65	0.475	0.361	0.208
6.6	Yes	Noncha	$17.14^{a,b}$	30.04	20.45	$0.786^{b,c,d}$	1.39	0.949	33.5	$78.56^{a,b}$	90.38	0.508	0.383	0.226
6.6	Yes	Cha	$17.75^{a,b}$	27.84	17.83	$0.8458^{a,b}$	1.35	0.849	35.81	$78.45^{a,b}$	85.063	0.495	0.367	0.209
20	No	Noncha	16.15^{b}	31.29	19.13	0.685^{d}	1.33	0.817	32.69	$81.94^{a,b}$	87.31	0.498	0.383	0.219
20	No	Cha	$17.36^{a,b}$	29.02	16.83	$0.785^{b,c,d}$	1.31	0.764	33.69	$82.25^{a,b}$	88.25	0.517	0.355	0.191
20	Yes	Noncha	$17.97^{a,b}$	34.10	22.53	$0.778^{b,c,d}$	1.49	0.977	33.75	84.63^{a}	91.63	0.546	0.415	0.247
20	Yes	Cha	15.70^{b}	25.81	16.64	$0.703^{c,d}$	1.16	0.743	31.63	74.63^{b}	82.44	0.502	0.351	0.203
SEM			0.889	1.388	1.063	0.0394	0.068	0.0501	1.040	2.909	2.377	0.0243	0.0204	0.0115
P-value														
Riboflavin			0.289	0.794	0.151	0.034	0.336	0.019	0.044	0.649	0.179	0.499	0.951	0.430
Bacillus			0.345	0.347	0.868	0.082	0.998	0.640	0.641	0.085	0.417	0.438	0.631	0.906
Cocci.			0.725	0.005	<0.0001	0.056	0.243	0.0006	0.379	0.785	0.063	0.794	0.012	<0.0001

Riboflavin × Bacillus[1]	0.852	0.770	0.150	0.552	0.992	0.391	0.656	0.679	0.753	0.958	0.615	0.115
Riboflavin × Cocci.	0.309	0.028	0.545	0.314	0.072	0.608	0.438	0.075	0.360	0.325	0.482	0.625
Bacillus × Cocci.	0.683	0.390	0.779	0.949	0.260	0.507	0.986	0.274	0.023	0.663	0.953	0.328
Riboflavin × Bacillus × Cocci.	0.027	0.066	0.124	0.023	0.082	0.149	0.170	0.050	0.101	0.133	0.482	0.307

[a–c]Means in a column not sharing a common superscript were different ($P < 0.05$).

Abbreviations: Cha, Challenged with cocci.; Duo, duodenum; Ile, ileum; Jej, jejunum; Noncha, Nonchallenged.

[1]Means of nonsignificant interaction is not listed.

challenge increased relative weight of proventriculus when birds were fed riboflavin at 6.6 ppm. On coccidial challenged birds, riboflavin at 20 ppm lowered relative proventriculus weight compared with birds fed riboflavin at 6.6 ppm. On coccidial challenged birds, supplementation of riboflavin at 20 ppm lowered relative liver weights compared with birds supplemented with 0.75 ppm riboflavin (Table 9).

Intestine on Day 36 The increase of duodenum and jejunum weights on day 27 by coccidia challenge was lost on day 36. However, coccidial challenge lowered relative and absolute weight of ileum ($P = 0.0006$; $P < 0.0001$, respectively) and relative weight by length of ileum and jejunum ($P < 0.0001$; $P = 0.012$, respectively). Riboflavin supplemented at 20 ppm reduced relative ileum weight ($P = 0.019$), and riboflavin at 20 ppm reduced duodenum length in comparison to riboflavin at 0.75 ppm ($P = 0.044$). Absolute jejunum weight was affected interactively by riboflavin and coccidial challenge. When birds were fed 20 ppm of riboflavin, coccidial challenge reduced jejunum weight ($P = 0.028$), and challenged birds fed 20 ppm riboflavin had lower jejunum weight than birds fed riboflavin at 0.75 ppm ($P = 0.028$). When birds were fed *B. subtilis*, coccidial challenge reduced the ileum length ($P = 0.023$; Table 10).

The 3-way interaction of riboflavin, *B. subtilis*, and coccidial challenge was observed in the absolute and relative weight of the duodenum ($P = 0.027$ and $P = 0.023$; Table 10). The absolute duodenum weight was higher on coccidial challenged birds when fed the diet with riboflavin at 0.75 ppm and *B. subtilis*. Birds fed riboflavin at 0.75 ppm had higher duodenum weight than birds fed riboflavin at 20 ppm, when birds were challenged with coccidial and fed the diet containing *B. subtilis*. When birds were challenged with coccidial and fed *B. subtilis*, riboflavin at 20 ppm reduced relative duodenum weight in comparison to that of birds fed riboflavin at 0.75 ppm ($P = 0.023$). Similar to relative and absolute duodenum weight, 3-way interaction affected jejunum length ($P = 0.050$). When birds were fed riboflavin at 6.6 ppm and diet with *B. subtilis*, coccidial challenge increased jejunum length, but coccidial challenge reduced jejunum length when birds were fed riboflavin at 20 ppm and diet with *B. subtilis*. *B. subtilis* supplementation lowered jejunum length when birds were fed riboflavin at 0.75 ppm and were not challenged with coccidial (Table 10).

Gut Histomorphology

As shown in Table 11, on day 27 dietary supplementation of *B. subtilis* did not affect the VH, CD, VW, and villus height to crypt depth ratio (**VH:CD**) of all 3 parts of intestine duodenum, jejunum, and ileum. On the other hand, coccidial challenge increased CD ($P = 0.001$) and reduced VH:CD ratio ($P = 0.003$) of duodenum. Similarly, to the duodenum, coccidial challenge increased CD ($P = 0.003$) and reduced VH:CD ($P = 0.041$) of ileum; interestingly, the VH of the ileum was higher in

Table 11. The small intestine histology on day 27 and day 36 of Ross 708 male broilers fed *Bacillus subtilis* and challenged with coccidiosis.

		Day 27												Day 36			
		Duodenum				Jejunum				Ileum				Jejunum			
Bacillus	Cocci	VH (µm)	CD (µm)	VW (µm)	VH:CD	VH (µm)	CD (µm)	VW (µm)	VH:CD	VH (µm)	CD (µm)	VW (µm)	VH:CD	VH (µm)	CD (µm)	VW (µm)	VH:CD
No		2,027	362	251	6.15	1,439	315	231	4.77	741	245	205	3.19	1,250	354	223	3.70
Yes		2,054	331	236	6.54	1,447	334	232	4.65	714	216	199	3.45	1,348	322	226	4.50
SEM		62.5	19.6	13.2	0.458	99.7	21.4	12.7	0.474	28.4	15.0	10.6	0.170	82.2	27.7	14.9	0.400
	Noncha	2,056	291^{b}	236	7.42^{a}	1,366	309	239	4.81	674^{b}	196^{b}	208	3.57^{a}	1,404	338	231	4.43
	Cha	2,024	402^{a}	251	5.27^{b}	1,520	340	224	4.60	781^{a}	265^{a}	196	3.06^{b}	1,194	338	219	3.77
	SEM	62.5	19.6	13.2	0.458	99.7	21.4	12.7	0.474	28.4	15.0	10.6	0.170	82.3	27.7	14.9	0.400
P-value																	
Bacillus		0.765	0.217	0.419	0.553	0.953	0.546	0.985	0.854	0.499	0.182	0.720	0.282	0.409	0.426	0.875	0.171
Cocci.		0.718	0.001	0.416	0.003	0.285	0.321	0.427	0.757	0.013	0.003	0.443	0.041	0.085	0.997	0.576	0.259
Bacillus × Cocci.[1]		0.247	0.859	0.971	0.811	0.343	0.380	0.300	0.865	0.780	0.651	0.331	0.417	0.107	0.328	0.581	0.667

[a-c] Means in a column not sharing a common superscript were different ($P < 0.05$).

Abbreviations: Cha, Challenged with cocci.; CD, Crypt depth; Noncha, Nonchallenged; VH, Villus height; VW, Villus width; VH:CD, Villus height to Crypt depth ratio

[1] Means of nonsignificant interaction is not listed.

Table 12. Intestinal lesion score due to *Eimeria acervulina* on day 27 Ross 708 male broilers fed riboflavin and *Bacillus subtilis* diet and challenged with coccidiosis.

			Duodenum				Jejunum			
Riboflavin	Bacillus	Cocci.	0	1	2	3	0	1	2	3
0.75			46.9	21.9	25.0	6.3	50.0	18.8	18.8	12.5
6.6			37.5	31.3	25.0	6.3	40.6	31.3	18.8	9.4
20			50.0	18.8	18.8	12.5	62.5	9.4	18.8	9.4
SEM[1]			7.64	7.73	6.79	4.23	7.62	7.09	6.62	4.60
	No		47.9	27.1	22.9	2.1	54.2	25.0	16.7	4.2^{b}
	Yes		41.7	20.8	22.9	14.6	47.9	14.6	20.8	16.7^{a}
	SEM[1]		6.24	6.31	5.55	3.46	6.22	5.78	5.40	3.75
		Noncha	72.9^{a}	25.0	2.08^{b}	0.00	79.2^{a}	18.8	2.1^{b}	0.0^{b}
		Cha	16.7^{b}	22.9	43.8^{a}	16.7	22.9^{b}	20.8	35.4^{a}	20.8^{a}
		SEM[1]	6.24	6.31	5.55	3.5	6.22	5.79	5.40	3.75
Bacillus × Cocci.										
	No	Noncha	75.0	25.0	0.0	0.0^{b}	79.2	20.8	0.0	0.0^{b}
	No	Cha	20.8	29.2	45.8	4.2^{b}	29.2	29.2	33.3	8.3^{b}
	Yes	Noncha	70.8	25.0	4.2	0.0^{b}	79.2	16.7	4.17	0.0^{b}
	Yes	Cha	12.5	16.7	41.7	29.2^{a}	16.7	12.5	37.5	33.3^{a}
		SEM[1]	8.82	8.92	7.85	4.89	8.80	8.19	7.64	5.31
P-value										
Riboflavin			0.488	0.496	0.755	0.487	0.133	0.098	1.000	0.858
Bacillus			0.481	0.486	1.000	0.013	0.480	0.207	0.587	0.021
Cocci.			<0.0001	0.816	<0.0001	0.001	<0.0001	0.800	<0.0001	0.0002
Riboflavin × Bacillus[2]			0.316	0.850	0.433	0.120	0.314	0.435	0.310	0.632
Riboflavin × Cocci[2].			0.316	0.103	0.329	0.487	0.846	0.298	0.744	0.858
Bacillus × Cocci.			0.814	0.486	0.597	0.013	0.480	0.448	1.000	0.021
Riboflavin × Bacillus × Cocci.[2]			0.946	0.324	0.755	0.120	0.846	0.824	0.414	0.632

[a-c]Means in a column not sharing a common superscript were different ($P < 0.05$).
Abbreviations: Cha, Challenged with cocci.; Noncha, Nonchallenged
[1]SEM = Standard error of mean, 0 = Normal, 1 = slight, 2 = moderate, 3 = severe lesion score.
[2]Means of nonsignificant interaction is not listed.

challenged birds than that of nonchallenged birds ($P = 0.013$). On day 36, only jejunum samples were taken for the morphological analysis as the strain used for the coccidial challenge was supposed to proliferate on the middle section of the intestine. The morphological results of the jejunum were not affected by supplementation of *B. subtilis* in diet or coccidial challenge (Table 11).

Table 13. Intestinal lesion scores because of *Eimeria maxima* on day 27 Ross 708 male broilers fed riboflavin and *Bacillus subtilis* diet and challenged with coccidiosis.

Treatments			Duodenum			Jejunum				Ileum			
Riboflavin	Bacillus	Cocci.	0	1	2	0	1	2	3	0	1	2	3
0.75			81.3	12.5	6.3	53.1	21.9	15.6	9.38	75.0	15.6	6.3	3.13
6.6			87.5	12.5	0.0	59.4	21.9	15.6	3.13	68.8	25.0	6.3	0.00
20			93.8	3.13	3.1	56.3	34.4	9.38	0.00	71.9	15.6	12.5	0.00
SEM[1]			5.64	5.12	3.17	7.59	7.97	6.03	3.441	7.64	6.79	5.00	1.804
	No		87.5	8.3	4.2	56.3	27.1	12.5	4.17	77.1	12.5	8.3	2.08
	Yes		87.5	10.4	2.1	56.3	25.0	14.6	4.17	66.7	25.0	8.3	0.00
	SEM[1]		4.60	4.18	2.58	6.20	6.50	4.93	2.809	6.24	5.55	4.09	1.473
		Noncha	100.0^{a}	0.0^{b}	0.0^{b}	83.3^{a}	14.6^{b}	2.1^{b}	0.0^{b}	87.5^{a}	10.4^{b}	2.1^{b}	0.00
		Cha	75.0^{b}	18.8^{a}	6.3^{a}	29.2^{b}	37.5^{a}	25.0^{a}	8.33^{a}	56.3^{b}	27.1^{a}	14.6^{a}	2.08
		SEM[1]	4.60	4.18	2.58	6.20	6.50	4.93	2.809	6.24	5.55	4.09	1.473
P-value													
Riboflavin			0.298	0.333	0.382	0.845	0.444	0.700	0.153	0.846	0.533	0.596	0.373
Bacillus			1.000	0.726	0.570	1.000	0.821	0.766	1.000	0.241	0.115	1.000	0.320
Cocci.			0.0003	0.002	0.091	<0.0001	0.015	0.002	0.039	0.001	0.037	0.034	0.320
Riboflavin × Bacillus[2]			1.000	0.884	0.724	0.118	0.244	0.700	0.442	0.134	0.234	0.217	0.373
Riboflavin × Cocci.[2]			0.298	0.333	0.382	0.483	0.444	0.700	0.153	0.316	0.178	1.000	0.373
Bacillus × Cocci.[2]			1.000	0.726	0.570	0.345	0.821	0.372	1.000	0.814	0.292	0.473	0.320
Riboflavin × Bacillus × Cocci.[4]			1.000	0.884	0.724	0.483	0.815	1.000	0.442	0.678	0.404	0.596	0.373

[a-c]Means in a column not sharing a common superscript were different ($P < 0.05$).
Abbreviations: Cha, Challenged with cocci.; Noncha = Nonchallenged.
[1]SEM = Standard error of mean, 0 = Normal, 1 = slight, 2 = moderate, 3 = severe lesion score (duodenum do not have lesion 3).
[2]Means of nonsignificant interaction is not listed.

Table 14. Intestinal lesion scores because of *Eimeria acervulina* on day 36 Ross 708 male broilers fed riboflavin and *Bacillus subtilis* diet and challenged with coccidiosis.

			Duodenum				Jejunum			
Riboflavin	Bacillus	Cocci.	0	1	2	3	0	1	2	3
0.75			78.1	12.5	9.4	0.00	59.4	21.9	18.8	0.0
6.6			75.0	18.8	3.1	3.13	62.5	12.5	15.6	9.4
20			65.6	34.4	0.0	0.00	53.1	37.5	6.3	3.1
SEM[1]			7.09	6.80	3.44	1.804	7.24	6.80	5.95	3.54
	No		72.9	20.8	6.3	0.00	62.5	22.9	12.5	2.1
	Yes		72.9	22.9	2.1	2.08	54.2	25.0	14.6	6.3
	SEM[1]		5.79	5.55	2.81	1.473	5.91	5.55	4.86	2.89
		Noncha	52.1^{b}	37.5^{a}	8.3^{a}	2.08	29.2^{b}	39.6^{a}	25.0^{a}	6.3
		Cha	93.8^{a}	6.25^{b}	0.0^{b}	0.00	87.5^{a}	8.3^{b}	2.1^{b}	2.1
		SEM[1]	5.77	5.55	2.81	1.473	5.91	5.55	4.86	2.89
Riboflavin × Bacillus										
0.75	No		68.8	18.8	12.5	0.00	56.3	25.0	18.8	0.0^{b}
0.75	Yes		87.5	6.25	6.3	0.00	62.5	18.8	18.8	0.0^{b}
6.6	No		81.3	12.5	6.3	0.00	75.0	12.5	12.5	0.0^{b}
6.6	Yes		68.8	25.0	0.0	6.25	50.0	12.5	18.8	18.8^{a}
20	No		68.8	31.3	0.0	0.00	56.3	31.3	6.3	6.2^{a,b}
20	Yes		62.5	37.5	0.0	0.00	50.0	43.8	6.3	0.0^{b}
SEM[1]			10.02	9.61	4.87	2.552	10.24	9.61	8.41	5.00
P-value										
Riboflavin			0.435	0.070	0.153	0.373	0.649	0.037	0.308	0.169
Bacillus			1.000	0.791	0.298	0.320	0.322	0.791	0.763	0.311
Cocci			<0.0001	0.0002	0.039	0.320	<0.0001	0.0002	0.001	0.311
Riboflavin × Bacillus			0.263	0.405	0.760	0.373	0.313	0.613	0.912	0.039
Riboflavin × Cocci.[2]			0.937	0.533	0.153	0.373	0.218	0.022	0.528	0.772
Bacillus × Cocci.[2]			0.612	0.428	0.298	0.320	0.322	0.791	0.366	1.000
Riboflavin × Bacillus × Cocci.[2]			0.047	0.070	0.760	0.373	0.940	0.613	0.760	0.462

[a-c]Means in a column not sharing a common superscript were different ($P < 0.05$).

Abbreviations: Cha, Challenged with cocci.; Noncha, Nonchallenged.

[1]SEM = Standard error of mean (0 = Normal, 1 = slight, 2 = moderate, 3 = severe lesion score).

[2]Means of nonsignificant interactions are not listed.

Coccidial Lesions

Lesion Score The *Eimeria* challenge on day 14 had increased the intestinal lesion score on the specific site of their proliferation. Based on the species of *Eimeria* spp colonization, the color of lesion is determined, as *E. acervulina* forms white colonies, whereas *E. maxima* forms red colonies in the intestine.

Eimeria acervulina Lesions

Duodenum Lesion On day 27, coccidial challenge increased the percentage of moderate lesion of *E. acervulina* ($P < 0.0001$) and decreased percentage of no-lesion ($P < 0.0001$). Severe lesion was interactively affected by *B. subtilis* and coccidial challenge ($P = 0.013$), and lesion scores were higher only in birds fed *B. subtilis* and challenged with coccidiosis (Table 12). On day 36, coccidial challenge increased the no-lesion ($P < 0.0001$) and lowered slight and moderate lesions ($P = 0.0002$, $P = 0.039$, respectively; Table 14)

Jejunum Lesion On day 27, coccidial challenge had decreased percentage of nonlesion score ($P < 0.0001$), no effects on slight ($P = 0.800$), increased moderate lesion score ($P < 0.0001$). *B. subtilis* and coccidial challenge interactively affected severe lesion score ($P = 0.021$), and coccidial challenged birds fed diet with *B. subtilis* had more severe lesions than others ($P = 0.021$; Table 12). On day 36, coccidial challenge increased no-lesion ($P < 0.0001$) and lowered slight, moderate ($P = 0.0002$, $P = 0.001$, respectively). Riboflavin and coccidial challenge interactively affected severe jejunum lesions ($P = 0.039$). *B. subtilis* supplementation increased severe lesion scores on jejunum when birds were fed riboflavin at 6.6 ppm (Table 14).

Ileum Lesion As the proliferation site of the *E. acervulina* was in the upper tract, there were no effects on percentage of lesion score because of *E. acervulina* proliferation in the ileum.

Eimeria maxima Lesions

Duodenum Lesion On day 27, coccidial challenge increased slight lesion of *E. maxima* ($P = 0.002$) and lowered no-lesion ($P = 0.0003$; Table 13). There was no difference in lesions because of *E. maxima* on day 36 (Table 15)

Jejunum Lesion On day 27, coccidial challenge lowered no-lesion percentage ($P < 0.0001$) and increased slight, moderate, and severe lesions ($P = 0.015$, $P = 0.002$, and $P = 0.039$, respectively) produced by *E. maxima* (Table 13). There was no difference in *E. maxima* lesion scores because of coccidial challenge on day 36. Although, there was interaction between riboflavin doses and coccidial challenge ($P^{2} = 0.034$; Table 15).

Ileum Lesion Ileum was the specific site for the proliferation of the *E. maxima*. On day 27, coccidial challenge

Table 15. Intestinal lesion scores because of *Eimeria maxima* on day 36 Ross 708 male broilers of fed riboflavin and *Bacillus subtilis* diet and challenged with coccidiosis.

			Duodenum				Jejunum				Ileum		
Riboflavin	Bacillus	Cocci.	0	1	2	3	0	1	2	3	0	1	2
0.75			78.1	18.8	3.13	0.00	31.3	34.4	18.8	15.63	65.6	28.1	6.25
6.6			71.9	21.9	6.25	3.13	43.8	25.0	21.9	9.38	81.3	15.6	3.13
20			71.9	28.1	0.00	0.00	50.0	31.3	15.6	3.13	90.6	6.25	3.13
SEM[1]			7.88	7.55	3.165	1.804	8.43	8.22	6.304	5.091	7.24	6.66	3.585
	No		68.8	27.1	4.17	0.00	39.6	33.3	16.7	10.42	83.3	12.5	4.17
	Yes		79.2	18.8	2.08	2.08	43.8	27.1	20.8	8.33	75.0	20.8	4.17
	SEM[1]		6.43	6.16	2.584	1.473	6.88	6.71	5.15	4.157	5.91	5.44	2.927
		Noncha	72.9	20.8	6.25	2.08	33.3	33.3	22.9	10.42	79.2	16.7	4.17
		Cha	75.0	25.0	0.00	0.00	50.0	27.1	14.6	8.33	79.2	16.7	4.17
		SEM[1]	6.43	6.16	2.584	1.473	6.88	6.71	5.14	4.157	5.91	5.44	2.927
P-value													
Riboflavin			0.811	0.672	0.382	0.373	0.283	0.715	0.783	0.228	0.054	0.072	0.777
Bacillus			0.256	0.342	0.570	0.320	0.670	0.512	0.569	0.724	0.322	0.282	1.000
Cocci.			0.820	0.634	0.091	0.320	0.091	0.512	0.256	0.724	1.000	1.000	1.000
Riboflavin × Bacillus[2]			0.237	0.128	0.724	0.373	0.099	0.866	0.086	0.141	0.649	0.600	0.471
Riboflavin × Cocci.[2]			0.237	0.479	0.382	0.373	0.484	0.368	0.034[3]	0.607	0.194	0.220	0.471
Bacillus × Cocci.[2]			0.820	1.000	0.570	0.320	0.670	0.128	1.000	0.080	0.322	1.000	0.048[3]
Riboflavin × Bacillus × Cocci.[2]			0.237	0.220	0.724	0.373	0.283	0.537	0.783	0.607	0.940	0.803	0.777

[a-c]Means in a column not sharing a common superscript were different ($P < 0.05$).

Abbreviations: Cha, Challenged with cocci; Noncha, Nonchallenged.

[1]SEM = Standard error of mean, 0 = Normal, 1 = slight, 2 = moderate, 3 = severe lesion score.

[2]Means of nonsignificant interactions are not listed.

[3]Fisher's LSD test was not able to separate means of challenge effects on jejunum and ileum coccidial moderate lesions (score 2) so only *P*-values were listed in the table with superscripts.

lowered no-lesion percentage ($P = 0.001$) and increased slight and moderate lesion percentage over non-challenged birds ($P = 0.037$, $P = 0.034$, respectively; Table 13). On day 36, there was no effect on lesion percentage because of coccidial challenge owing to proliferation of *E. maxima*. Although, there was interaction between *B. subtilis* and coccidial challenge ($P^3 = 0.048$; Table 15).

Relationship Between Intestinal Histomorphology, Intestinal Measurements, and Lesion Score

The VH of jejunum was positively correlated with jejunum weight ($P = 0.013$), whereas VH of ileum was negatively correlated with ileum length ($P = 0.021$) on day 27. Although the jejunum histology of day 36 was

Table 16. Partial correlation of the intestinal histomorphology, intestinal measurement, and intestinal lesion score produced by *Eimeria acervulina* and *Eimeria maxima* on Ross 708 male broilers sampled on day 27 and day 36.

	Villus height		Crypt depth		Villus width		VH:CD[1]	
	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value	Coefficients	*P*-value
Duodenum[2]								
Weight (g)	0.0366	0.072	0.209	0.315	0.184	0.377	−0.099	0.637
Length(cm)	0.087	0.677	0.200	0.336	0.116	0.579	−0.171	0.413
Jejunum[2]								
Weight (g)	0.509	0.013	0.2808	0.194	0.239	0.270	0.198	0.364
Length(cm)	−0.001	0.965	0.122	0.577	−0.085	0.699	−0.162	0.457
Ileum[2]								
Weight (g)	0.154	0.424	0.155	0.420	0.151	0.433	−0.209	0.275
Length(cm)	−0.426	0.021	−0.228	0.233	−0.0370	0.848	0.046	0.81
Jejunum[3]								
Weight (g)	0.405	0.067	0.341	0.130	0.153	0.506	0.086	0.709
Length(cm)	−0.401	0.071	0.159	0.491	−0.613	0.0031	−0.439	0.046
Duodenum[2]								
E. acervulina	−0.186	0.343	0.512	0.005	0.072	0.715	−0.492	0.008
Jejunum[2]								
E. acervulina	−0.086	0.780	0.358	0.230	−0.302	0.316	0.297	0.325
E. maxima	0.198	0.517	−0.242	0.426	−0.097	0.753	0.464	0.110
Ileum[2]								
E. maxima	−0.127	0.497	−0.001	0.998	0.427	0.017	−0.042	0.821
Jejunum[3]								
E. acervulina	0.288	0.183	−0.121	0.582	0.065	0.769	0.147	0.504
E. maxima	0.148	0.502	−0.328	0.126	0.273	0.208	0.378	0.076

[1]VH:CD = Villus height: Crypt depth.

[2]Duodenum, Jejunum, Ileum = Intestinal measurements of day 27.

[3]Jejunum = Intestinal measurements of day 36.

Table 17. Partial correlation of the intestinal measurement and intestinal lesion score produced by *Eimeria acervulina* and *Eimeria maxima* on Ross 708 male broilers sampled on day 27 and day 36.

	Eimeria acervulina		Eimeria maxima	
	Coefficient	*P*-value	Coefficient	*P*-value
Duodenum[1]				
Weight (g)	0.050	0.639	0.027	0.800
Length(cm)	0.167	0.117	0.166	0.121
Jejunum[1]				
Weight (g)	0.304	0.004	0.335	0.001
Length(cm)	0.259	0.015	0.181	0.091
Ileum[1]				
Weight (g)	-	-	0.233	0.026
Length(cm)	-	-	0.113	0.288
Duodenum[2]				
Weight (g)	0.022	0.839	−0.021	0.841
Length(cm)	−0.144	0.164	0.0135	0.897
Jejunum[2]				
Weight (g)	0.170	0.103	−0.031	0.765
Length(cm)	−0.088	0.402	−0.0775	0.460
Ileum[2]				
Weight (g)	-	-	0.121	0.245
Length(cm)	-	-	0.056	0.592

[1] Duodenum, Jejunum, Ileum = Intestinal measurements of day 27.
[2] Duodenum, Jejunum, Ileum = Intestinal measurements of day 36.

not affected by either coccidial challenge or supplementation of *B. subtilis*, jejunum VW and VH:CD were negatively correlated with jejunum length ($P = 0.031$ and $P = 0.046$, respectively). Duodenum CD on day 27 was positively correlated and VH:CD ratio was negatively correlated with coccidial lesion produced by *E. acervulina* ($P = 0.005$; $P = 0.008$, respectively). Ileum VW of day 27 was positively associated with coccidial lesion produced by *E. acervulina* ($P = 0.017$; Table 16). The jejunum weight was positively correlated with coccidial lesion produce by both *E. acervulina* and *E. maxima* ($P = 0.004$ and $P = 0.001$, respectively), whereas jejunum length was positively correlated only with coccidial lesion produced by *E. acervulina* ($P = 0.015$). Ileum weight was positively correlated with coccidial lesion produced by *E. maxima* ($P = 0.026$; Table 17).

DISCUSSION

Immune Organs (Spleen and Bursa)

Spleen, bursa, and thymus are the hub to produce immune cells where immune cell production and maturation occurs effectively in healthy animals in comparison to that of diseased or immune-compromised animals. Increased weight of immune organs can be correlated with increased production of immune cells and better immunity in birds (Teo and Tan 2007; Sikandar et al., 2017).

Coccidial challenge increased relative weight of spleen; however, the absolute weight of spleen was not affected on day 27. One possibility for the increase in relative weight was decreased BW because the relative weight was ratio of absolute organ weight and BW. Decrease in BW because of coccidial challenge may have influenced the ratio of organ weight and BW, which leads to reduction of relative spleen weight. Another possibility was inflammation due to infection. The increase in the relative weight of spleen may be because of increased inflammation and increased secretion of inflammatory cytokines (Tadayon et al., 2016). In previous studies, coccidial challenge increased lysis of red blood cells because of infection (Herrick et al., 1936; Natt and Herrick, 1955). The increased red blood cell lysis increased the workload of the spleen, leading to hypertrophy of the spleen (Pivkin et al., 2016; Li et al., 2018). The increased activity of the spleen during infection and process of recovery increases workload of the organs, which ultimately leads to hypertrophy (Panda and Combs, 1964).

Bursa of Fabricius is a vital lymphoid organ which is involved in differentiation of B-lymphocytes and immunity of birds (Schat and Skinner, 2013). Although various modern technology has been used for assessing immune status of the bird, lymphoid organ weight is still the important marker to reflect immune status, as heavier lymphoid organs have a higher ability to produce immune cells (Heckert et al., 2002). The immune status of the animals can be predicted using the relative weight of lymphoid organs (Abdel-Fattah et al., 2008). Weights of lymphoid organs is reduced in immune-compromised animals, which indicates that there is relationship between lymphoid organ weight and production of immune cells (Rose and Hesketh, 1979; Fan et al., 2013). In this experiment, the coccidial challenge reduced the absolute weight of the bursa on day 27; however, the relative bursal weight was not affected by coccidial challenge. Bursa and BW are positively associated with each other (Alloui et al., 2005; Cazaban et al., 2015), which indicates that the reduction of absolute bursal weight in this experiment may be because of reduction in BW of sampled birds because of coccidial challenge. In our study, the relative weight of bursa to BW was 0.167% in challenged and 0.181% in nonchallenged birds at day 27, which indicates there may have been compromised immune function of birds because of coccidial challenge which was not alleviated by any of the dietary supplementation strategies. Although the exact mechanism of coccidial challenge reducing lymphoid organ's weight is unknown, a possible explanation may be because of necrosis occurring in the lymphoid organs (Awais et al., 2013) and atrophy of the lymphoid organs (Palo, 1987). Supplementation of riboflavin and *B. subtilis* was expected to reduce inflammation provoked by the coccidial challenge, help to enhance antioxidant capacity of the birds, and help proper development of immune organs. In a companion study, dietary *B. subtilis* supplementation reduced Ross 708 male broiler mortality from day 35 to 41 (Poudel and Zhai, 2019). However, supplementation of riboflavin and *Bacillus subtills* did not accelerate the bursa recovery from the coccidial challenge.

Liver

The role of the liver and pancreas for digestion of feed material is crucial. In broilers, the role of the liver is multifunctional as it is involved in synthesis (bile salt), metabolism (lipid, carbohydrate, and protein), excretion, and detoxification processes (Zaefarian et al., 2019). Birds fed diets with *B. subtilis* had reduced absolute weight of liver; however, BW and relative liver weight to BW was not affected on day 27. In previous research, *B. subtilis* reduced hepatic lipid content in Zebrafish (Falcinelli et al., 2015) and *Lactobacillus rhamnosus* PL60 lowered hepatic steatosis in mice (Lee et al., 2006). From these results, it can be inferred that *B. subtilis* can lower fat accumulation in the liver and reduce liver weight. The result was consistent with Molnar et al. (2011) who reported decrease in liver weight while supplementing *B. subtilis* at 3.635×10^{11} CFU/kg of finished feed.

Pancreas

The relative pancreas weight was higher in birds fed diets with *B. subtilis*, when riboflavin was supplemented at 6.6 ppm on day 13. The exact mechanism of how the supplementation of probiotics increases the pancreas weight is still unknown. This result is in corroboration with results of Olnood et al. (2015) who reported increased weight of pancreas while feeding probiotic *Lactobacillus johnsonii* to broilers. Sugiharto et al. (2018) also reported that feeding of probiotics consisting of multiple strains of *Bacillus* spp tends ($P = 0.07$) to increase pancreas weight. Increase in the weight of the pancreas can be correlated with increase in the production of pancreatic enzymes like protease and amylase because of supplementation of *B. subtilis* (Wang and Gu, 2010), but increased weight of pancreas was lost on the next phases of the growth.

Proventriculus

The proventriculus is a glandular stomach which secretes hydrochloric acid and pepsinogen, whereas the gizzard is the muscular part which has an important function of grinding the feed material. For effective digestion of feed material, the role of the proventriculus and gizzard is very important. Coccidial challenge increased the relative weight of the proventriculus on day 27. This may be because of the inflammation caused by the *Eimeria* challenge, but the exact mechanism of how the *Eimeria* challenge caused the increase in weight of the proventriculus is still unknown.

Small Intestine

Coccidial infection increased relative and absolute weights of the duodenum, jejunum, and ileum, and weight per cm length of ileum on day 27, 14-day postinfection. The increase in the relative and absolute weight of intestine may be because of the increase in the synthesis of the mucosa (Bozkurt et al., 2014). Quintana-Hayashi et al. (2018) reported that the co-challenge of *Eimeria* spp and *Clostridium* spp in broilers resulted in increase of up to 54% in MUC5AC mRNA level in comparison to nonchallenged birds. MUC5AC production generally increases during severe infection and protects mucosa from infection (Perez-Vilar et al., 2004; Quintana-Hayashi et al., 2015). In another study, birds challenged with coccidia increased mucus thickness in comparison to unchallenged birds even though challenged birds were fed an antibiotic Salinomycin (Nabian et al., 2018). Another reason for the increase in the weight of the parts of the intestine may be because of inflammation caused by proliferation of *Eimeria* spp in the epithelial cells which caused accumulation of fluid in the epithelial lining (Friend and Stockdale, 1980). After 21 d postchallenge, that is, on day 36, the birds may have recovered as the inflammation caused by proliferation of *Eimeria* and damage caused by multiplication of the *Eimeria* was lower, and the fluctuation in relative weight of the duodenum and jejunum was lost. However, the relative and absolute weight of the ileum and relative weight per cm of the ileum and jejunum was higher in nonchallenged birds. The inability of the intestine to develop properly due to *Eimeria* infection may be the reason behind the reduction of relative and absolute weight of the ileum even after 21 d postinfection.

Along with an increase of the relative intestinal weight, *Eimeria* challenge also increased the length of duodenum and jejunum on day 27. Shorter intestinal length can relate to efficient absorption of nutrients, as turkey poults fed diets with semipurified lectin had shorter intestinal length and better feed efficiency (Fasina et al., 2004, 2006), and intestinal length was reduced in birds fed antimicrobials (Miles et al., 2006; Wang et al., 2016). Intestinal length may have increased to compensate for reduction of intestinal nutrient absorption because of coccidial challenge.

In broilers, *Eimeria* spp proliferate in a specific region of intestine. Depending on the magnitude of infection, the damage to epithelial of intestine varies. If magnitude of infection is high, it may lead to mortality (Chapman 2014; Chapman et al., 2016). Among the oocysts which were orally gavaged on day 14, *E. acervulina* and *E. mivati* proliferate in the upper gastrointestinal tract, primarily in the duodenum. *E. maxima* proliferates especially in the jejunum and upper part of ileum, and *E. tenella* proliferates in the ceca (McDougald and Fitz-coy, 2013). *Eimeria* proliferation causes injury and infection in the intestine, which induces increased division of stem cells to repair the damaged epithelial cells. Damage increases the weight of duodenum, jejunum, and ileum because of inflammation of the mucosal wall and concurrent bacterial infection followed by damage in intestine (Williams, 2002, 2005).

Histomorphology of Small Intestine

The structural changes in the small intestine because of coccidial challenge can disclose important information about intestinal damage caused by coccidial challenge.

Increased villus height of the small intestine is often connected with increased growth performance of broiler. In this study, coccidial challenge increased CD and reduced ratio of VH:CD of duodenum and ileum on day 27. The results were similar to previous studies, in which birds challenged with *E. acervulina* had longer CD and lower VH:CD ratio (Sun et al., 2016; Oikeh et al., 2019). The increased CD due to coccidial challenge may be because of increased mitotic activity in crypts to maintain villus height damaged by proliferation of coccidiosis. Short CD is associated with lower epithelial cell turnover in the gut, which means energy required for proliferation of epithelial cells of CD can be preserved. The increase in VH:CD ratio indicates the decrease in the turnover ratio of intestinal mucosa. In this study, coccidial challenge reduced VH:CD in comparison to that of nonchallenged birds, which indicates that there was higher intestinal mucosal turnover in challenged birds. Higher turnover ratio in intestinal epithelium means higher energy required to maintain the gut, which can result in reduction in growth efficiency of animals. Interestingly in this study, coccidial challenge increased the VH of the ileum, which is inconsistent with that of the various previous studies which found that the challenge had either no effect or reduced villus height of ileum (Calik et al., 2019; Oikeh et al., 2019). Supplementation of *B. subtilis* and increased doses of riboflavin did not reduce inflammation of intestine and enhanced speedy recovery from the damage caused by coccidial challenge on intestinal epithelium as indicated by increased intestinal weight and increased CD. The detrimental effects of coccidial challenge were diminished over time.

Lesion Scores

In this study, we grew the birds on commercial farm litter to mimic commercial broiler production settings. Birds were orally gavaged with 20 × dose of commercial coccidiosis vaccine to experimentally induce coccidiosis to challenged group of birds on day 14. Lesion scoring was conducted to validate coccidial challenge success. Oral gavage of coccidial vaccine increased *E. acervulina* lesion in duodenum and jejunum on day 27. On day 36, challenged birds had lower percentage of slight and moderate lesion than nonchallenged birds, but no difference in severe lesion condition due to *E. acervulina* and any of the lesions condition due to *E. maxima*. This indicates that those challenged birds developed coccidiosis much earlier and then developed resistance by day 36 and their lesions started to reduce. Whereas those birds which were not challenged may have received lower concentration of oocyst naturally and develops slight and moderate lesion.

Relationship Between Intestinal Histomorphology, Intestinal Measurements, and Lesion Score

The length of ileum and jejunum was negatively correlated with VH and VH:CD, respectively. This indicates that higher VH and VH:CD are associated with shorter intestine length. The structure of the intestine is very important to reveal the nutritional absorption capacity of intestine. Higher VH and VH:CD of intestine is often connected with better intestinal health and better nutritional absorption capacity of intestine (Onderci et al., 2006). From these results, it can be concluded that when intestinal health condition is suboptimal, intestinal length may increase. This result is further supported by our result as there was a positive correlation between the jejunum length and coccidial lesions, which indicates that increase in severity of coccidial challenge can further increase the length of intestine.

CONCLUSION

In this study, coccidial challenge negatively impacted Ross 708 male broiler immune organ development, small intestine morphology, and histomorphology at an early age. Supplementation of riboflavin and *B. subtilis* at tested level under current experimental conditions did not benefit birds to recover from coccidial challenge. However, effects of the coccidial challenge subside along with the age of birds as the effects on internal organs because of coccidial challenge were lost along with an increase in age of birds.

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DISCLOSURES

The authors declare no conflicts of interest.

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