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 supplement-ed 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*®-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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... characterize a potent BSH inhibitor with potential as a novel alternative to AGPs to improve antibo... 
... increased oxidative stress can increase woody breast (WB) [31]. WB is a meat quality problem, which... 
... it also has an antioxidant protection function [30]. Increased oxidative stress can increase woody br... 
... was estimated a global cost of $12.10 billion in 2016 [11]. Among the total, estimated economic losses... 
... the inclusion of AGPs from feed has increased the risk of enteric diseases, causing significant eco... 
... demand by consumers forced the industry to shift broiler production from conventional to antibo... 

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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.
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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.

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].
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 m²/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® 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 × 10⁸ 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
verified viable \(4.1 \times 10^7 - 1.5 \times 10^8\) 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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| 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 (%) | Starter d 0–14  | Grower d 14–28  | Finisher 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 | 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 | - | - | - |
| Calculated composition (%) | | | |
| 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 sulfur 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 |

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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; 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 \(1.1 \times 10^8\) 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.
(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°C to extract serum. Collected serum samples were stored in a −80°C 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

| 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 |
| SEM1)      | 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 | Bacillus | 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.
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

| 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 | p-value |
|------------|----------|-------------|------|------|------|---------|---------|---------|--------|---------|---------|---------|---------|--------|
| 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* | 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* | 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* | 1.222  | 4.328 |
| SEM        | 0.011    | 0.015      | 0.018 | 0.0097 | 0.0081 | 0.0093  | 0.019   | 0.0003 | 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* |
| 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* |
| 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* | 2.08* | 2.73 | 1.063* | 0.680* | 0.657   | 2.70* | 1.477* | 1.742* | 1.801   | 1.603  | 1.575*   | 1.179   | 1.200  | 4.380 |
| Challenge  | 1.27b   | 1.98b | 2.67b | 0.932b | 0.712b | 0.686   | 2.63b | 1.557b | 1.687b | 1.784   | 1.616  | 1.468b   | 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

| Riboflavin | Coccidiosis | d 28 | d 35 | d 41 | d 14–28 | d 28–35 | d 35–41 | d 0–41 | p-value |
|------------|-------------|------|------|------|---------|---------|---------|--------|--------|
| 0.75       | Non-challenge | 1.43 | 2.11 | 2.78 | 1.085   | 0.687   | 0.668* | 2.74   | 1.468   | 1.748   | 1.852* | 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* | 2.64   | 1.551   | 1.702   | 1.724* | 1.610  | 1.457   | 1.220  | 1.214  | 4.311 |
| 6.6        | Non-challenge | 1.38 | 2.07 | 2.69 | 1.046   | 0.692   | 0.618b | 2.65   | 1.484   | 1.730   | 1.835* | 1.606  | 1.564   | 1.184  | 1.147  | 4.318 |
| 6.6        | Challenge    | 1.28 | 1.99 | 2.67 | 0.929   | 0.707   | 0.687b | 2.63   | 1.565   | 1.713   | 1.775* | 1.624  | 1.478   | 1.212  | 1.217  | 4.340 |
| 20         | Non-challenge | 1.40 | 2.06 | 2.75 | 1.060   | 0.663   | 0.686a | 2.71   | 1.480   | 1.750   | 1.727* | 1.591  | 1.568   | 1.157  | 1.215  | 4.365 |
| 20         | Challenge    | 1.29 | 2.00 | 2.67 | 0.945   | 0.713   | 0.670a | 2.63   | 1.556   | 1.648   | 1.823* | 1.615  | 1.472   | 1.173  | 1.228  | 4.290 |
| SEM        | 0.016       | 0.021 | 0.026 | 0.0436 | 0.0418 | 0.048a | 0.187 | 0.597   | 0.442   | 0.727   | 0.402  | 0.924   | 0.020   | 0.121  | 0.306 |

p-value

| Riboflavin | Bacillus | Coccediosis | Riboflavin × Bacillus | Riboflavin × Coccidiosis | Bacillus × Coccidiosis | Riboflavin × Bacillus × Coccidiosis |
|------------|----------|-------------|-------------------|--------------------------|------------------------|-----------------------------------|
| 0.532      | 0.660    | 0.186       | < 0.001           | < 0.001                  | < 0.001                | < 0.001                           |
| 0.079      | 0.142    | 0.142       | 0.114             | 0.763                    | 0.549                  | 0.140                             |
| 0.008      | 0.190    | 0.239       | 0.149             | 0.334                    | 0.009                  | 0.239                             |
| 0.356      | 0.685    | 0.992       | 0.276             | 0.621                    | 0.504                  | 0.988                             |
| 0.305      | 0.627    | 0.640       | 0.305             | 0.379                    | 0.916                  | 0.635                             |

1) n = 8.
2) Means of non-significant interactions are not listed.
3) Means in a column not sharing a common superscript are different (p < 0.05).

https://www.ejast.org
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,
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 | Drumstick | Thigh | Car-cass | Fat pad | Wing | Breast | Tender | Drumstick | Thigh |
| 0.75       |          |             | 2.753   | 1.903    | 32.3    | 20.56 | 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   | 567    | 113    | 242       | 317   | 69.39    | 1.10    | 10.87   | 29.81  | 5.94   | 12.72  | 16.59   |
| SEM        |          |             | 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    | 31.7    | 207   | 575    | 113    | 246a       | 314   | 69.29    | 1.18    | 10.80   | 29.98  | 5.87   | 12.81  | 16.59   |
|            | Yes      |             | 2.748   | 1.883    | 31.5    | 204   | 566    | 112    | 241b       | 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    | 208   | 577    | 114    | 245        | 315   | 69.33    | 1.12    | 10.84   | 29.92  | 5.95   | 12.80  | 16.52   |
|            | Challenge |          | 2.751   | 1.888    | 32.6    | 204   | 564    | 111    | 242        | 312   | 69.19    | 1.29    | 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

| Riboflavin | Bacillus | Coccidiosis | Absolute weight (g) | Weight / BW (%) | Weight / carcass weight (%) |
|------------|----------|-------------|---------------------|----------------|---------------------------|
| 0.75       | No       |             | 2.723   | 1.955    | 32.3    | 210   | 585a   | 114    | 250       | 316   | 69.39    | 1.14    | 10.82   | 29.88  | 5.84   | 12.85  | 16.37   |
| 6.6        | No       |             | 2.729   | 1.901    | 31.6    | 204   | 557c   | 111    | 245       | 307   | 68.78    | 1.36    | 10.87   | 30.26  | 6.01   | 13.03  | 16.65   |
| 6.6        | Yes      |             | 2.706   | 1.908    | 31.6    | 207   | 580a   | 112    | 243       | 310   | 69.58    | 1.24    | 10.84   | 29.79  | 5.89   | 12.75  | 16.10   |
| 20         | No       |             | 2.797   | 1.920    | 31.2    | 208   | 582c   | 114    | 243       | 320   | 69.46    | 1.11    | 10.84   | 30.06  | 5.94   | 12.68  | 16.84   |
| 20         | Yes      |             | 2.754   | 1.890    | 30.6    | 205   | 571bc  | 112    | 241       | 315   | 69.31    | 1.10    | 10.89   | 29.56  | 5.94   | 12.76  | 16.35   |
| 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 | Bacillus | Coccidiosis | Riboflavin × Bacillus | Riboflavin × Coccidiosis | Bacillus × Coccidiosis |
|------------|----------|-------------|-----------------------|--------------------------|-----------------------|
| 0.14       | 0.995    | 0.427       | 0.613                 | 0.613                    | 0.613                 |
| 0.947      | 0.024    | 0.834       | 0.284                 | 0.284                    | 0.284                 |
| 0.822      | 0.074    | 0.024       | 0.052                 | 0.052                    | 0.052                 |
| 0.189      | 0.053    | 0.940       | 0.367                 | 0.367                    | 0.367                 |
| 0.650      | 0.198    | 0.980       | 0.570                 | 0.570                    | 0.570                 |
| 0.541      | 0.655    | 0.615       | 0.940                 | 0.940                    | 0.940                 |
| 0.693      | 0.287    | 0.725       | 0.634                 | 0.634                    | 0.634                 |

1) *n = 8*.
2) Means of non-significant interactions are not listed.
3) 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.
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 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       | 0.644   | 0.289       | 0.442   | 1.038   |         |        |
| No        | 1.20    | 0.16        | 1.34*   | 6.16    |         |        |
| Yes       | 1.32    | 0.49        | 0.32*   | 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 | 0.399 | 0.372 | 0.375 | 0.647 |
| Riboflavin × Coccidiosis | 0.673 | 0.387 | 0.935 | 0.998 |
| Bacillus × Coccidiosis | 0.952 | 0.968 | 0.463 | 0.112 |
| Riboflavin × Bacillus × Coccidiosis | 0.922 | 0.998 | 0.541 | 0.679 |

*1)n = 8.*

*Means of non-significant interactions are not listed.

*Means in a column not sharing a common superscript are different (*p* < 0.05).
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 pindolepesii*, *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.

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       | 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^a  |
|                         | 20 Non-challenge   | 0.836 | 18.1^ab |
|                         | 20 Challenge       | 0.851 | 19.2^ab |
| SEM                    | 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 | 0.557 | 0.038 |
| Bacillus × Coccidiosis | 0.311 | 0.389 |
| Riboflavin × Bacillus × Coccidiosis | 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).
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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