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Riboflavin and *Bacillus subtilis* effects on growth performance and woody-breast of Ross 708 broilers with or without *Eimeria* spp. challenge

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Abstract

This study was conducted to assess the effects of the dietary supplementation of riboflavin (as a bile salt hydrolase [BSH] inhibitor) and *Bacillus subtilis* on growth performance and woody breast of male broilers challenged with *Eimeria* spp. Intestinal bacteria, including supplemented probiotics, can produce BSH enzymes that deconjugate conjugated bile salts and reduce fat digestion. A 3 × 2 × 2 (riboflavin × *Bacillus subtilis* × *Eimeria* spp. challenge) factorial arrangement of treatments in randomized complete block design was used. On d 14, birds were gavaged with 20× doses of commercial cocci vaccine (Coccivac^{R}-B52, Merck Animal Health, Omaha, NE). Dietary treatment of riboflavin and *B. subtilis* did not affect body weight (BW), body weight gain (BWG), and feed conversion (FCR) d 0 to 14 and overall d 0 to 41. *Eimeria* spp challenge reduced BWG, feed intake (FI), and increased FCR between d 14 to 28, but increased BWG and lowered FCR between d 28 to 35. There were no effects of the *Eimeria* spp. challenge on the overall d 0 to 41 FCR and FI, but BWG was reduced. *Eimeria* spp. challenge increased the abdominal fat pad weight and slight woody breast incidences on processed birds on d 42. Dietary inclusion of *B. subtilis* and riboflavin at tested levels did not help birds to mitigate the negative impact of *Eimeria* spp. challenge to enhance the growth performance.

Keywords: Riboflavin, *Bacillus subtilis*, Coccidiosis, Growth performance

INTRODUCTION

Antibiotics have been used to control enteric diseases and promote growth in broilers. However, concurrent use of antibiotics to control the sub-clinical infection and enhance growth in food animals has been associated with the emergence of antibiotic resistance [1]. In order to reduce antibiotic resistance, European Union banned use of antibiotic growth promoters (AGPs) in broiler diets from 2006 [2], and FDA announced the voluntary withdrawal in the USA [3]. Although the removal of AGPs from animals' diet was voluntary in the USA, the intense market competition and increasing

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Availability of data and material
Upon a reasonable request, the datasets of this study can be available from the corresponding author or first author.

Authors' contributions
Conceptualization: Lin J, Zhai W.
Data curation: Poudel S, Zhai W, Zhang L.
Formal analysis: Poudel S, Zhai W.
Methodology: Poudel S, Zhai W.
Validation: Zhai W.
Investigation: Zhai W, Poudel S.
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Ethics approval and consent to participate
The Institutional Animal Care and Use Committee of Mississippi State University approved the bird's husbandry and handling methods used in this study with protocol number 16-542.

demand by consumers forced the industry to shift broiler production from conventional to antibiotic-free broiler production. The shift of production system caused a reduction in broiler production [4]. The withdrawal of AGPs from feed has increased the risk of enteric diseases, causing significant economic losses to the broiler industry [5–7]. Among the various pathogens to cause enteric disease, coccidiosis, caused by a protozoan parasite of genus *Eimeria*, is the major issue. *Eimeria* spp. not only causes intestinal damage but also provoke growth of other pathogens, such as *Clostridium perfringens* [8]. Coccidiosis causes intestinal lesions, bloody diarrhea, interruption of the digestive process, impaired nutrient absorption, and increased mortality, ultimately reducing growth rate, decreasing feed digestion, and increasing feed conversion ratio (FCR) [8–10]. The economic losses caused by coccidiosis was estimated a global cost of $ 12.10 billion in 2016 [11]. Among the total, estimated economic losses caused by sub-clinical coccidiosis-related poor feed conversion was 65.2% of losses [12]. In previous studies, the sub-therapeutic doses of antibiotics have increased growth, enhanced intestinal morphology, modulated microbial diversity, and reduced pathogenic bacterial numbers in the intestine [13,14]. Along with this, extensive gut microbiome studies have shown that AGPs usage significantly reduces microbial populations that can produce powerful bile salt hydrolase (BSH) in the intestine [15]. Thus, inhibition of BSH activity, the gateway enzyme controlling downstream microbial and host bile acid metabolism in the intestine, is a promising approach to enhance host lipid metabolism and body weight gain (BWG) in food animals [15,16]. Recently, we have identified several novel BSH inhibitors (e.g., riboflavin) [17] and evaluated *in vivo* efficacy of the BSH inhibitors for modulating host bile profile and physiology in broilers [18].

In addition to the recently discovered BSH inhibitors with potential as an alternative to AGPs, probiotics have been considered as a feasible and attractive non-antibiotic approach for poultry production [19]. *Bacillus subtilis* (*B. subtilis*) is a common probiotic used in the poultry industry. *Bacillus* is a spore-forming Gram-positive bacterium that can withstand the feed pelleting process and can recover to an active functional vegetative cell in gastrointestinal tract of poultry [20]. In previous studies, the inclusion of *B. subtilis* in the feed improved BWG and FCR in the broiler [21–24]. The *Bacillus*-based diet helped increase villus height to crypt depth [25]. However, supplementation of *Bacillus* is unable to change cecal microbial composition of *Lactobacillus* spp. [23,24], *Escherichia coli* [21,23], or *Clostridium* spp. [23] between birds fed control diet and birds fed diet supplemented with *Bacillus*. The supplementation of *Bacillus* did not produce a consistent increase in BWG and FCR from d 0 to 54 when birds were challenged with coccidiosis [26]. The reason behind the inconsistency may be the production of BSH enzymes by probiotics as well as other intestinal microflora, which could reduce host lipid metabolism and growth performance. In particular, Song et al. [27] recently reported that *Bacillus* has the highest number of strains with BSH paralogs based on exhaustive analysis of the worldwide human gut microbiome.

Riboflavin (7, 8 dimethyl-10-ribityl-isoalloxazine) is an essential water-soluble vitamin required for the utilization of dietary protein and energy [28]. Flavin mononucleotide and flavin adenine dinucleotide is the coenzyme derivatives of riboflavin which participate in various redox reactions [29]. Riboflavin is not only essential for the enzymatic reaction for nutritional utilization, but it also has an antioxidant protection function [30]. Increased oxidative stress can increase woody breast (WB) [31]. WB is a meat quality problem, which makes the breast fillet hard and pale in color when severely affected. It is also reported that a riboflavin deficient diet reduces the superoxide dismutase (SOD) and glutathione and increases malondialdehyde and lipid peroxidation [32,33]. So, riboflavin can be helpful in reducing oxidative stress in birds. Recently, riboflavin also has been characterized as a potent BSH inhibitor with potential as a novel alternative to AGPs to improve growth performance and feed efficiency in food animals [16–18,34].

In this experiment, we hypothesized that the dietary inclusion of *B. subtilis*, along with the higher

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doses of riboflavin, could enhance the growth performance and reduce WB incidence in broilers experimentally induced with coccidiosis. Therefore, the objective was to determine the effects of supplementation of *B. subtilis* and riboflavin on broilers challenged with coccidiosis pathogen on growth performance, processing yield, and WB condition.

MATERIALS AND METHODS

Bird management

The Institutional Animal Care and Use Committee of Mississippi State University approved the bird's husbandry and handling methods used in this study with protocol number 16-542. The experiment was conducted in an environmentally controlled house located at Mississippi State University, Poultry Research Unit. The day-old chicks were purchased from a commercial hatchery and were vaccinated against Marek's disease, Newcastle disease, and Infectious Bronchitis at the hatchery. The chicks did not receive coccidiosis vaccination. Chicks were feather-sexed upon arrival. A total of 1,248-day-old Ross 708 male broiler chicks were weighed and randomly allocated to 96-floor pens (13 birds/pen) with a stocking density of 0.084 m^2/bird. Each pen was equipped with a commercial tube feeder and a nipple drinker line consisting of 3 nipple drinkers per pen. The temperature was adjusted according to the commercial temperature program of Aviagen, which was adjusted to the age of the birds. Twenty-four hours light was provided for the first 24 hours after arrival, then a 23L:1D photoperiod was provided from d 1 to 7 and 20L:4D photoperiod was provided from d 8 to 41. The birds received crumbled starter feed from d 0 to 14 and pelleted grower and finisher feed from d 14 to 28 and d 28 to 41, respectively.

Diet formulation

Corn-soybean meal-based basal starter, grower, and finisher diets were formulated according to the nutrient recommendation of Ross × Ross 708, except for riboflavin [35]. Before formulating the diets, all major raw ingredients were analyzed using near-infrared spectroscopy (NIR system, model: XDS-XM-1100 series, FOSS, Hilleröd, Sweden), and a commercial database (Precise Nutrition Evaluation, Adisseo, Alpharetta, GA, USA) for determination of proximate analysis, digestible amino acids, and metabolizable energy values. The feed was formulated using least-cost software from Creative Formulation Concepts, Educational version LLC (Pierz, MN, USA). Except for riboflavin and *B. subtilis*, all the raw ingredients were first mixed in a vertical screw mixer. Different levels of riboflavin and *B. subtilis* were mixed according to the treatments in 25-lb mixers first and then mixed in a batch using a 2-ton capacity horizontal ribbon mixer. The diet was then pelleted, cooled in the vertical cooler, and sacked off into properly labeled bags. The starter diet was crumbled after pelleting, and grower and finisher diets were pelleted.

Experimental design and dietary treatments

Ninety-six experimental units (floor pens) were divided into 8 blocks (served as replicates) based on location in the house. Twelve different treatments were randomly assigned to the experimental unit within each block. The treatment design consisted of a three-factor 3 × 2 × 2 factorial arrangement. Three levels of riboflavin (Lutavit^R Riboflavin SG 80, BASF, Ludwigshafen, Germany) 0.75, 6.6 (recommended), and 20 ppm, were added to the basal diet (Table 1). Different doses of riboflavin for this study were chosen based on the previous dosimetric study [36]. Diet with or without *Bacillus subtilis* PB6 (CLOSTAT® Dry, Kemin Industries, Iowa, USA) at the rate of 1.1 × 10^8 CFU/kg of diet was prepared. The viable plate count was conducted as described by [37]. A selective agar Mannitol yolk polymyxin agar was used to enumerate *B. subtilis*. Actual plate count

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

Ingredients[1] (%)	Starter	Grower	Finisher
	d 0–14	d 14–28	d 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
ME (kcal/kg)	3000	3099	3196
Digestible lysine (%)	1.28	1.15	1.02
Digestible methionine (%)	0.71	0.64	0.59
Digestible total sulfer amino acid (%)	0.95	0.87	0.80
Digestible threonine (%)	0.86	0.77	0.68
Riboflavin (ppm)	1.477	1.433	1.344
Choline chloride (ppm)	771	725.75	680.4
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 B_{12}, 0.01 mg; folic acid, 0.6 μg; choline, 379 mg; D-pantothenic acid, 8.8 mg; riboflavin, 5.0 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 replacement of sand on diet without these additives.

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

verified viable 4.1 × 10^7–1.5 × 10^8 CFU /kg in the finished feed. The third factor was a *Eimeria* spp. challenge to the birds. To induce coccidiosis, on d 14 the birds belonging to challenge groups were orally gavaged with the 20× doses of commercial vaccine (COCCIVAC®-B52, Merck Animal Health, Omaha, NE, USA) consisting of five different strains of *Eimeria: E. acervulina*, *E. maxima*, *E maxima MFP*, *E. mivati*, and *E. tenella* in 1ml of sterilized distilled water [38]. Birds belonging to non-challenged groups were orally gavaged with 1 mL of sterilized distilled water. In order to verify that the coccidial challenge was successful, the coccidial lesion was scored and reported in a companion study [39]. Scoring was conducted according to modified methods described by Conway and Elizabeth McKenzie [9], which is based on scores ranging from 0 (no gross lesion), 1

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(0 to 4 petechiae on serosa per cm^2), 2 (4 to 10 petechiae on serosa per cm^2), and 3 (10 to numerous petechiae on serosa per cm^2).

Growth performance

Body weight (BW) was determined on d 0, 14, 28, 35, and 41. The average BW of birds was calculated by dividing the pen weights by number of birds present in each pen. The average BW, and BWG were calculated during each period. Growth rate was calculated by dividing the BWG between intervals by average BW at the initial age. Mortality and mortality weight were recorded daily. Feed intake (FI) was measured on d 14, 28, 35, and 41 and was corrected for mortality.

Processing measurement

Five broilers per pen were randomly selected, weighed, tagged, and cooped on d 41. After 16 hours of feed withdrawal, birds were processed in a small-scale commercial-type processing plant capable of processing 1,080 birds per hour. Hot carcass and fat pad weights were measured immediately after processing. The carcasses were chilled for 4 hours and then manually deboned. The weights of the wing, thigh, drumstick, breast (pectoralis major), and tender (pectoralis minor) were recorded.

Woody breast scoring

The WB scoring was performed on birds selected for intestinal lesion scoring on d 36 (birds were euthanized using CO_2 asphyxiation) and processed birds on d 42. Palpation was done in skinless breast muscle. The WB scoring was done following the modified palpation technique rather than the visual scoring technique [40]. The scoring was done on a scale of 0 to 3; muscle with no hardness was considered as normal and scored 0; muscle with slight hardness mainly on the cranial part of breast muscle was considered as slight WB and scored 1; muscle with a moderate hardness on the cranial part and slight hardness throughout the caudal portion was scored 2, and muscle with severe hardness throughout the whole fillet was scored as WB score 3.

Blood sample collection

Blood samples were collected from the brachial vein in tubes without anticoagulants on d 35; birds selected for blood sample collection were later used for sampling and WB scoring in d 36. After allowing the blood to clot (2 h period), samples were centrifuged (Beckman Coulter, Inc., model J-6B) at 3,424 × g (3,500 rpm) for 20 minutes at 4℃ to extract serum. Collected serum samples were stored in a −80℃ freezer until further analysis was performed. The serum was used to determine serum SOD activity using Superoxide Dismutase Assay Kit (Item no. 706002, Cayman chemicals, Ann Arbor, MI, USA). Along with the collection of the serum, the blood smear was prepared at the time of blood collection and stained with the Giemsa stain. The Heterophils and lymphocytes present in the blood smear were counted to determine the heterophil: lymphocyte (H:L) ratio.

Statistical analysis

A randomized complete block design with factors of 3 × 2 × 2 (riboflavin × *B. subtilis* × coccidiosis) as the fixed effects and eight replicating blocks were used as a random effect. As for the *Eimeria* spp. challenge, the third factor of the treatment was applied only after the d 14, data collected before d 14, when the *Eimeria* spp. challenge was applied, were analyzed using a 2-way ANOVA. The data after d 14 were analyzed using 3-way ANOVA in the PROC GLM procedure of SAS version 9.4 [41]. The significance level was set at ($p \leq 0.05$). If the main effects or interaction effects among the treatments were significant, then Fisher's least significant difference test was conducted to separate

the means. The categorical data of the WB score were converted to the percentage of birds with the WB and the data were analyzed as the quantitative data for each of the categories using the Proc GLM procedure of SAS 9.4 [41]. Spearman partial correlation was used to analyze the relationship between the H:L ratio and serum SOD with WB and WB score with live BW, carcass weight (CW), and breast weight.

RESULTS

Growth performance

Body weight

Supplementation of the different doses of riboflavin and *B. subtilis* did not affect the BW on d 14 (Table 2). The *Eimeria* spp. challenge reduced BW of birds on d 28 ($p < 0.0001$), d 35 ($p < 0.0001$), and d 41 ($p = 0.004$; Table 3).

Body weight gain

The BWG was not significantly affected by dietary treatment of riboflavin and *B. subtilis* during the starter phase d 0 to 14 (Table 2). *Eimeria* spp. challenge reduced BWG on d 14 to 28 ($p < 0.0001$) but increased BWG during d 28 to 35 ($p = 0.001$). Between d 35 and d 41, *Eimeria* spp. challenge increased BWG when birds were fed riboflavin at 6.6 ppm ($p = 0.009$). However, overall BWG was lower in challenged birds during d 0 to 41 ($p = 0.004$; Table 3).

Feed intake

Dietary supplementation of riboflavin and *B. subtilis* did not affect FI on d 0 to 14 (Table 2). The *Eimeria* spp. challenge reduced the FI between d 14 and d 28 ($p < 0.0001$), after d 28, FI was not affected by *Eimeria* spp. challenge, i.e., there was no difference in FI on challenged and non-challenged birds on d 28 to 35 ($p = 0.076$), d 35 to 41 ($p = 0.304$), and overall FI d 0 to 41 ($p = 0.056$). Riboflavin supplementation did not reduce FI in other phases except for d 28 to 35 ($p = 0.020$). Riboflavin supplemented at 20 ppm of the basal diet reduced FI compared to birds

Table 2. The growth performance of male broilers fed riboflavin and *Bacillus subtilis* from d 0–14

Riboflavin	*Bacillus*	Body weight (g)		BWG (g)	FCR	FI (g)	Mortality%
		d 0	d 14	d 0–14	d 0–14	d 0–14	d 0–14
0.75		40.1	339	299	1.408	424	1.92
6.6		40.0	340	300	1.405	429	3.79
20		40.3	339	299	1.405	422	2.40
SEM[1)]		0.15	3.56	3.52	0.0072	4.84	0.850
	No	40.0	342	302	1.406	430	3.45
	Yes	40.2	337	297	1.405	420	1.96
	SEM	0.12	2.90	2.88	0.0059	3.95	0.694
p-value							
Riboflavin		0.479	0.976	0.965	0.951	0.568	0.233
Bacillus		0.202	0.256	0.230	0.951	0.063	0.106
Riboflavin × *Bacillus*[2)]		0.696	0.092	0.087	0.917	0.158	0.497

[1)]n = 8.

[2)]Means of non-significant interaction is not listed.

BWG, body weight gain; FCR, feed conversion ratio; FI, feed intake.

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Table 3. The body weight, body weight gain, feed conversion ratio and feed intake of Ross 708 male broilers fed riboflavin and *Bacillus subtilis* and challenged with coccidiosis from d 14–41

			Body weight (kg)			Body weight gain (kg)				Feed conversion ratio				Feed intake (kg)			
Riboflavin	*Bacillus*	Coccidiosis	d 28	d 35	d 41	d 14–28	d 28–35	d 35–41	d 0–41	d 14–28	d 28–35	d 35–41	d 0–41	d 14–28	d 28–35	d 35–41	d 0–41
0.75			1.34	2.05	2.73	1.003	0.702	0.685	2.69	1.510	1.725	1.789	1.611	1.527	1.208[a]	1.226	4.384
6.6			1.33	2.03	2.68	0.987	0.700	0.653	2.64	1.524	1.722	1.806	1.615	1.521	1.198[a]	1.182	4.329
20			1.34	2.03	2.71	1.003	0.688	0.678	2.67	1.518	1.699	1.775	1.603	1.520	1.165[b]	1.222	4.328
SEM[1)]			0.011	0.015	0.018	0.0097	0.0081	0.0093	0.019	0.0030	0.0110	0.0150	0.0180	0.0130	0.0109	0.0166	0.0296
	No		1.35	2.05	2.72	1.007	0.698	0.675	2.68	1.518	1.716	1.805	1.615	1.535	1.194	1.225	4.384[a]
	Yes		1.33	2.02	2.69	0.989	0.695	0.669	2.65	1.517	1.714	1.775	1.604	1.510	1.187	1.194	4.310[b]
	SEM		0.009	0.012	0.015	0.0080	0.0066	0.0077	0.016	0.0081	0.0128	0.0210	0.0053	0.0107	0.0090	0.0130	0.0242
		Non-challenge	1.39[a]	2.08[a]	2.73[a]	1.063[a]	0.680[b]	0.657	2.70[a]	1.477[b]	1.742[a]	1.801	1.603	1.575[a]	1.179	1.200	4.380
		Challenge	1.27[b]	1.98[b]	2.67[b]	0.932[b]	0.712[a]	0.686	2.63[b]	1.557[a]	1.687[b]	1.784	1.616	1.468[b]	1.202	1.220	4.314
		SEM	0.010	0.012	0.015	0.0078	0.0066	0.0077	0.015	0.0082	0.0167	0.0240	0.0050	0.0105	0.0104	0.0148	0.0242
Riboflavin × Coccidiosis																	
0.75		Non-challenge	1.43	2.11	2.78	1.085	0.687	0.668[a]	2.74	1.468	1.748	1.852[a]	1.612	1.596	1.196	1.239	4.457
0.75		Challenge	1.26	1.98	2.68	0.922	0.717	0.700[a]	2.64	1.551	1.702	1.724[c]	1.610	1.457	1.220	1.214	4.311
6.6		Non-challenge	1.38	2.07	2.69	1.046	0.692	0.618[b]	2.65	1.484	1.730	1.835[ab]	1.606	1.564	1.184	1.147	4.318
6.6		Challenge	1.28	1.99	2.67	0.929	0.707	0.687[a]	2.63	1.565	1.713	1.775[abc]	1.624	1.478	1.212	1.217	4.340
20		Non-challenge	1.40	2.06	2.75	1.060	0.663	0.686[a]	2.71	1.480	1.750	1.727[bc]	1.591	1.568	1.157	1.215	4.365
20		Challenge	1.29	2.00	2.67	0.945	0.713	0.670[a]	2.63	1.556	1.648	1.823[abc]	1.615	1.472	1.173	1.228	4.290
SEM			0.016	0.021	0.026	0.0137	0.0114	0.0132	0.022	0.0140	0.0223	0.3700	0.0089	0.0183	0.0155	0.0230	0.0419
p-value																	
Riboflavin			0.532	0.660	0.186	0.436	0.418	0.048	0.187	0.597	0.442	0.727	0.402	0.924	0.020	0.121	0.306
Bacillus			0.079	0.142	0.142	0.114	0.763	0.549	0.140	0.976	0.909	0.070	0.152	0.101	0.585	0.106	0.034
Coccidiosis			< .0001	< .0001	0.004	< .0001	0.001	0.011	0.004	< .0001	0.004	0.328	0.075	< .0001	0.076	0.304	0.056
Riboflavin × *Bacillus*[2)]			0.607	0.872	0.665	0.871	0.906	0.433	0.667	0.291	0.856	0.107	0.554	0.768	0.996	0.915	0.910
Riboflavin × Coccidiosis			0.098	0.190	0.239	0.149	0.334	0.009	0.239	0.965	0.165	0.013	0.333	0.307	0.931	0.130	0.143
Bacillus × Coccidiosis[2)]			0.356	0.685	0.992	0.276	0.621	0.504	0.988	0.308	0.339	0.362	0.858	0.678	0.085	0.451	0.979
Riboflavin × *Bacillus* × Coccidiosis[2)]			0.305	0.627	0.640	0.305	0.379	0.916	0.635	0.234	0.639	0.790	0.177	0.400	0.194	0.581	0.337

[1)]n = 8.

[2)]Means of non-significant interactions are not listed.

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

supplemented with 0.75 and 6.6 ppm riboflavin between d 28 to 35 (p = 0.020). Although, the inclusion of *B. subtilis* in the feed did not affect FI during the different phases of growth, i.e., d 14 to 28 (p= 0.101), d 28 to 35 (p = 0.585), and d 35 to 41 (p = 0.106), overall FI d 0 to 41 was reduced by supplementation of *B. subtilis* (p = 0.034; Table 3).

Feed conversion ratio

After the *Eimeria* spp. challenge on d 14, the challenge increased FCR during the growth phase of d 14 to 28 (p < 0.0001), but FCR was reduced in the challenged birds on d 28 to 35 (P = 0.004). As the days progressed, the challenge did not affect the FCR of birds, i.e., there was no significant difference between challenged and non-challenged birds on d 35 to 41 (p = 0.328) and overall FCR d 0 to 41 (p = 0.075). The interaction of riboflavin and *Eimeria* spp. challenge affected the FCR on d 35 to 41, and *Eimeria* spp. challenge reduced FCR on d 35 to 41 when birds were fed riboflavin at 0.75 ppm (p = 0.013; Table 3).

Processing carcass yield and abdominal fat pat

Absolute weight

There was no 3-factor interaction effect of dietary additives and *Eimeria* spp. challenge on the processing yield. There was no difference in BW of processed birds by any of the treatments. The supplementation of *B. subtilis* reduced the CW (p = 0.024) and drumstick weights (p = 0.041). However, *Eimeria* spp. challenge increased fat pad weight (p = 0.024) and decreased tender weight (p = 0.008). The riboflavin and *B. subtilis* interactively affected breast weight (p = 0.005). For birds fed riboflavin at 0.75 ppm, supplementation of *B. subtilis* reduced breast meat weight (Table 4).

Relative weight

Eimeria spp. challenge increased the relative fat pad weight to BW in comparison to that of non-challenged birds (p = 0.045). The relative thighs to CW were interactively affected by the riboflavin and *B. subtilis*. On birds fed riboflavin at the rate of 6.6 ppm and 20 ppm, *B. subtilis* supplementation reduced relative thighs to CW (p = 0.024; Table 4).

Woody breast condition

Eimeria spp. challenge reduced the normal breast percentage (p = 0.009) and increased slight WB condition and presence of WB condition (p = 0.040, p = 0.009, respectively) compared to that of non-challenged birds. Riboflavin and *B. subtilis* interactively affected the normal breast percentage (p = 0.004). *B. subtilis* supplementation increased the percentage of normal breast when birds were fed riboflavin at 0.75 ppm. However, for birds fed riboflavin at 6.6 ppm, *B. subtilis* supplementation reduced the percentage of normal breast. Increasing the doses of riboflavin supplementation in the diet could not increase the percentage of normal breast (p = 0.872) or decrease the percentage of slight WB (p = 0.720), percentage of moderate WB (p = 0.876), and percentage of severe WB (p = 0.822; Table 5).

WB score was positively correlated with live BW (r = 0.350, p < 0.0001), CW (r = 0.434, p < 0.0001), breast weight (r = 0.522, p < 0.0001).

Mortality

The mortality was not affected by different levels of riboflavin and *B. subtilis* up to d 14. Although the birds were challenged with *Eimeria* spp. on d 14, there was no significant increase in mortality between challenged birds and non-challenged birds in the growth phase of d 14 to 28 (p = 0.313), d 28 to 35 (p = 0.360), d 35 to 41(p = 0.606), and overall mortality d 0 to 41 (p = 0.259). However,

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Table 4. The absolute processing weight (g) of Ross 708 male broilers processed on d 42 fed riboflavin and *Bacillus subtilis* and challenged with coccidiosis

Riboflavin	*Bacillus*	Coccidiosis	Absolute weight (g)								Weight / BW (%)		Weight / carcass weight (%)				
			BW	Car-cass	Fat pad	Wing	Breast	Tender	Drum-stick	Thigh	Car-cass	Fat pad	Wing	Breast	Tender	Drum-stick	Thigh
0.75			2,753	1,903	32.3	206	566	112	246	315	69.09	1.25	10.84	30.07	5.92	12.94	16.51
6.6			2,717	1,904	31.6	205	569	112	244	309	69.30	1.26	10.79	29.90	5.86	12.83	16.32
20			2,776	1,905	30.9	206	576	113	242	317	69.39	1.10	10.87	29.81	5.94	12.72	16.59
SEM[1)]			20.8	15.9	0.75	1.8	6.5	1.1	2.0	3.5	0.224	0.073	0.044	0.192	0.050	0.068	0.109
	No		2,750	1,925^{a}	31.7	207	575	113	246^{a}	314	69.29	1.18	10.80	29.98	5.87	12.81	16.59
	Yes		2,748	1,883^{b}	31.5	204	566	112	241^{b}	313	69.22	1.23	10.87	29.87	5.95	12.85	16.37
	SEM		17.0	13.0	0.61	1.5	5.3	0.9	1.6	2.9	0.183	0.060	0.036	0.157	0.040	0.056	0.089
		Non-challenge	2,746	1,921	30.6^{b}	208	577	114^{a}	245	315	69.33	1.12^{b}	10.84	29.92	5.95	12.80	16.52
		Challenge	2,751	1,888	32.6^{a}	204	564	111^{b}	242	312	69.19	1.29^{a}	10.83	29.93	5.86	12.86	16.43
		SEM	17.0	13.0	0.61	1.5	5.3	0.9	1.6	2.9	0.183	0.060	0.036	0.157	0.041	0.056	0.089
Riboflavin × *Bacillus*																	
0.75	No		2,723	1,955	32.3	210	585^{a}	114	250	316	69.39	1.14	10.82	29.88	5.84	12.85	16.37^{bc}
0.75	Yes		2,783	1,852	32.3	201	547^{c}	111	241	314	68.78	1.36	10.87	30.26	6.01	13.03	16.65^{ab}
6.6	No		2,729	1,901	31.6	204	557^{bc}	111	245	307	69.02	1.28	10.75	30.00	5.83	12.91	16.55^{ab}
6.6	Yes		2,706	1,908	31.6	207	580^{ab}	112	243	310	69.58	1.24	10.84	29.79	5.89	12.75	16.10^{c}
20	No		2,797	1,920	31.2	208	582^{ab}	114	243	320	69.46	1.11	10.84	30.06	5.94	12.68	16.84^{a}
20	Yes		2,754	1,890	30.6	205	571^{abc}	112	241	315	69.31	1.10	10.89	29.56	5.94	12.76	16.35^{bc}
SEM			29.5	22.5	1.05	2.6	9.2	1.6	2.8	5.0	0.317	0.103	0.062	0.272	0.070	0.096	0.153
p-value																	
Riboflavin			0.144	0.995	0.427	0.918	0.539	0.613	0.390	0.204	0.621	0.245	0.481	0.621	0.523	0.076	0.204
Bacillus			0.947	0.024	0.834	0.144	0.260	0.284	0.041	0.724	0.792	0.507	0.212	0.631	0.195	0.674	0.085
Coccidiosis			0.822	0.074	0.024	0.052	0.078	0.008	0.145	0.525	0.583	0.045	0.863	0.955	0.128	0.508	0.492
Riboflavin × *Bacillus*			0.189	0.053	0.940	0.116	0.005	0.367	0.313	0.719	0.181	0.375	0.953	0.260	0.496	0.209	0.024
Riboflavin × Coccidiosis[2)]			0.650	0.198	0.980	0.409	0.570	0.720	0.606	0.970	0.118	0.543	0.876	0.233	0.733	0.393	0.416
Bacillus × Coccidiosis[2)]			0.541	0.565	0.615	0.076	0.940	0.160	0.405	0.362	0.903	0.930	0.649	0.863	0.330	0.647	0.090
Riboflavin × *Bacillus* × Coccidiosis[2)]			0.693	0.287	0.725	0.339	0.634	0.559	0.136	0.847	0.511	0.104	0.698	0.125	0.784	0.802	0.735

[1)]n = 8.

[2)]Means of non-significant interactions are not listed.

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

Table 5. The woody breast condition percentage of processed Ross 708 male birds on d 42 fed riboflavin and *Bacillus subtilis* and challenged with coccidiosis

Riboflavin	*Bacillus*	Coccidiosis	Normal	Slight	Moderate	Severe
0.75			67.7	17.7	12.2	2.50
6.6			65.2	20.8	10.9	3.13
20			67.2	20.6	10.2	2.03
SEM[1)]			3.62	3.04	2.81	1.237
	No		64.5	21.4	12.0	2.08
	Yes		68.9	17.9	10.2	3.02
	SEM		2.96	2.49	2.30	1.010
	Non-challenge		72.3[a]	16.0[b]	8.7	3.02
	Challenge		61.0[b]	23.3[a]	13.5	2.08
	SEM		2.95	2.48	2.30	1.010
Riboflavin × *Bacillus*						
0.75	No		59.1[bc]	21.6	15.6	3.75
0.75	Yes		76.3[a]	13.8	8.8	1.25
6.6	No		73.1[ab]	16.9	8.8	1.25
6.6	Yes		57.2[c]	24.7	13.1	5.00
20	No		61.4[bc]	25.8	11.6	1.25
20	Yes		73.1[ab]	15.3	8.8	2.81
SEM			5.12	4.31	3.98	1.749
p-value						
Riboflavin			0.872	0.720	0.876	0.822
Bacillus			0.302	0.321	0.587	0.514
Coccidiosis			0.009	0.040	0.136	0.514
Riboflavin × *Bacillus*			0.004	0.077	0.363	0.200
Riboflavin × Coccidiosis[2)]			0.824	0.445	0.111	0.895
Bacillus × Coccidiosis[2)]			0.461	0.510	0.239	0.277
Riboflavin × *Bacillus* × Coccidiosis[2)]			0.843	0.315	0.708	0.895

[1)]n = 8.

[2)]Means of non-significant interactions are not listed.

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

supplementation of *B. subtilis* reduced mortality on d 35 to 41 ($p = 0.050$); there was no significant difference in overall d 0 to 41 mortality due to any of the treatments (Table 6).

Blood cell counts and superoxide dismutase activity

The serum SOD activity was interactively affected by the riboflavin and *Eimeria* spp. challenge in which birds fed with 6.6 ppm of riboflavin and non-challenged had higher enzyme assay than that of challenged birds with the same level of riboflavin ($p = 0.038$; Table 7). Although there was no difference among the treatments for the heterophil to lymphocyte (H:L) ratio, the H:L ratio was positively correlated with WB ($p = 0.037$, r = 0.23).

DISCUSSION

Although the main aim of this experiment was to determine the dual properties of riboflavin other than as a vitamin, i.e., BSH inhibitor and an antioxidant with *B. subtilis* during the *Eimeria* spp.

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Table 6. The mortality (%) of Ross 708 male birds fed riboflavin and *Bacillus subtilis* and challenged with coccidiosis

Riboflavin	*Bacillus*	Coccidiosis	d 14–28	d 28–35	d 35–41	d 0–41
0.75			1.70	0.48	0.48	4.57
6.6			1.11	0.50	0.77	6.30
20			0.96	0	1.24	4.57
SEM[1)]			0.644	0.289	0.442	1.038
	No		1.20	0.16	1.34[a]	6.16
	Yes		1.32	0.49	0.32[b]	4.13
	SEM		0.533	0.236	0.361	0.859
		Non-challenge	0.88	0.48	0.70	5.84
		Challenge	1.64	0.17	0.96	4.45
		SEM	0.533	0.236	0.361	0.859
p-value						
Riboflavin			0.694	0.387	0.472	0.418
Bacillus			0.869	0.320	0.050	0.100
Coccidiosis			0.313	0.360	0.606	0.259
Riboflavin × *Bacillus*[2)]			0.399	0.372	0.375	0.647
Riboflavin × Coccidiosis[2)]			0.673	0.387	0.935	0.998
Bacillus × Coccidiosis[2)]			0.952	0.968	0.463	0.112
Riboflavin × *Bacillus* × Coccidiosis[2)]			0.922	0.998	0.541	0.679

[1)]n = 8.

[2)]Means of non-significant interactions are not listed.

[a,b]Means in a column not sharing a common superscript are different ($p < 0.05$).

challenged condition; however, due to lack of the interaction between riboflavin and *B. subtilis* here in main results, we discussed more on the impact we find due to the *Eimeria* spp. challenge.

Growth performance

In the current study, supplementation of riboflavin along with or without *B. subtilis* was unable to reduce the negative impact produced by *Eimeria* spp. challenge on BW and BWG. The challenged birds had lower BW and BWG than non-challenged birds between d 0 to 41. The reduction of BW and BWG due to the *Eimeria* spp. challenge was expected. In a companion study, we found that the *Eimeria* spp. challenge reduced villus height to crypt depth ratio and increased crypt depth in duodenum and ileum on d 27 [39]. The damage in the intestinal structure due to *Eimeria* proliferation can reduce absorption of carbohydrates and protein, as it was found that *Eimeria* spp. challenge reduced secretion of an endogenous enzyme-like sucrase and isomaltose [42]. *Eimeria* spp. challenge also reduced ileal digestible energy and apparent ileal digestibility of amino acids [43–45]. Although challenged birds had lower BW than non-challenge; other than the period of d 14 to 28, challenge birds continue to feed same amount of feed as non-challenge birds meaning that either challenge birds had lower absorption or birds were spending their energy in immunomodulation and maintenance of damaged intestinal villi [45], which subsequently reduces BW and BWG. In this study, *Eimeria* spp. challenge reduced FI during d 14 to 28; during this phase, *Eimeria* spp. were rapidly multiplying in intestinal epithelial of challenged birds [45]. In previous study, the birds challenged with coccidiosis increased expression of Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in duodenum and jejunum [46]. Expression of IL-1β and TNF-α can lead to reduction of FI when IL-1β and TNF-α were injected; it reduced FI in mice [47]. Thus, reduced FI might be associated with increased expression of the aforementioned cytokines due to *Eimeria* spp.

Table 7. The heterophil to lymphocyte (H:L) ratio and superoxide dismutase (SOD) enzyme activity in serum of male broilers on d 35 fed riboflavin and *Bacillus subtilis* and challenged with coccidiosis

Riboflavin	*Bacillus*	Coccidiosis	H:L	SOD U/mL
0.75			0.979	18.1
6.6			0.884	18.7
20			0.843	18.7
SEM[1)]			0.0544	1.37
	No		0.936	17.8
	Yes		0.868	19.2
	SEM		0.0446	1.12
	Non-challenge		0.876	17.8
	Challenge		0.928	19.2
	SEM		0.0447	1.12
Riboflavin × Coccidiosis				
0.75		Non-challenge	0.981	17.9[ab]
0.75		Challenge	0.976	18.3[ab]
6.6		Non-challenge	0.811	22.7[a]
6.6		Challenge	0.958	14.7[b]
20		Non-challenge	0.836	18.1[ab]
20		Challenge	0.851	19.2[ab]
SEM[1)]			0.0769	1.94
p-value				
Riboflavin			0.199	0.946
Bacillus			0.278	0.382
Coccidiosis			0.407	0.168
Riboflavin × *Bacillus*			0.057	0.971
Riboflavin × Coccidiosis[2)]			0.557	0.038
Bacillus × Coccidiosis[2)]			0.311	0.389
Riboflavin × *Bacillus* × Coccidiosis[2)]			0.448	0.441

[1)]n = 8.

[2)]Means of non-significant interactions are not listed.

[a,b]Means in a column not sharing a common superscript are different ($p < 0.05$).

challenge.

In this study, supplementation of *Bacillus* did not improve BW and BWG; these results are in agreement with results of Wang et al. [26], in which *B. subtilis* supplementation did not show difference in BWG as compared to birds fed control diet without supplemented probiotics or antibiotics. Similarly, this result was accompanied by several other research in which supplementation of multi-strain (*Lactobacillus plantarum, L. rhamnosus, Enterococcus faecium, Candida pintolepesii, Bifidobacterium bifidum,* and *A. oryzae*) [48], *Lactobacillus* spp. [25], and *B. subtilis strain BS8* [49] did not affect BW and BWG in comparison to birds fed control diets. However, in the current study, *B. subtilis* supplementation reduced FI from d 0 to 41 without affecting FCR from d 0 to 41. Amerah et al. [49] also observed that supplementation of *Bacillus*-based probiotics at 10^5 and 10^6 CFU/g reduced FI d 1 to 42. Although researchers have been observing reduced FI due to supplementation of *Bacillus* spp., there is no exact mechanism known to our knowledge of how *B. subtilis* supplementation can reduce FI, which is currently unknown.

In our study, BWG during d 28 to 35 was higher in challenged birds; this may be due to compensatory growth after recovery of *Eimeria* spp. challenge. Compensatory growth is rapid

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growth following growth retardation due to reduction in nutrient composition in feed [50]. Male broilers exhibit greater compensatory growth after a period of undernutrition compared to females [51]. In this study, only male broiler was used. Another possible reason for growth might be shift of energy utilization from immunity to growth. However, there was intestinal inflammation in d 27, which was in the path of the recovery until d 36. Since the birds were on the path of recovery, we did not observe any changes in jejunum histology on d 36 in a companion study [39] and challenge birds, had lower FCR d 28 to 35 compared to non-challenged. The lower in FCR of challenged birds from d 28 to 35 in this study; might be due to challenged birds still having a lower BW, and the nutritional requirement for maintenance was lower than that of the heavier non-challenged birds for the same period. However, challenged birds continue to have lower BW than non-challenged birds to other phases of growth might be due to the carry-over effects of retarded BW during the d 14 to 28 when *Eimeria* spp. were rapidly multiplying and causing damage to intestine. In this study, *Eimeria* spp. challenge reduction BW, BWG, and FI during d 14 to 28, which hampered overall (d 0 to 41) BW and BWG of challenged birds.

Processing and carcass yield

The BW of birds selected for processing did not differ due to dietary treatments of riboflavin and *B. subtilis* and *Eimeria* spp. challenge. In this study, Eimeria spp. challenge increased the abdominal fat pad weight was increased and decreased the tender weight. *Eimeria* proliferation in intestine can impair osmolarity of gut and hampered the absorption of sodium and potassium [45]. Decreased sodium and potassium content can reduce protein synthesis [52], reduction in protein synthesis might have subsequently reduced tender weights. Along with this, the reduction in tender weight might be linked to a reduction in absorption of glucose [25] and downregulation of gene associated with absorption of amino acid transporter [42] due to *Eimeria* spp. proliferation in epithelium of intestine. The increase in fat deposition in the challenged broilers might be due to inability of the challenged broiler to absorb dietary energy and protein due to the damage caused by the *Eimeria* spp. challenge in the intestine. As Kassim et al. [53] and Collin et al. [54] reported that dietary energy and protein reduction can increase abdominal fat pad deposition. Additionally, an increase of oxidative stress and a decrease of antioxidants (SOD) may increase the deposition of fat pad in birds [55]. We also observed *Eimeria* spp. challenge reduced SOD level, when birds were fed recommended doses of riboflavin (6.6 ppm) in the serum and increased WB incidences. Increased WB incidences also indicated increased oxidative stress.

In this study, supplementation of *B. subtilis* reduced the weight of the carcass and drumsticks, which was opposite to the results obtained by Deniz et al. [56], who found that supplementation of probiotics (*B. subtilis* DSM 17299) increased hot CW. Supplementation of the *B. subtilis* reduced the breast weight of broiler only at 0.75 ppm doses of riboflavin; this may be due to the enhanced lipid digestion by reducing BSH enzyme (produced by the intestinal microflora and the *B. subtilis*) activity by the higher doses of riboflavin. Lower doses of riboflavin supplementation may not be able to post the same effects. As riboflavin was found to inhibit the BSH enzyme produced by different strains of *Lactobacillus* during the *in vitro* studies [17,34].

Woody breast

In this experiment, the *Eimeria* spp. challenge reduced the percentage of normal breast and increased the percentage of slight WB. Although the exact etiology of WB formation is still unknown, it is often connected with higher growth rate, dietary nutrition, genetic line of birds, sex, age, and oxidative stress [57–59]. Due to the intracellular multiplication of *Eimeria* spp., the parasite produces metabolites, which attributes to the release of excessive free radicals (superoxide)

during the infection [60]. Free radicals can interfere with homeostasis and make cells prone to damage [61]. The increase in free radicals and decrease in the antioxidant enzyme in blood [62] due to *Eimeria* challenge may have increased WB condition in the birds. Based on the literature, we hypothesized that riboflavin could increase antioxidant parameters like SOD, malondialdehyde, glutathione peroxidase, glutathione and help reduce WB [63,64]. However, in our study, increased doses of riboflavin up to 20 ppm did not increase the serum SOD activity, perhaps due to prominent effects of coccidiosis infection rather than that of riboflavin effects on reduction of oxidative stress. Furthermore, partial correlation analysis showed that WB score was positively correlated with live BW, CW, and breast weight representing heavier the live BW, CW, and breast weight higher will be the probability of having severe WB.

The Heterophil to Lymphocyte (H:L) ratio is an indicator of stress measurement in poultry [65]. Stress factors like food or water deprivation, extreme temperature, exposure to new social situations, and interaction with disease can increase heterophil counts and reduce lymphocyte counts in blood [65–67]. In our study, H:L ratio was not affected by dietary treatments and *Eimeria* spp. challenge. However, the overall H:L ratio reported in this study was higher than other studies [68]. The dissimilarity in results among the studies may be due to stress, which altered adrenocorticotropic hormone (ACTH) [68]. Heterophil to Lymphocyte ratio obtained in our study is approximately similar to H:L ratio of birds fed 20 ppm corticosterone in the diet to induce stress in birds [66].

Mortality

There was no significant increase in mortality of the birds due to the *Eimeria* spp. challenge, although the challenged birds exhibited an increased percentage of *Eimeria* spp. lesion scores on d 27. Supplementation of *B. subtilis* reduced the mortality of the birds d 35 to 41. The reduction in mortality due to supplementation of *B. subtilis* might be due to its ability to enhance host immunity by inhibiting the pathogens and stabilizing the intestinal microbiome [69]. Still, in our study, the effects of *B. subtilis* was only seen after the birds were recovered from the *Eimeria* spp. challenge.

CONCLUSION

The results obtained in this study showed the proposed hypothesis riboflavin would help reduce BSH enzyme produced by the intestinal microflora and probiotics (*B. subtilis*) and subsequently enhance growth performance of birds was failed since increased doses of riboflavin (20 ppm) was not able to enhance BW, and BWG. However, supplementation of riboflavin (20 ppm) reduced FI from d 28 to 35. Along with this negative impact of *Eimeria* spp. challenge on BW, BWG, GR cannot be overcome by supplementation *B. subtilis* along with increased doses of riboflavin. However, supplementation of *B. subtilis* shows some promising results in reducing FI and mortality. Furthermore, the increased supplementation of the riboflavin at the tested level did not help birds to reduce the WB conditions.

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