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Original Research Article

Effects of phytase and 25-hydroxycholecalciferol supplementation in broilers fed calcium-phosphorous deficient diets, with or without *Eimeria* challenge, on growth performance, body composition, bone development, and gut health

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ABSTRACT

The study evaluated the effects of nutritional strategies on broilers challenged with *Eimeria* from d 14 to 26. A total of 840 Cobb male broilers were fed five diets in a 2 × 5 factorial arrangement: 1) nutrient adequate diet (PC; 0.84% calcium [Ca], 0.42% available phosphorus [avP]); 2) Ca-P deficient diet (NC; 0.64% Ca, 0.22% avP); 3) NC + 1500 FTU/kg phytase of diet (NC + PHY); 4) NC + 5000 IU/kg 25-hydroxycholecalciferol of diet (NC + 25OHD); and 5) NC with both supplements (NC + PHY + 25OHD), with and without *Eimeria* challenge. All treatments had six replicate cages with 14 birds per cage. At 5 days post inoculation (DPI), the challenged birds exhibited higher serum fluorescein isothiocyanated (FITC-d) levels than the unchallenged birds ($P < 0.001$). The NC + PHY and NC + PHY + 25OHD groups exhibited lower FITC-d levels compared to the NC + 25OHD group ($P = 0.012$). Significant interaction effects between *Eimeria* challenge and dietary treatments were observed on various parameters. During 0 to 6 and 0 to 12 DPI, *Eimeria* challenge resulted in decreased the body weight gain (BWG) ($P < 0.05$) but had a negative effect on the feed conversion ratio (FCR) in birds compared to the unchallenged group ($P < 0.05$). Reducing Ca and avP levels in the diet (NC) did not adversely affect BWG, but negatively impacted FCR, bone ash weight, ash concentration, and femur bone microstructure parameters ($P < 0.05$). On 12 DPI, *Eimeria* challenge led to decreased tibia bone weight, bone volume, fat-free bone weight (FFBW), and ash weight of birds ($P < 0.05$). Supplementation with phytase alone or in combination with 25OHD improved growth performance, gut permeability, bone ash and bone microstructure parameters in birds ($P < 0.05$). However, the group fed 25OHD alone showed enhancements on growth performance, mineral apposition rate (MAR), bone ash concentration and ash percentage of the birds ($P < 0.05$). In conclusion, lowering Ca and avP levels in the diet negatively affected FCR and bone development but did not affect intestinal integrity in broilers. Dietary supplementation of phytase, 25OHD, or phytase in combination of 25OHD could enhance the growth performance and bone quality of broilers infected with *Eimeria*. Notably, the benefits of phytase supplementation were generally more pronounced than those associated with 25OHD supplementation; however, the combination of phytase and 25OHD could induce optimum effects.

1. Introduction

Numerous strategies and additives are utilized to optimize performance, sustain animal health and welfare, all while managing costs effectively. However, the poultry industry has undergone significant changes, especially in response to the prohibition of antibiotic use (Powers and Angel, 2008). Coccidiosis stands out as a significant concern in poultry, leading to an estimated annual

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global economic loss of up to $14 billion (Blake et al., 2020). The disease is caused by protozoan parasites of the genus *Eimeria* (Fatoba and Adeleke, 2018). By targeting the intestinal tract of broiler chickens, *Eimeria* causes gastrointestinal damage, which can profoundly compromise gut integrity, impact nutrition digestion and absorption, and enhance inflammation, immune response, and oxidative stress, leading to inhibited growth performance and decreased overall flock health (Choi and Kim, 2022; Lopes et al., 2023; Sharma et al., 2024). Although the poultry industry has attained success in controlling coccidiosis by good management, anticoccidials and vaccination, emerging concerns revolve around the potential development of resistance among *Eimeria* species to anticoccidials as well as identified gaps in vaccination protocols, coupled with constraints on antibiotic use (Chapman and Jeffers, 2014; Chapman, 2018). These concerns are driving continued research endeavors and the adoption of alternative strategies to improve coccidiosis control in the poultry industry.

The predominant form of phosphorus (P) in a typical corn-soybean meal diet is phytate (Bedford, 2000). However, phytate P is largely indigestible to poultry due to limited phytase activity in the chicken gastrointestinal tract (Humer et al., 2015). This inefficiency in hydrolyzing dietary phytate P into inorganic phosphate results in substantial P excretion in manure, contributing to P wastage and environmental pollution (Humer et al., 2015). Furthermore, as a polyanionic molecule, phytate can chelate positively charged cations, with a particular affinity for calcium (Ca), iron, and zinc. Numerous studies have highlighted the potential benefits of incorporating exogenous phytase into poultry diets. By adding phytase, inorganic phosphate can be released from phytate, enhancing P availability, and subsequently reducing P excretion (Woyengo and Nyachoti, 2013; Wang et al., 2021). Beyond its impact on P utilization, phytase also plays a vital role in releasing other nutrients bound by phytic P. This includes improvements in the digestibility and retention of protein (Cowieson et al., 2006), amino acids (Cowieson et al., 2017), and Ca (Bedford and Rousseau, 2017). Furthermore, phytase has been shown to markedly enhance the availability of various trace minerals such as zinc, iron, magnesium, and copper (Lönnerdal, 2002; Rimbach et al., 2008; Moss et al., 2018). The utilization of phytase in poultry nutrition not only addresses P management but also contributes to overall nutrient efficiency and environmental sustainability. Currently, it is reported that about 90% of poultry and about 70% of pig diets include exogenous phytase (MarketsandMarkets, 2019).

Vitamin D, a fat-soluble nutrient, has long been recognized for its crucial role in preventing or treating rickets (Dittmer and Thompson, 2011). In both birds and mammals, vitamin D_3 (cholecalciferol) is the exclusive form of vitamin D synthesized through the conversion of 7-dehydrocholesterol (provitamin D) in the skin exposed to ultraviolet irradiation (Fraser, 1980). Alternatively, it can be obtained through dietary sources and absorbed in the intestine (Świątkiewicz et al., 2017). In its initial form, cholecalciferol is biologically inactive in animal organisms. To become active, it undergoes a sequential process of hydroxylation, first transforming into 25-hydroxycholecalciferol (25OHD) in the liver and then further converting to 1,25-dihydroxcholecalciferol [1,25$(OH)_2D_3$] in the kidney (DeLuca, 2004). Vitamin D_3 is essential for promoting Ca absorption in the intestine and maintaining proper serum Ca and P levels, which are crucial for normal bone function. Additionally, it plays a key role in bone growth and remodeling (Ross et al., 2013). Supplementation of vitamin D_3 and its metabolites in broiler diets has been shown to enhance growth performance (Han et al., 2017), increase maximum bone breaking strength and bone mineral content (Kim et al., 2011; Kheiri and Landy, 2019; Leyva-Jimenez et al., 2019b), and lower occurrence of bone pathologies, such as tibial dyschondroplasia (Khan et al., 2010), especially when Ca and P levels in diet are unbalanced (Zhang et al., 2020). Traditionally, the poultry industry has relied on vitamin D_3 as a primary source of vitamin D. However, since 2006, 25OHD has been approved and extensively utilized as an alternative vitamin D source in poultry farming (Adhikari et al., 2020). Dietary 25OHD has demonstrated greater effectiveness than vitamin D_3 in enhancing the overall performance of broilers (Yarger et al., 1995). Its use as a replacement or supplement to vitamin D_3 proves effective in promoting performance, improving bone mineralization (Leyva-Jimenez et al., 2019a), and modifying avian immunity (Chou et al., 2009).

While previous studies have examined the effects of phytase and 25OHD separately, their combined impact on broilers fed a diet low in Ca and P while challenged with coccidiosis remains unexplored. Therefore, this study aimed to evaluate the effects of phytase and 25OHD supplementation in broilers fed a Ca and P deficient diet under *Eimeria* challenge. The hypothesis of this research was that the dietary inclusion of phytase in combination of 25OHD could mitigate the adverse effects of coccidiosis, leading to positive interactions in terms of performance and overall health of broilers.

2. Materials and methods

2.1. Animal ethics statement

The study received approval from the Institutional Animal Care and Use Committee at the University of Georgia (A2021 12–012), and was conducted at the Poultry Research Center of the University of Georgia.

2.2. Animals, housing, experimental design, and diet

A total of 840 male Cobb 500 broilers at 14 days of age were assigned to 10 treatments using a completely randomized design. These treatments were arranged in a 2 × 5 factorial design, with six replicated pens per treatment and 14 birds per pen. The main factors were *Eimeria* challenge and dietary treatments. The study lasted for 26 days, during which the chickens were housed in battery cages with free access to feed and water. The environmental conditions, including temperature and lighting, were managed according to the Cobb Broiler Management Guide (Cobb, 2021).

All birds received the same starter diet from d 0 to 13. From d 14 onwards, the birds were fed one of five experimental grower diets: 1) a positive control with a standard diet (PC; 0.84% Ca and 0.42% available phosphorus [avP]); 2) a negative control with a low Ca and P diet (NC; 0.64% Ca and 0.22% avP); 3) NC + 1500 FTU/kg of phytase (NC + PHY; Quantum Blue, AB Vista, Marlborough, UK); 4) NC + 5000 IU/kg of 25OHD (NC + 25OHD; BioD, Huvepharma, Sofia, Bulgaria); and 5) NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD (NC + PHY + 25OHD). The diets, which were primarily based on corn and soybean meal, were formulated to meet, or exceed the nutritional requirements of broilers for all nutrients, except for Ca and avP, as detailed in Table 1. Feed samples were sent to the Feed and Environmental Water Laboratory (Athens, GA, USA) to measure total Ca, total P (ICP-OES method, method 968.08; AOAC, 1996), crude fiber (Ankom 200, method 962.09; AOAC, 2005), and total N (Combustion technique, method 993.13; AOAC, 1996). The crude protein was calculated by total N × 6.25. The 25OHD concentrations were measured via LC-MS/MS method (Certificate Number 3969.01; ISO/IEC 17025:2017; Heartland Assays, Ames, LA, USA). On d 14, birds in the challenged groups were orally inoculated with a solution containing distilled water and 12,500 sporulated *Eimeria maxima*, 12,500 sporulated *Eimeria tenella*, and 62,500 sporulated *Eimeria acervulina* oocysts suspended in 1 mL amount. The challenge dose was established based on a prior

Table 1
The ingredient (as-fed basis, %) calculated and analyzed compositions of starter and grower diets (dry matter basis, %).

Item	Starter (d 0 to13)	Grower (d 14 to 26 [0 to 12 DPI])[1]				
		PC	NC	NC + PHY	NC+25OHD	NC + PHY+25OHD
Ingredients						
Corn	58.57	61.17	61.17	61.17	61.17	61.17
Soybean meal	36.06	31.49	31.49	31.49	31.49	31.49
Soybean oil	1.58	2.74	2.74	2.74	2.74	2.74
Dicalcium phosphate	1.57	1.45	0.38	0.38	0.38	0.38
Limestone	1.18	1.12	1.20	1.20	1.20	1.20
Sand		1.00	2.00	2.00	2.00	2.00
Common salt	0.34	0.35	0.34	0.34	0.34	0.34
DL-Methionine	0.29	0.28	0.28	0.28	0.28	0.28
Vitamin premix[2]	0.10	0.10	0.10	0.10	0.10	0.10
Mineral premix[3]	0.08	0.08	0.08	0.08	0.08	0.08
L-Lysine HCl	0.15	0.18	0.18	0.18	0.18	0.18
Threonine	0.08	0.04	0.04	0.04	0.04	0.04
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated nutrient levels[4]						
ME, kcal/kg	3008	3086	3086	3086	3086	3086
Crude protein	22.00	20.00	20.00	20.00	20.00	20.00
Crude fiber	2.19	2.11	2.11	2.11	2.11	2.11
Total calcium	0.90	0.84	0.64	0.64	0.64	0.64
Total phosphorus	0.72	0.67	0.47	0.47	0.47	0.47
avP	0.45	0.42	0.22	0.22	0.22	0.22
25OHD, IU/kg					5000	5000
Analyzed nutrient levels						
Crude protein		19.38	19.88	20.38	20.00	19.00
Crude fiber		2.43	2.56	2.21	2.55	2.38
Total calcium		0.82	0.66	0.86	0.84	0.70
Total phosphorus		0.57	0.38	0.44	0.41	0.37
25OHD, IU/kg		220	220	124	3980	3459

DPI = days post inoculation; ME = metabolizable energy; avP = available phosphorus; 25OHD = 25-Hydroxycholecalciferol.
[1] PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY+25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.
[2] Vitamin premix supplemented as per kilogram of diets: thiamin mononitrate 2.4 mg; nicotinic acid 44 mg; riboflavin 4.4 mg; D-Ca pantothenate 12 mg; vitamin B_{12} (cobalamin) 12.0 mg; pyridoxine HCl, 4.7 mg; D-Biotin 0.11 mg; folic acid 5.5 mg; menadione sodium bisulfite complex 3.34 mg; choline chloride 220 mg; cholecalciferol 27.5 mg; transretinyl acetate 1.892 mg; α tocopheryl acetate 11 mg; ethoxyquin 125 mg.
[3] Mineral premix supplemented as per kilogram of diets: manganese ($MnSO_4 \cdot H_2O$) 60 mg; iron ($FeSO_4 \cdot 7H_2O$) 30 mg; zinc (ZnO) 50 mg; copper ($CuSO_4 \cdot 5H_2O$) 5 mg; iodine (ethylene diaminedihydroiodide) 0.15 mg; selenium ($NaSeO_3$) 0.3 mg.
[4] Nutrient levels were calculated using analyzed values from the latest batches of key ingredients and reference values from Feedstuffs (2019). The feed formulation was completed using WUFFDA, a Microsoft Excel workbook designed for least-cost feed formulation based on the latest Feedstuffs table.

study conducted in our lab, aiming to induce a mild coccidiosis infection (Teng et al., 2020). Birds in the unchallenged and *Eimeria*-challenged groups were housed in separate batteries within the same environmentally controlled room to prevent cross-contamination.

2.3. Growth performance and sample collection

Body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) were measured on d 14, 20, and 26 by weighing the birds and feed in each pen. Daily FI from d 14 to 26 (0 to 12 DPI) was also measured. Daily mortality was recorded and used for the calculation of corrected FCR. On 6 DPI (d 20), six birds from each pen were humanely euthanized through cervical dislocation for sample collection. Additionally, on 12 DPI (d 26), another four birds per pen were euthanized to collect further samples.

2.4. Gut permeability and lesion score assays

On 5 DPI (d 19), one bird per cage (six birds/treatment) was inoculated with 1 mL of a fluorescein isothiocyanate–dextran (FITC-d; Sigma–Aldrich Co., St. Louis, MO, USA) solution (2.2 mg/mL) to assess gut permeability, as described by Castro et al. (2020). Blood was collected and centrifuged to extract serum 2 h-post oral gavage. The serum concentration of FITC-d was measured using a microplate reader (Spectramax M5, Molecular Devices, San Jose, CA, USA), following the method outlined by Teng et al. (2020). On 6 DPI (d 20), intestinal lesion scores for *E. maxima*, *E. tenella*, and *E. acervulina* was conducted at the duodenum, jejunum, and ceca, which was according to Johnson and Reid (1970) and Choi et al. (2023).

2.5. Analysis of mineral apposition rate (MAR)

The calcein injection method was used to measure MAR for bone formation. Calcein, a fluorescent dye, binds to newly mineralized bone and serves as a marker for mineral apposition, indicating the rate of bone formation between two calcein injection gaps (Chen et al., 2020; Sharma et al., 2023). One bird from each pen was injected with 20 mg/kg of calcein solution intraperitoneally (Sigma Aldrich, St. Louis, MO, USA) based on body weight on 4 DPI (d 18). On 8 DPI (d 22), the same birds received a second injection following the earlier protocol, and on 12 DPI (d 26), the right tibias of the injected birds were collected, cleared of all attached tissues, and preserved in 70% ethanol. For analysis, a 0.5 mm bone section was cut from the mid-diaphysis using a circular saw (Ryobi, Anderson, SC, USA). These sections were then observed under a compound microscope (BZ-X810, Keyence Corp., Itasca, IL, USA), and images were captured using a BZ-X810 All-in-one Fluorescence Microscope (Keyence Inc., Itasca, IL, USA). The MAR was determined by measuring the distance between two calcein labels using the BZ-X800 Analyzer (Keyence Corp., Itasca, IL, USA).

2.6. Tibial bone ash analysis

Bone ash was described by the methodologies by Zhang and Coon (1997) and Kim et al. (2004). Right tibias were collected from 2 birds per pen on both 6 DPI (d 20) and 12 DPI (d 26). The initial weight of tibia bones was measured after removing adhering muscles. The bone volume was measured by immersing the bones in water and weighing them, assuming a water specific gravity of 1 g/cm^3 at room temperature. The bones were then dried for 24 h and subjected to a Soxhlet apparatus using hexane (Fisher Scientific International Inc., Waltham, MA, USA) to extract fat for 48 h. After fat removal, the bones were re-dried for another 24 h and re-weighed to obtain the fat-free bone weight (FFBW). The ash weight was determined by incinerating the fat-free bones in a furnace (600 °C) overnight. The ash concentration and ash percentage were then calculated using the following formulas:

Ash concentration (g/cm^3) = ash weight (g)/bone volume (cm^3);

Ash percentage (%) = 100 × ash weight (g)/FFBW (g).

2.7. Microstructure analysis of femur bones

At 6 DPI (d 20) and 12 DPI (d 26), the right femurs were collected from one bird per pen and scaned using the Skyscan 1275 X-ray Microtomograph (micro-CT; Bruker MicroCT, Billerica, MA, USA). The scanning protocol was based on the methodology described by Chen and Kim (2020), with the X-ray source at 70 kV, 142 μA for 6 DPI (d 20) and 80 kV, 125 μA for 12 DPI (d 26), using a 0.5-mm aluminum filter to reduce beam-hardening effects. The pixel size remained constant at 25 μm. Scanning was conducted over a 180° rotation with a rotation angle of 0.4°, and four images were captured per rotation. Images were reconstructed into 3D using N-Recon (Bruker MicroCT, Billerica, MA, USA), aligned to a vertical position using Data Viewer (Bruker MicroCT, Billerica, MA, USA), and transferred to CTAn program (Bruker MicroCT, Billerica, MA, USA) to select volume of interest (VOI). Bone mineral density (BMD) calibration was performed using two phantoms made from calcium hydroxyapatite with the densities of 0.25 and 0.75 g/cm^3.

Morphometric analysis was focused on the metaphyseal region of bones, covering a total of 200 slides (5 mm). Subsequently, the 3D model underwent customized processing to separate the trabecular bone from the cortical bone, following the methodology of Chen and Kim (2020). Various parameters were analyzed and outlined in Table 2, adhering to the definitions provided by White et al. (2023) and Sharma et al. (2023). The bone mineral content (BMC; mg) was calculated by BMD (g/cm^3) times tissue volume (TV; mm^3).

2.8. Body composition analysis

Bird body composition was measured using dual energy X-ray absorptiometry (DEXA). On 6 DPI (d 20) and 12 DPI (d 26), two randomly chosen birds from each pen were euthanized and scanned by a DEXA scanner (GE Healthcare, Chicago, IL, USA) with a small animal module by encore software (Lunar Prodigy from GE, version 12.20.023). BMD, BMC, total body fat, and muscle of the birds were calculated according to Castrol et al. (2019). Two birds were selected from each pen and considered as an experimental unit.

2.9. Gene expression analysis

After collecting the junctions between jejunum and ileum, they were cleansed of digesta with 1× phosphate-buffered saline solution, then snap-frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. Homogenization of the samples was performed using QiAzol lysis reagent (Qiagen Inc., Valencia, CA, USA) and a bead beater (Biospec Products, Bartlesville, OK, USA), followed by total RNA extraction as per the manufacturer's instructions. The purity and concentration of RNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA concentrations were normalized, and cDNA synthesis was conducted using a high-capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA).

Quantitative real-time PCR was carried out on a Step One thermocycler (Applied Biosystems, Foster City, CA, USA) using SYBR Green on Master mix (Bio-Rad Laboratories, Hercules, CA, USA) to quantify mRNA expression. The PCR conditions were set as follows: 95 °C for 10 min, followed by 95 °C for 15 s, annealing temperature for 20 s, and 72 °C for 15 s for 40 cycles, as established in our previous study (Tompkins et al., 2023a). Duplicate samples were processed and target gene expression was measured using the $2^{-\triangle\triangle Ct}$ method, in accordance with the guidelines provided by Livak and Schmittgen (2001). The mean ΔCt of each marker gene from the control group was used to calculate the ΔΔCt value. The expression levels in the treatment groups were expressed as fold changes. Primer sequences for both housekeeping and target genes are listed in Table 3.

2.10. Statistical analysis

The experimental data are presented as the mean and standard error of the mean (SEM) and were analyzed using a two-way ANOVA with a general linear model (GLM) in SAS Studio (SAS Institute Inc., Cary, NC, USA). The data were subjected to two-way ANOVA to obtain results for each factor (dietary treatments and *Eimeria* challenge) as well as their interactions. In the case of significant differences, the treatments were compared using Tukey's test. The level of significance was set at $P \leq 0.05$. The mathematical model is listed below:

$$y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \epsilon_{ijk}$$

where μ is the grand mean, α_i is the effect of *Eimeria* challenge, β_j is the dietary treatment, $(\alpha\beta)_{ij}$ is the effect due to any interaction between the *Eimeria* challenge and the dietary treatment, and ε_{ijk} is the random error.

3. Results

3.1. Growth performance and daily feed intake

The results of growth performance are presented in Table 4. The BWG showed a decrease in *Eimeria*-challenged groups compared to unchallenged groups ($P < 0.001$). In addition, an interaction effect of *Eimeria* challenge and diets was observed on BWG ($P = 0.027$) during 0 to 6 DPI (d 14 to 20). In unchallenged groups, the NC and NC + PHY+25OHD groups exhibited significantly lower BWG compared to the PC group ($P = 0.027$). However, in *Eimeria*-challenged groups, no significant difference was found on BWG between the NC and PC birds ($P > 0.05$), whereas the

Table 2
Definition and description of microstructure properties of tibia bones from micro-computed tomography.

Abbreviations	Variables	Description of variables	Standard unit
TV	Tissue volume	Volume of the entire region of interest	mm^3
BV	Bone volume	Volume of the bone segment	mm^3
BV/TV	Bone volume/tissue volume ratio	Bone volume as a fraction of tissue volume from the region of interest	%
TS	Tissue surface	Surface of the entire region of interest	mm^2
BS	Bone surface	Surface of the bone segment	mm^2
BS/BV	Bone surface/bone volume ratio	Ratio of bone surface/bone volume	mm^{-1}
BS/TV	Bone surface/tissue volume ratio	Ratio of bone surface/tissue volume	mm^{-1}
BMD	Bone mineral density	Measure the bone mineral content per unit of volume	g/cm^3
BMC[1]	Bone mineral content	Measure the bone mineral content of the tissue	mg
Tb.Th	Trabecular thickness	Mean thickness of trabeculae measured using 3D methods	mm
Tb.Sp	Trabecular separation	Mean distance between trabeculae measured using 3D methods	mm
Tb.N	Trabecular number	Average number of trabeculae per unit of length	mm^{-1}
Tb.Pf	Trabecular pattern factor	Indicate the degree of trabecular branching	mm^{-1}
SMI	Structure model index	An indicator of the structure of trabeculae	
Po.N (cl)	Number of closed pores	Number of closed pores	
Po.V (cl)	Volume of closed pores	Volume of closed pores	mm^3
Po.S (cl)	Surface of closed pores	Surface of closed pores	mm^2
Po (cl)	Closed porosity percentage	Volume of closed pores (Po.V[cl], mm^3)/total volume of bone (BV, mm^3)	%

[1] BMC (mg) = BMD (g/cm^3) × TV(mm^3).

Table 3
List of primers for quantitative real-time PCR.

Gene symbol	Accession number	Forward primer (5' to 3')	Reverse primer (5' to 3')
Housekeeping gene			
GAPDH	NM_204305.1	CCTCTCTGGCAAAGTCCAAG	GGTCACGCTCCTGGAAGATA
β-Actin	NM_205518.2	CAACACAGTGCTGTCTGGTGGTA	ATCGTACTCCTGCTTGCTGATCC
Tight junction proteins			
CLDN1	NM_001013611.2	TGGAGGATGACCAGGTGAAGA	CGAGCCACTCTGTTGCCATA
JAM2	XM_025149444.1	AGCCTCAAATGGGATTGGATT	CATCAACTTGCATTCGCTTCA
OCLN	XM_025144248.1	ACGGCAGCACCTACCTCAA	GGCGAAGAAGCAGATGAG
ZO1	XM_015278981.2	CAACTGGTGTGGGTTTCTGAA	TCACTACCAGGAGCTGAGAGGTAA
Mucin			
MUC2	JX_284122.1	ATGCGATGTTAACACAGGACTC	GTGGAGCACAGCAGACTTTG

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; *CLDN1* = claudin 1; *JAM2* = junctional adhesion molecule 2; *OCLN* = occludin; *ZO1* = zonula occludens 1; *MUC2* = mucin 2.

NC + PHY+25OHD group had higher BWG than the PC group ($P = 0.027$).

Eimeria challenge significantly suppressed FI, BWG and FCR of chickens ($P < 0.05$). During 0 to 6 DPI (d 14 to 20), *Eimeria*-challenged birds experienced decreased FI ($P < 0.001$) and increased FCR ($P = 0.001$) compared to non-challenged birds. In the 7 to 12 DPI (d 21 to 26) period, *Eimeria*-challenged birds had a higher FCR ($P = 0.037$). Over the entire period (0 to 12 DPI; d 14 to 26), *Eimeria*-challenged birds exhibited lower BWG and FI, as well as higher FCR ($P < 0.05$).

Furthermore, dietary treatments significantly influenced the growth performance of chickens. During 0 to 6 DPI (d 14 to 20), no significant difference was observed between the NC and PC groups on FCR ($P > 0.05$). However, the birds in the NC + 25OHD group displayed an elevated FCR compared to the NC + PHY group (1.48 vs. 1.40; $P = 0.034$). During 7 to 12 DPI (d 21 to 26), no significant difference on BWG was found between the NC and PC groups ($P > 0.05$), whereas the NC + PHY + 25OHD group had increased BWG ($P = 0.009$) compared to the NC group. For FCR, the birds in the NC group showed an increased FCR compared to the PC group ($P < 0.001$). During the entire period (0 to 12 DPI; d 14 to 26), no significant difference was observed between the NC and PC groups on BWG ($P > 0.05$), but the birds in the NC + PHY + 25OHD group showed higher BWG than the NC group (957 vs. 893 g; $P = 0.007$). For FCR, the birds in the NC group displayed an increased FCR compared to the PC group ($P < 0.001$).

Daily FI results are presented in Table 5. An interaction effect of *Eimeria* challenge and diets was observed only on 2 DPI (d 16; $P = 0.005$). *Eimeria* challenge significantly decreased the FI of the PC birds (78.17 vs. 81.37 g) but significantly increased the FI of NC + PHY + 25OHD birds (82.92 vs. 77.92 g; $P = 0.005$). For the main effect, *Eimeria* challenge significantly decreased ($P < 0.05$) the daily FI of birds on 4, 5, 6 and 7 DPI (d 18, 19, 20 and 21), while it significantly increased ($P < 0.05$) the daily FI of birds on 9 and 12 DPI (d 23 and 26). Additionally, dietary treatments significantly influenced the daily FI of chickens. On 1 DPI (d 15), the birds in the NC and NC + PHY + 25OHD groups showed significantly increased FI compared to birds in the PC group ($P = 0.035$). On 5 DPI (d 19), no significant difference between the NC and PC groups on FI was observed ($P > 0.05$), but the NC + 25OHD birds had higher FI than the NC and NC + PHY birds ($P = 0.012$). Moreover, on 10 DPI (d 24), the birds in the NC group exhibited decreased FI compared to the PC group ($P = 0.013$). The overall mortality in the current study was 0.36 %, with no difference among different treatments ($P > 0.05$).

3.2. Gut permeability and intestinal lesion scores

The gut permeability results revealed that *Eimeria* challenge significantly increased ($P < 0.001$) the serum FITC-d level of birds on 5 DPI (d 19; Table 6). No significant difference was observed between the NC and PC groups ($P > 0.05$). However, the NC + 25OHD group exhibited a higher FITC-d level than the NC + PHY and NC + PHY + 25OHD groups ($P = 0.012$).

No significant interaction between *Eimeria* challenge and diets was observed on lesion scores of the duodenum, jejunum, or ceca of broilers on 6 DPI (d 20; $P > 0.05$). However, *Eimeria*-challenged birds had significantly higher duodenal, jejunal and cecal lesion scores compared to the unchallenged birds ($P < 0.05$), as shown in Table 6.

Table 4
Effects of phytase and 25-Hydroxycholecalciferol and their combination on growth performance of broilers infected with mixed *Eimeria* spp. during 0 to 12 days post inoculation (DPI) (d 14 to 26)[1].

Item[2]		0 to 6 DPI (d 14 to 20)			7 to 12 DPI (d 21 to 26)			0 to 12 DPI (d 14 to 26)		
		BWG, g	FI, g	FCR, g/g	BWG, g	FI, g	FCR, g/g	BWG, g	FI, g	FCR, g/g
Interaction										
UNCHA	PC	406^{a}	561	1.38	566	777	1.38	973	1338	1.38
	NC	382^{bc}	550	1.44	547	770	1.41	930	1320	1.42
	NC + PHY	398^{ab}	546	1.38	567	789	1.39	965	1334	1.38
	NC + 25OHD	386^{abc}	560	1.45	543	770	1.42	930	1330	1.43
	NC + PHY + 25OHD	381^{bcd}	553	1.46	592	765	1.30	973	1318	1.36
CHA	PC	344^{f}	517	1.51	560	764	1.37	904	1282	1.42
	NC	357^{ef}	526	1.48	499	759	1.52	855	1284	1.50
	NC + PHY	359^{def}	511	1.43	549	760	1.39	908	1271	1.40
	NC + 25OHD	356^{ef}	537	1.51	539	781	1.46	919	1318	1.45
	NC + PHY + 25OHD	369^{cde}	535	1.45	572	772	1.35	941	1307	1.39
SEM		3.4	3.2	0.009	5.6	4.1	0.011	6.7	5.8	0.007
P-value		0.027	0.513	0.136	0.666	0.587	0.195	0.323	0.445	0.441
Challenge effect										
UNCHA		391^{a}	554^{a}	1.42^{b}	563	774	1.38^{b}	954^{a}	1328^{a}	1.39^{b}
CHA		357^{b}	525^{b}	1.47^{a}	544	767	1.42^{a}	905^{b}	1292^{b}	1.43^{a}
P-value		<0.001	<0.001	0.001	0.061	0.422	0.037	<0.001	0.002	0.002
Diet effect										
PC		375	539	1.44^{ab}	563^{ab}	771	1.37^{bc}	938^{ab}	1310	1.40^{bc}
NC		370	538	1.46^{ab}	523^{b}	764	1.47^{a}	893^{b}	1302	1.46^{a}
NC + PHY		379	529	1.40^{b}	558^{ab}	774	1.39^{abc}	937^{ab}	1303	1.39^{bc}
NC + 25OHD		371	549	1.48^{a}	541^{ab}	775	1.44^{ab}	925^{ab}	1324	1.44^{ab}
NC + PHY + 25OHD		375	544	1.45^{ab}	582^{a}	768	1.33^{c}	957^{a}	1313	1.37^{c}
P-value		0.769	0.124	0.034	0.009	0.926	<0.001	0.007	0.721	<0.001

BWG = body weight gain; FI = feed intake; FCR = feed conversion ratio.
[a-f] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] *n* (d 14) = 6 replicate cages × 14 birds/cage = 84; *n* (d 20) = 6 replicate cages × 13 birds/cage = 78; *n* (d 26) = 6 replicate cages × 5 birds/cage = 30.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC+25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY+25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

Table 5
Effects of phytase and 25-Hydroxycholecalciferol and their combination on daily feed intake of broilers infected with mixed *Eimeria* spp. from 1 to 12 days post inoculation (DPI) (d 15 to 26)[1].

Item[2]		1 DPI	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI	7 DPI	8 DPI	9 DPI	10 DPI	11 DPI	12 DPI
Interaction													
UNCHA	PC	76.11	81.37^{ab}	88.34	99.52	104.63	110.62	133.10	110.50	127.23	134.13	137.43	134.80
	NC	78.54	79.98^{bcd}	87.32	94.95	99.72	109.08	134.33	107.77	129.40	130.30	137.57	130.83
	NC + PHY	77.10	78.09^{d}	89.19	95.90	99.90	109.49	135.62	110.05	131.33	134.58	138.82	138.11
	NC + 25OHD	77.56	81.17^{abc}	89.72	97.32	104.19	110.24	132.30	109.74	127.61	131.50	136.49	132.00
	NC + PHY + 25OHD	78.51	77.92^{d}	90.70	96.63	98.95	110.77	131.46	106.97	131.65	130.59	136.45	127.77
CHA	PC	77.12	78.17^{cd}	87.98	96.73	88.50	90.69	94.42	110.83	134.48	143.12	139.89	146.12
	NC	79.50	79.24^{bcd}	88.00	95.60	87.04	96.21	108.53	109.00	134.27	125.17	135.60	145.97
	NC + PHY	76.96	77.93^{d}	86.07	92.48	85.64	92.21	98.87	107.33	131.80	138.04	136.33	143.93
	NC + 25OHD	78.23	81.77^{ab}	88.22	95.04	92.16	101.54	110.57	112.13	139.50	132.10	140.90	145.83
	NC + PHY + 25OHD	78.67	82.92^{a}	90.11	94.77	91.03	97.81	114.20	111.97	134.64	137.92	137.47	137.17
SEM		0.280	0.376	0.452	0.492	1.012	1.343	2.319	0.637	0.938	1.123	1.021	1.328
P-value		0.951	0.005	0.742	0.705	0.159	0.415	0.064	0.442	0.316	0.194	0.832	0.696
Challenge effect													
UNCHA		77.56	79.70	89.05	96.86^{a}	101.53^{a}	110.04^{a}	133.36^{a}	109.01	129.44^{b}	132.22	137.35	132.70^{b}
CHA		78.10	80.07	88.07	94.92^{b}	88.87^{b}	95.69^{b}	105.32^{b}	110.25	134.95^{a}	135.08	137.91	143.80^{a}
P-value		0.330	0.642	0.286	0.045	<0.001	<0.001	<0.001	0.341	0.003	0.139	0.756	<0.001
Diet effect													
PC		76.61^{c}	79.91^{ab}	88.16	98.13	96.56^{ab}	100.65	113.76	110.66	130.85	138.63^{a}	138.42	140.46
NC		79.02^{a}	79.61^{ab}	87.66	95.27	93.38^{b}	102.64	121.43	108.38	131.83	127.73^{b}	136.58	138.40
NC + PHY		77.03^{bc}	78.01^{b}	87.63	94.19	92.12^{b}	100.85	117.24	108.69	131.57	136.15^{ab}	137.58	141.02
NC + 25OHD		77.89^{abc}	81.47^{a}	88.97	96.18	98.17^{a}	105.89	121.43	110.94	133.55	131.80^{ab}	138.70	138.92
NC + PHY + 25OHD		78.59^{ab}	80.42^{a}	90.41	95.70	94.99^{ab}	104.29	122.83	109.47	133.01	133.92^{ab}	136.96	132.47
P-value		0.035	0.023	0.280	0.129	0.012	0.380	0.206	0.647	0.862	0.013	0.960	0.126

[a-f] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] *n* (1-5 DPI) = 6 replicate cages × 14 birds/cage = 84; *n* (6 DPI) = 6 replicate cages × 13 birds/cage = 78; *n* (7-12 DPI) = 6 replicate cages × 5 birds/cage = 30.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC+25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

3.3. Mineral apposition rate

The results of MAR are presented in Table 6. An interaction effect between *Eimeria* challenge and diets was observed ($P = 0.019$). *Eimeria* challenge significantly decreased the MAR of the PC and NC + 25OHD birds ($P = 0.019$). In both unchallenged and *Eimeria*-challenged groups, reducing the levels of Ca and avP in the diet (NC groups) significantly decreased the MAR compared to the PC

Table 6
Effects of phytase and 25-Hydroxycholecalciferol and their combination on fluorescein isothiocyanate-dextran level on 5 days post inoculation (DPI) (d 19), lesion scores on 6 DPI (d 20), and mineral apposition rate during 4 to 8 DPI (d 18 to 22) of broilers infected with mixed *Eimeria* spp[1].

Item[2]		FITC-d level, ng/mL	Lesion scores			MAR, μm
			Duodenum	Jejunum	Ceca	
Interaction						
UNCHA	PC	33	0.00	0.17	0.08	438.63^{ab}
	NC	26	0.00	0.33	0.17	267.77^{ef}
	NC + PHY	12	0.00	0.33	0.08	406.16^{bc}
	NC + 25OHD	39	0.00	0.50	0.00	527.20^{a}
	NC + PHY + 25OHD	8	0.00	0.17	0.00	376.35^{bcd}
CHA	PC	101	1.75	1.17	0.95	297.68^{cde}
	NC	86	1.50	1.11	0.50	174.74^{f}
	NC + PHY	84	1.33	1.28	0.53	360.23^{bcde}
	NC + 25OHD	135	1.97	1.61	0.58	284.03^{de}
	NC + PHY + 25OHD	67	1.47	0.86	0.67	395.33^{bc}
SEM		7.0	0.119	0.088	0.057	16.614
P-value		0.685	0.443	0.856	0.358	0.019
Challenge effect						
UNCHA		23^{b}	0.00^{b}	0.30^{b}	0.07^{b}	398.69^{a}
CHA		97^{a}	1.61^{a}	1.21^{a}	0.64^{a}	302.40^{b}
P-value		<0.001	<0.001	<0.001	<0.001	<0.001
Diet effect						
PC		60^{ab}	0.88	0.67	0.51	368.16^{a}
NC		59^{ab}	0.75	0.72	0.33	221.25^{b}
NC + PHY		45^{b}	0.67	0.81	0.31	381.10^{a}
NC + 25OHD		87^{a}	0.99	1.06	0.29	394.56^{a}
NC + PHY + 25OHD		31^{b}	0.74	0.51	0.33	385.84^{a}
P-value		0.012	0.443	0.133	0.481	<0.001

FITC-d = fluorescein isothiocyanate-d; MAR = mineral apposition rate.
[a-f] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] *n* (FITC-d) = 6 replicate cages × 1 bird/cage = 6; *n* (Lesion scores) = 6 replicate cages × 3 birds/cage = 18; *n* (MAR) = 6 replicate cages × 1 bird/cage = 6.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

(unchallenged: 267.77 vs. 438.63 μm; *Eimeria* challenge: 174.74 vs. 297.68 μm; $P < 0.001$). However, the supplementation of phytase, 25OHD, or both, increased the MAR of birds to the same levels as the PC groups had ($P < 0.001$).

3.4. Bone ash

The results of bone ash parameters are detailed in Tables 7 and 8. On 6 DPI (d 20), no interaction effect between *Eimeria* challenge and diets was observed regarding bone ash parameters ($P > 0.05$; Table 7). For FFBW, no significant difference was found between the NC and PC groups ($P > 0.05$), whereas the NC + 25OHD group exhibited a lower FFBW compared to the NC + PHY group ($P = 0.004$). Additionally, birds in the NC group had significantly reduced ash weight and ash concentration compared to the PC birds; however, supplementing with phytase, or a combination of phytase and 25OHD to the NC diet improved ash weight and ash concentration to the levels equivalent to the PC group had ($P < 0.05$). For ash percentage, the birds in the NC group displayed significantly lower ash percentage compared to the birds in the PC group (43.67% vs. 48.10%; $P < 0.001$). Nonetheless, supplementing with phytase or 25OHD was effective in improving the ash percentage of birds. Moreover, when supplementing phytase and 25OHD together, the ash percentage of birds was elevated to the same level as PC birds ($P < 0.001$). Notably, *Eimeria* challenge did not show any impact on bone ash parameters on 6 DPI (d 20; $P > 0.05$).

On 12 DPI (d 26), an interaction effect between *Eimeria* challenge and diets was observed on ash percentage (Table 8; $P = 0.024$). *Eimeria* challenge significantly compromised the ash percentage of the PC and NC + 25OHD birds ($P = 0.024$). In both unchallenged and *Eimeria*-challenged groups, reducing the levels of Ca and avP in the diet (NC groups) significantly decreased the ash percentage of birds compared to the PC (unchallenged: 45.72% vs. 52.10%; *Eimeria* challenge: 46.66% vs. 50.88%; $P < 0.001$); however, supplementing with phytase, 25OHD, or both was able to improve the ash percentage of birds ($P < 0.001$). Importantly, phytase or a combination of phytase and 25OHD showed better improvement than 25OHD alone, achieving the same ash percentage (NC + PHY group) as the PC group had or higher levels (NC + PHY + 25OHD group) than the PC group ($P < 0.001$).

Eimeria challenge significantly suppressed bone ash parameters in chickens on 12 DPI (d 26; Table 8; $P < 0.05$). *Eimeria*-challenged birds experienced significantly decreased initial bone weight (13.91 vs. 14.95 g; $P < 0.001$), bone volume (11.69 vs. 12.53 cm^3; $P < 0.001$), FFBW (4.96 vs. 5.37 g; $P < 0.001$), ash weight (2.47 vs. 2.70 g; $P < 0.001$), and ash percentage (49.59% vs. 50.20%; $P = 0.013$). In addition, dietary treatments significantly influenced the FFBW, ash weight, and ash concentration of chickens on 12 DPI (d 26; $P < 0.05$; Table 8). Reducing Ca and avP in the diet (NC group) significantly decreased the FFBW, ash weight, ash percentage, and ash concentration of birds compared to the PC ($P < 0.05$); however, supplementing with phytase or a combination of phytase and 25OHD was able to improve the FFBW, ash weight, ash percentage, and ash concentration to the same level as the PC group had ($P < 0.05$). In addition, 25OHD alone significantly increased ash concentration compared to the NC group ($P < 0.001$).

3.5. Micro-CT

3.5.1. Total volume of interest

The impact of *Eimeria* challenge and dietary factors on microstructure changes within the total VOI of the femur on 6 DPI (d 20) and 12 DPI (d 26) is shown in Tables 9 and 10. On 6 DPI (d 20), interaction effects between *Eimeria* challenge and diets were observed for bone surface/bone volume (BS/BV; $P = 0.023$) and

Table 7
Effects of phytase and 25-Hydroxycholecalciferol and their combination on tibia bone ash parameters of broilers infected with mixed *Eimeria* spp. on 6 days post inoculation (DPI) (d 20)[1].

Item[2]		Initial bone weight, g	Bone volume, cm^3	FFBW, g	Ash weight, g	Ash percentage, %	Ash concentration, g/cm^3
Interaction							
UNCHA	PC	8.86	7.31	3.59	1.73	48.17	0.24
	NC	8.68	7.14	3.23	1.40	43.38	0.20
	NC + PHY	9.27	7.69	3.67	1.73	47.25	0.23
	NC + 25OHD	8.53	7.31	3.20	1.42	44.40	0.19
	NC + PHY + 25OHD	8.57	7.07	3.51	1.69	48.15	0.24
CHA	PC	8.97	7.42	3.58	1.72	48.03	0.23
	NC	8.77	7.37	3.28	1.44	43.95	0.20
	NC + PHY	8.94	7.37	3.53	1.65	46.76	0.22
	NC + 25OHD	8.73	7.37	3.25	1.38	45.14	0.20
	NC + PHY + 25OHD	9.12	7.49	3.66	1.75	47.73	0.23
SEM		0.094	0.082	0.044	0.028	0.256	0.003
P-value		0.712	0.738	0.854	0.861	0.243	0.921
Challenge effect							
UNCHA		8.78	7.30	3.44	1.60	46.27	0.22
CHA		8.91	7.41	3.46	1.59	46.36	0.22
P-value		0.521	0.550	0.814	0.863	0.800	0.493
Diet effect							
PC		8.91	7.37	3.58^{ab}	1.72^{a}	48.10^{a}	0.23^{a}
NC		8.73	7.26	3.26^{ab}	1.42^{b}	43.67^{d}	0.20^{b}
NC + PHY		9.10	7.53	3.60^{a}	1.69^{a}	47.00^{b}	0.22^{a}
NC + 25OHD		8.63	7.34	3.22^{b}	1.40^{b}	44.74^{c}	0.20^{b}
NC + PHY + 25OHD		8.84	7.28	3.59^{ab}	1.72^{a}	47.94^{ab}	0.24^{a}
P-value		0.591	0.866	0.004	<0.001	<0.001	<0.001

FFBW = fat free bone weight.
[a,b] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] $n = 6$ replicate cages × 2 birds/cage = 12.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

Table 8
Effects of phytase and 25-Hydroxycholecalciferol and their combination on tibia bone ash parameters of broilers infected with mixed *Eimeria* spp. on 12 days post inoculation (DPI) (d 26)[1].

Item[2]		Initial bone weight, g	Bone volume, cm^3	FFBW, g	Ash weight, g	Ash percentage, %	Ash concentration, g/cm^3
Interaction							
UNCHA	PC	14.66	12.27	5.40	2.81	52.10^{ab}	0.23
	NC	15.28	13.09	5.05	2.31	45.72^{f}	0.18
	NC + PHY	15.42	12.81	5.67	2.91	51.29^{bc}	0.23
	NC + 25OHD	14.48	12.19	5.13	2.54	49.41^{d}	0.21
	NC + PHY + 25OHD	14.94	12.31	5.60	2.94	52.51^{a}	0.24
CHA	PC	13.82	11.46	5.12	2.61	50.88^{c}	0.23
	NC	13.47	11.62	4.50	2.10	46.66^{f}	0.18
	NC + PHY	14.17	11.87	5.13	2.59	50.41^{cd}	0.22
	NC + 25OHD	13.52	11.57	4.65	2.23	48.00^{e}	0.19
	NC + PHY + 25OHD	14.58	11.92	5.41	2.81	52.00^{ab}	0.24
SEM		0.143	0.116	0.066	0.045	0.314	0.003
P-value		0.455	0.557	0.728	0.804	0.024	0.205
Challenge effect							
UNCHA		14.95^{a}	12.53^{a}	5.37^{a}	2.70^{a}	50.20^{a}	0.22
CHA		13.91^{b}	11.69^{b}	4.96^{b}	2.47^{b}	49.59^{b}	0.21
P-value		<0.001	<0.001	<0.001	<0.001	0.013	0.102
Diet effect							
PC		14.24	11.87	5.26^{ab}	2.71^{a}	51.49^{ab}	0.23^{ab}
NC		14.37	12.36	4.77^{c}	2.20^{b}	46.19^{d}	0.18^{d}
NC + PHY		14.79	12.34	5.40^{a}	2.75^{a}	50.85^{b}	0.22^{b}
NC + 25OHD		14.00	11.88	4.89^{bc}	2.38^{b}	48.70^{c}	0.20^{c}
NC + PHY + 25OHD		14.76	12.12	5.50^{a}	2.88^{a}	52.26^{a}	0.24^{a}
P-value		0.217	0.397	<0.001	<0.001	<0.001	<0.001

FFBW = fat free bone weight.
[a-f] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] $n = 6$ replicate cages × 2 birds/cage = 12.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20 % Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC+25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY+25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

BMD ($P = 0.038$; Table 9). In terms of BS/BV, *Eimeria* challenge led to a significant decrease in BS/BV for birds in the NC and NC + PHY + 25OHD groups, whereas it increased BS/BV for birds in the NC + PHY group ($P = 0.023$). Among the unchallenged groups, both NC and NC + 25OHD groups exhibited significantly higher BS/BV ratios compared to PC birds ($P = 0.023$). However, in *Eimeria*-challenged groups, no significant difference in BS/BV between the NC and PC groups was observed ($P > 0.05$). Interestingly,

supplementing phytase to the NC diet in *Eimeria*-challenged birds resulted in a higher BS/BV ratio compared to the PC group (14.54 vs. 11.97 mm^{-1}; $P = 0.023$). Regarding BMD, *Eimeria* challenge led to a significant decrease in BMD for birds in the PC and NC + 25OHD groups ($P = 0.038$). In unchallenged groups, the NC group showed a significantly lower BMD compared to PC birds (0.191 vs. 0.263 g/cm^3; $P = 0.038$). However, supplementation with phytase (NC + PHY), 25OHD (NC + 25OHD), or both (NC + PHY + 25OHD) improved the BMD of birds in unchallenged groups ($P = 0.038$). Notably, the NC + 25OHD and NC + PHY + 25OHD groups achieved BMD levels similar to the PC group ($P = 0.038$). In *Eimeria*-challenged groups, no significant difference in BMD between the NC and PC groups was observed ($P > 0.05$). Nevertheless, supplementing with a combination of phytase and 25OHD to the NC diet (NC + PHY + 25OHD) in *Eimeria*-challenged groups resulted in higher BMD compared to the PC group (0.259 vs. 0.227 g/cm^3; $P = 0.038$).

Additionally, *Eimeria* challenge exhibited a significant reduction only in bone volume as a fraction of tissue volume (BV/TV; 32.06% vs. 34.67%; $P = 0.037$) on 6 DPI (d 20). The dietary treatments had significant influence on bone volume (BV), BV/TV, BMD, and BMC in the microstructure of femurs on 6 DPI (d 20; Table 9; $P < 0.05$). Among the dietary groups, the NC birds displayed significantly lower BV, BV/TV, BMD, and BMC compared to PC birds ($P < 0.05$). However, supplementation with phytase alone (NC + PHY) or a combination of phytase and 25OHD (NC + PHY + 25OHD) resulted in increased levels of BV, BV/TV, BMD, and BMC, aligning closely with the values observed in the PC group ($P < 0.05$).

On 12 DPI (d 26), no significant interaction was noted between *Eimeria* challenge and diets concerning femoral microstructural architecture, as indicated in Table 10. The *Eimeria* challenge, however, had a significant suppressive effect on tissue volume (TV; $P = 0.008$) and tissue surface (TS) in birds ($P = 0.012$). Additionally, the dietary treatments exerted an influence on the microstructural architecture parameters of femur bones. Among the dietary groups, the NC birds exhibited significantly lower BV, BV/TV, BMD, and BMC compared to PC birds ($P < 0.05$). Nevertheless, supplementation with phytase alone (NC + PHY) or a combination of phytase and 25OHD (NC + PHY + 25OHD) led to increased levels of BV, BV/TV, BMD, and BMC, to similar levels of the values observed in the PC group ($P < 0.05$).

3.5.2. Trabecular bone

The effects of *Eimeria* challenge and diets on femoral microstructural architectural changes of the trabecular bone are shown in Tables 11 and 12. On 6 DPI (d 20), significant interaction effects between *Eimeria* challenge and diets were observed on BMD ($P = 0.028$) and trabecular separation (Tb.Sp; $P = 0.027$; Table 11). Regarding BMD, the *Eimeria*-challenged PC and *Eimeria*-challenged NC groups exhibited lower BMD compared to the unchallenged NC + 25OHD and *Eimeria*-challenged NC + PHY + 25OHD groups ($P = 0.028$). In terms of Tb.Sp, *Eimeria* challenge increased Tb.Sp in the NC and NC + 25OHD groups ($P = 0.027$). No significant difference was found among unchallenged groups ($P > 0.05$). However, in *Eimeria*-challenged groups, the NC + PHY and NC + PHY + 25OHD birds showed lower Tb.Sp values compared to other *Eimeria*-challenged groups ($P = 0.027$).

Furthermore, the *Eimeria* challenge exhibited a significant increase in trabecular pattern factor (Tb.Pf; 16.02 vs. 14.83 mm^{-1}; $P = 0.044$) on 6 DPI (d 20; Table 11). The dietary treatments had a significant influence on the structure model index (SMI) in the femoral microstructural architectural of trabecular bones on 6 DPI (d 20; $P = 0.015$; Table 11). Among the dietary groups, the NC birds displayed a significantly higher SMI compared to PC birds (2.58 vs. 2.44; $P = 0.015$). However, supplementation with a combination of phytase and 25OHD (NC + PHY+25OHD) resulted in a decreased SMI to a level similar to that of the PC group ($P = 0.015$).

Table 9
Effects of phytase and 25-Hydroxycholecalciferol and their combination on femur metaphysis total bone of broilers infected with mixed *Eimeria* spp. on 6 days post inoculation (DPI) (d 20)[1].

Item[2]		TV, mm^3	BV, mm^3	BV/TV, %	TS, mm^2	BS, mm^2	BS/BV, mm^{-1}	BS/TV, mm^{-1}	BMD, g/cm^3	BMC, mg
Interaction										
UNCHA	PC	244.95	97.38	39.71	266.91	1087.47	11.13^{de}	4.38	0.263^{a}	64.34
	NC	224.08	64.28	28.47	291.56	930.99	14.84^{a}	4.11	0.191^{f}	43.41
	NC + PHY	239.03	82.89	34.84	268.55	952.39	11.45^{de}	3.96	0.230^{bcde}	54.90
	NC + 25OHD	220.47	78.77	33.44	261.06	1047.36	13.23^{abc}	4.35	0.234^{abcd}	55.44
	NC + PHY + 25OHD	260.05	94.63	36.88	277.64	1174.60	12.40^{abcd}	4.49	0.240^{abc}	61.54
CHA	PC	245.65	83.03	33.81	264.83	993.19	11.97^{cde}	4.04	0.227^{cde}	55.71
	NC	237.78	71.46	30.20	258.99	861.09	12.13^{cde}	3.64	0.205^{def}	48.65
	NC + PHY	252.69	77.46	30.83	318.78	1113.17	14.54^{ab}	4.40	0.218^{cdef}	54.67
	NC + 25OHD	236.35	66.25	28.13	285.37	859.22	13.11^{abc}	3.64	0.200^{ef}	47.25
	NC + PHY + 25OHD	249.43	97.34	37.31	277.01	1013.03	10.04^{e}	4.15	0.259^{ab}	67.89
SEM		3.587	2.109	0.742	5.408	30.189	0.332	0.078	0.0044	1.350
P-value		0.738	0.165	0.185	0.185	0.368	0.023	0.159	0.038	0.069
Challenge effect										
UNCHA		238.31	83.59	34.67^{a}	273.14	1038.56	12.61	4.26	0.232	55.92
CHA		244.21	79.11	32.06^{b}	279.69	966.38	12.44	3.97	0.222	54.83
P-value		0.353	0.169	0.037	0.471	0.238	0.680	0.059	0.164	0.604
Diet effect										
PC		245.30	90.20^{ab}	36.76^{a}	265.87	1040.32	11.55	4.21	0.245^{ab}	60.03^{ab}
NC		230.93	67.87^{c}	29.34^{b}	275.27	896.04	13.49	3.88	0.198^{c}	46.03^{c}
NC + PHY		245.86	80.17^{bc}	32.84^{ab}	291.38	1032.78	13.00	4.18	0.224^{abc}	54.78^{bc}
NC+25OHD		229.13	72.51^{c}	30.79^{b}	273.21	953.29	13.17	3.99	0.217^{bc}	51.35^{bc}
NC + PHY+25OHD		255.22	95.98^{a}	37.09^{a}	277.32	1101.16	11.32	4.32	0.250^{a}	64.71^{a}
P-value		0.127	<0.001	<0.001	0.605	0.255	0.075	0.332	<0.001	<0.001

TV = tissue volume; BV = bone volume; BV/TV = bone volume/tissue volume ratio; TS = tissue surface; BS = bone surface; BS/BV = bone surface/bone volume ratio; BS/TV = bone surface/tissue volume ratio; BMD = bone mineral density; BMC = bone mineral content.
[a-f] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] $n = 6$ replicate cages × 1 bird/cage = 6.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25-hydroxycholecalciferol; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25-hydroxycholecalciferol.

Table 10
Effects of phytase and 25-Hydroxycholecalciferol and their combination on femur metaphysis total bone of broilers infected with mixed *Eimeria* spp. on 12 days post inoculation (DPI) (d 26)[1].

Item[2]		TV, mm^3	BV, mm^3	BV/TV, %	TS, mm^2	BS, mm^2	BS/BV, mm^{-1}	BS/TV, mm^{-1}	BMD, g/cm^3	BMC, mg
Interaction										
UNCHA	PC	422.73	175.77	41.66	375.35	1497.26	8.52	3.50	0.257	108.27
	NC	421.70	142.12	33.91	372.50	1459.29	10.25	3.48	0.182	76.21
	NC + PHY	431.56	175.84	40.78	382.54	1610.40	9.20	3.74	0.256	110.29
	NC + 25OHD	401.02	145.72	36.50	359.02	1329.70	9.14	3.30	0.213	85.18
	NC + PHY + 25OHD	420.95	180.25	42.92	376.02	1424.69	7.98	3.37	0.263	110.15
CHA	PC	381.80	161.27	43.88	347.20	1391.43	9.41	4.13	0.263	97.35
	NC	392.47	145.09	37.18	351.51	1321.42	9.95	3.68	0.211	80.12
	NC + PHY	408.43	163.56	40.58	367.63	1438.53	9.25	3.76	0.247	102.35
	NC + 25OHD	380.96	135.19	35.52	345.04	1272.27	9.44	3.33	0.216	82.04
	NC + PHY + 25OHD	418.37	175.55	42.34	373.83	1547.89	8.92	3.68	0.260	107.78
SEM		4.442	3.110	0.690	3.265	32.395	0.208	0.076	0.0049	2.209
P-value		0.699	0.801	0.727	0.750	0.611	0.857	0.702	0.520	0.563
Challenge effect										
UNCHA		419.59[a]	163.94	39.15	373.09[a]	1464.27	9.02	3.48	0.234	98.02
CHA		397.22[b]	155.88	39.90	357.61[b]	1395.63	9.39	3.72	0.239	93.87
P-value		0.008	0.114	0.529	0.012	0.285	0.372	0.115	0.464	0.159
Diet effect										
PC		406.36	169.97[a]	42.77[a]	364.09	1454.93	8.96	3.82	0.260[a]	103.90[a]
NC		408.41	143.47[b]	35.55[b]	362.96	1396.62	10.10	3.58	0.196[b]	77.99[b]
NC + PHY		421.05	170.26[a]	40.68[ab]	375.76	1532.27	9.23	3.75	0.252[a]	106.68[a]
NC + 25OHD		390.99	140.45[b]	36.01[b]	352.03	1300.98	9.29	3.32	0.214[b]	83.61[b]
NC + PHY + 25OHD		419.66	177.90[a]	42.63[a]	374.93	1486.29	8.45	3.53	0.261[a]	108.97[a]
P-value		0.146	<0.001	<0.001	0.087	0.207	0.173	0.248	<0.001	<0.001

TV = tissue volume; BV = bone volume; BV/TV = bone volume/tissue volume ratio; TS = tissue surface; BS = bone surface; BS/BV = bone surface/bone volume ratio; BS/TV = bone surface/tissue volume ratio; BMD = bone mineral density; BMC = bone mineral content.
[a,b] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] $n = 6$ replicate cages × 1 bird/cage = 6.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25-hydroxycholecalciferol; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25-hydroxycholecalciferol.

Table 11
Effects of phytase and 25-Hydroxycholecalciferol and their combination on femur metaphysis trabecular bone of broilers infected with mixed *Eimeria* spp. on 6 days post inoculation (DPI) (d 20)[1].

Item[2]		TV, mm^3	BV, mm^3	BMD, g/cm^3	BMC, mg	Tb.Th, mm^{-1}	Tb.Sp, mm	Tb.N, mm	Tb.Pf, mm^{-1}	SMI
Interaction										
UNCHA	PC	123.91	6.79	0.107[ab]	13.28	0.102	2.52[abc]	0.54	13.92	2.41
	NC	141.47	5.54	0.100[ab]	12.73	0.099	2.12[c]	0.53	15.50	2.58
	NC + PHY	135.88	6.52	0.086[ab]	12.03	0.101	2.46[abc]	0.47	15.36	2.53
	NC + 25OHD	137.60	6.42	0.119[a]	16.68	0.100	2.16[c]	0.54	15.09	2.52
	NC + PHY + 25OHD	139.43	7.44	0.096[ab]	12.98	0.099	2.42[abc]	0.54	14.38	2.43
CHA	PC	141.09	6.48	0.091[b]	12.67	0.099	2.81[a]	0.47	14.89	2.46
	NC	148.12	5.70	0.090[b]	13.30	0.099	2.80[a]	0.39	16.06	2.59
	NC + PHY	151.31	7.90	0.108[ab]	16.03	0.097	2.13[c]	0.54	17.18	2.63
	NC + 25OHD	152.45	5.65	0.095[ab]	14.66	0.095	2.70[ab]	0.39	17.14	2.61
	NC + PHY + 25OHD	127.95	7.81	0.117[a]	17.16	0.102	2.25[bc]	0.52	14.84	2.50
SEM		2.883	0.272	0.0029	0.590	0.0010	0.062	0.016	0.294	0.018
P-value		0.523	0.770	0.028	0.365	0.657	0.027	0.221	0.857	0.908
Challenge effect										
UNCHA		135.66	6.62	0.102	13.54	0.100	2.34	0.52	14.83[b]	2.50
CHA		144.74	6.71	0.100	14.76	0.098	2.54	0.46	16.02[a]	2.56
P-value		0.145	0.761	0.821	0.304	0.426	0.081	0.059	0.044	0.075
Diet effect										
PC		132.50	6.63	0.099	12.98	0.100	2.67	0.50	14.40	2.44[c]
NC		144.79	5.63	0.094	13.01	0.099	2.46	0.46	15.81	2.58[a]
NC + PHY		143.59	7.21	0.097	14.03	0.099	2.29	0.51	16.27	2.58[a]
NC + 25OHD		145.03	6.00	0.107	15.67	0.098	2.43	0.47	16.12	2.56[ab]
NC + PHY + 25OHD		134.21	7.63	0.106	15.07	0.100	2.33	0.53	14.61	2.47[bc]
P-value		0.446	0.156	0.528	0.507	0.907	0.274	0.564	0.124	0.015

TV = tissue volume; BV = bone volume; BMD = bone mineral density; BMC = bone mineral content; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; Tb.N = trabecular number; Tb.Pf = trabecular pattern factor; SMI = structure model index.
[a–c] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] $n = 6$ replicate cages × 1 bird/cage = 6.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

On 12 DPI (d 26), no significant interaction was noted between *Eimeria* challenge and diets concerning femoral microstructural architecture, as detailed in Table 12. However, the *Eimeria* challenge exhibited a significant decrease on TV of birds (215.81 vs. 231.77 mm^3; $P = 0.049$) on 12 DPI (d 26). Additionally, the dietary treatments exerted impact on the microstructural architecture

Table 12
Effects of phytase and 25-Hydroxycholecalciferol and their combination on femur metaphysis trabecular bone of broilers infected with mixed *Eimeria* spp. on 12 days post inoculation (PDI) (d 26)[1].

Item[2]		TV, mm^3	BV, mm^3	BMD, g/cm^3	BMC, mg	Tb.Th, mm	Tb.Sp, mm	Tb.N, mm^{-1}	Tb.Pf, mm^{-1}	SMI
Interaction		223.07	15.76	0.124	27.62	0.137	3.00	0.50	7.07	1.93
UNCHA	PC	256.81	14.47	0.083	21.53	0.126	3.34	0.46	9.50	2.22
	NC	231.52	17.90	0.131	30.18	0.133	2.91	0.59	7.27	1.94
	NC + PHY	231.88	12.55	0.090	21.21	0.129	3.53	0.41	8.77	2.12
	NC + 25OHD	215.57	14.07	0.121	25.90	0.150	3.03	0.44	6.71	1.97
	NC + PHY + 25OHD	189.95	9.60	0.107	20.28	0.127	3.47	0.47	8.80	2.14
CHA	PC	224.47	12.51	0.077	17.28	0.127	3.22	0.43	8.73	2.13
	NC	221.85	15.83	0.123	27.35	0.140	3.39	0.52	6.75	1.92
	NC + PHY	222.08	11.29	0.097	21.47	0.133	3.51	0.38	9.62	2.26
	NC + 25OHD	214.53	15.38	0.121	26.08	0.145	3.44	0.50	6.24	1.86
	NC + PHY + 25OHD	223.07	15.76	0.124	27.62	0.137	3.00	0.50	7.07	1.93
SEM		4.357	0.628	0.0043	1.084	0.0018	0.067	0.017	0.251	0.028
P-value		0.675	0.453	0.911	0.768	0.464	0.442	0.798	0.287	0.136
Challenge effect										
UNCHA		231.77[a]	14.95	0.111	25.42	0.135	3.16	0.48	7.87	2.03
CHA		215.81[b]	13.08	0.105	22.68	0.134	3.41	0.46	8.03	2.06
P-value		0.049	0.101	0.528	0.190	0.846	0.069	0.524	0.702	0.582
Diet effect										
PC		209.82	13.30	0.117[a]	24.68	0.132[b]	3.24	0.49	7.93[ab]	2.04[ab]
NC		242.11	13.58	0.080[b]	19.41	0.126[b]	3.28	0.44	9.12[a]	2.17[a]
NC + PHY		227.12	16.96	0.127[a]	28.89	0.136[ab]	3.15	0.55	7.01[b]	1.93[b]
NC + 25OHD		226.98	11.92	0.094[ab]	21.34	0.131[b]	3.52	0.40	9.20[a]	2.19[a]
NC + PHY + 25OHD		215.05	14.73	0.121[a]	25.99	0.147[a]	3.24	0.47	6.48[b]	1.91[b]
P-value		0.148	0.101	0.002	0.057	0.002	0.463	0.065	<0.001	<0.001

TV = tissue volume; BV = bone volume; BMD = bone mineral density; BMC = bone mineral content; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation; Tb.N = trabecular number; Tb.Pf = trabecular pattern factor; SMI = structure model index.
[a,b] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] $n = 6$ replicate cages × 1 bird/cage = 6.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

parameters of femur bones on 12 DPI (d 26). Birds fed with a reduced Ca and avP diet (NC) displayed lower BMD compared to PC birds (0.080 vs. 0.117 g/cm^3; $P = 0.002$), whereas supplementing phytase, or 25OHD, or both, improved BMD to same level as the PC group ($P = 0.002$). No significant difference was found between the NC and PC groups in trabecular thickness (Tb.Th; $P > 0.05$), but birds in the NC + PHY + 25OHD group showed higher Tb.Th than the birds from other groups ($P = 0.002$). The NC + PHY and NC + PHY + 25OHD groups showed significantly decreased Tb.Pf and SMI values compared to the NC and NC + 25OHD groups on 12 DPI (d 26; $P < 0.05$).

3.5.3. Cortical bone

The impact of *Eimeria* challenge and dietary variations on femoral microstructure changes of the cortical bone is detailed in Tables 13 and 14. On 6 DPI (d 20), an interaction effect between *Eimeria* challenge and diets was observed regarding bone surface (BS; $P = 0.009$; Table 13). The unchallenged NC, *Eimeria*-challenged NC, and *Eimeria*-challenged NC + 25OHD groups exhibited lower BS compared to the unchallenged PC, unchallenged NC + PHY + 25OHD, and *Eimeria*-challenged NC + PHY + 25OHD groups ($P < 0.05$).

While no significant effect was observed from *Eimeria* challenge on the cortical bone microstructure on 6 DPI (d 20), the dietary treatments exhibited significant influence on TV, BV, BMC, volume of closed pores (Po.V(cl)), and surface of closed pores (Po.S(cl)) in the femoral microstructure of cortical bones on 6 DPI (d 20; $P < 0.05$; Table 13). Among the dietary groups, the NC birds displayed significantly decreased TV, BV, BMC, Po.V(cl), and Po.S(cl) compared to PC birds ($P < 0.05$). However, supplementation with phytase alone (NC + PHY), or a combination of phytase and 25OHD (NC + PHY + 25OHD) increased these parameters to values similar to those of the PC birds ($P < 0.05$).

On 12 DPI (d 26), no significant interaction was noted between *Eimeria* challenge and diets concerning femoral microstructure of cortical bones, as detailed in Table 14. However, the *Eimeria* challenge exhibited a significant decrease on TS of birds (456.91 vs. 475.16 mm^2; $P = 0.024$) on 12 DPI (d 26). Additionally, the dietary treatments exerted impact on cortical bone parameters on 12 DPI (d 26). Birds fed with a reduced Ca and avP diet (NC) displayed lower TV, BV, BMC, Po.V(cl), Po.S(cl), and closed porosity percentage (Po(cl)) compared to PC birds (Table 14; $P < 0.05$), while supplementing phytase alone (NC + PHY) or a combination of phytase and 25OHD (NC + PHY + 25OHD) improved TV, BV, BMC, Po.V(cl), Po.S(cl), and Po(cl) to the same level as the PC group ($P < 0.05$). The birds in the NC + 25OHD group showed a decreased number of closed pores (Po.N(cl)) compared to the PC group (910 vs. 1153; $P = 0.028$).

3.6. Body composition

The results of body composition are summarized in Tables 15 and 16. On 6 DPI (d 20), there was no significant interaction between *Eimeria* challenge and diets regarding broiler body composition, as detailed in Table 15. However, the *Eimeria* challenge resulted in a significant decrease in bone area (64.82 vs. 68.5 cm^2; $P = 0.045$), fat percentage (11.93% vs. 12.85%; $P = 0.041$), total tissue (738.51 vs. 769.01 g; $P = 0.040$), and fat mass (88.38 vs. 99.09 g; $P = 0.014$) on 6 DPI (d 20). Additionally, the dietary treatments had an impact on body composition parameters on 6 DPI (d 20). Birds fed with a reduced Ca and avP diet (NC) exhibited lower BMD, BMC, and bone area compared to PC birds ($P < 0.05$; Table 15), whereas supplementing phytase alone (NC + PHY) or a combination of phytase and 25OHD (NC + PHY + 25OHD), but not supplementing 25OHD alone, improved BMD, BMC, and bone area to the same level as the PC group ($P < 0.05$).

Table 13
Effects of phytase and 25-Hydroxycholecalciferol and their combination on femur metaphysis cortical bone of broilers infected with mixed *Eimeria* spp. on 6 days post inoculation (DPI) (d 20)[1].

Item[2]		TV, mm^3	BV, mm^3	TS, mm^2	BS, mm^2	BMD, g/cm^3	BMC, mg	Po.N(cl)	Po.V(cl), mm^3	Po.S(cl), mm^2	Po(cl), %
Interaction											
UNCHA	PC	113.55	378.17	378.17	832.96^{a}	0.421	47.05	439	0.280	16.92	0.307
	NC	74.51	395.94	395.94	624.51^{b}	0.325	24.43	261	0.098	6.72	0.173
	NC + PHY	95.32	379.38	379.38	699.77^{ab}	0.411	38.89	316	0.191	11.24	0.250
	NC + 25OHD	92.21	378.21	378.21	747.49^{ab}	0.357	32.40	306	0.142	9.01	0.197
	NC + PHY + 25OHD	112.58	398.91	398.91	878.48^{a}	0.381	42.61	409	0.222	13.71	0.258
CHA	PC	96.57	378.40	378.40	743.96^{ab}	0.400	38.62	313	0.177	10.72	0.235
	NC	81.45	366.94	366.94	630.35^{b}	0.369	30.21	309	0.149	9.40	0.230
	NC + PHY	93.06	445.10	445.10	785.17^{ab}	0.340	31.74	297	0.141	8.87	0.200
	NC + 25OHD	75.54	375.74	375.74	615.63^{b}	0.360	27.37	293	0.112	7.68	0.189
	NC + PHY + 25OHD	111.87	401.81	401.81	823.52^{a}	0.417	46.72	380	0.212	13.14	0.224
SEM		2.569	2.022	6.553	21.860	0.0085	1.334	15.3	0.0121	0.680	0.0108
P-value		0.243	0.120	0.225	0.009	0.168	0.075	0.466	0.270	0.253	0.353
Challenge effect											
UNCHA		97.63	74.20	386.12	756.64	0.379	37.08	346	0.187	11.52	0.237
CHA		91.70	70.10	393.60	719.73	0.377	34.93	318	0.158	9.96	0.216
P-value		0.140	0.183	0.565	0.477	0.909	0.275	0.350	0.193	0.207	0.138
Diet effect											
PC		105.06^{ab}	81.33^{ab}	378.29	788.46	0.411	42.84^{ab}	376	0.228^{a}	13.82^{a}	0.271
NC		77.98^{c}	59.13^{c}	381.44	627.43	0.347	27.32^{c}	285	0.124^{b}	8.06^{b}	0.201
NC + PHY		94.19^{bc}	70.68^{bc}	412.24	742.47	0.375	35.31^{bc}	307	0.166^{ab}	10.06^{ab}	0.225
NC + 25OHD		83.88^{c}	63.65^{c}	376.97	681.56	0.359	29.89^{c}	300	0.127^{b}	8.34^{b}	0.193
NC + PHY + 25OHD		112.23^{a}	85.98^{a}	400.36	851.00	0.399	44.67^{a}	394	0.217^{ab}	13.43^{ab}	0.241
P-value		<0.001	<0.001	0.330	0.364	0.081	<0.001	0.077	0.006	0.006	0.313

TV = tissue volume; BV = bone volume; TS = tissue surface; BS = bone surface; BMD = bone mineral density; BMC = bone mineral content; Po.N(cl) = number of closed pores; Po.V (cl) = volume of closed pores; Po.S (cl) = surface of closed pores; Po (cl) = closed porosity percentage.
[a-c]Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] $n = 6$ replicate cages × 1 bird/cage = 6.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

Table 14
Effects of phytase and 25-Hydroxycholecalciferol and their combination on femur metaphysis cortical bone of broilers infected with mixed *Eimeria* spp. on 12 days post inoculation (DPI) (d 26)[1].

Item[2]		TV, mm^3	BV, mm^3	TS, mm^2	BS, mm^2	BMD, g/cm^3	BMC, mg	Po.N(cl)	Po.V(cl), mm^3	Po.S(cl), mm^2	Po(cl), %
Interaction											
UNCHA	PC	189.63	156.47	480.94	1116.30	0.438	82.83	1092	0.980	51.79	0.620
	NC	154.30	124.25	476.91	1039.72	0.398	61.18	905	0.597	35.14	0.473
	NC + PHY	189.84	154.41	489.05	1147.71	0.435	82.43	1111	0.869	47.72	0.559
	NC + 25OHD	159.08	129.83	457.34	989.48	0.419	66.46	824	0.528	31.67	0.403
	NC + PHY + 25OHD	195.61	162.61	471.58	1111.10	0.443	86.26	891	0.760	39.80	0.607
CHA	PC	182.77	148.69	445.76	1135.82	0.431	78.93	1245	1.164	63.75	0.768
	NC	157.99	129.26	447.44	981.55	0.413	65.19	917	0.688	36.99	0.533
	NC + PHY	176.62	144.21	463.72	1079.44	0.437	77.14	1015	0.941	49.38	0.636
	NC + 25OHD	149.03	120.66	442.93	961.27	0.422	62.87	748	0.501	29.44	0.417
	NC + PHY + 25OHD	194.00	156.56	480.53	1194.15	0.431	83.70	1089	0.978	52.60	0.603
SEM		3.487	2.992	4.262	23.282	0.0044	1.713	39.2	0.0460	2.264	0.0267
P-value		0.885	0.866	0.453	0.821	0.890	0.804	0.650	0.876	0.681	0.899
Challenge effect											
UNCHA		177.69	145.51	475.16^{a}	1080.86	0.427	75.83	967	0.746	41.27	0.532
CHA		171.63	139.44	456.91^{b}	1068.49	0.427	73.34	987	0.834	45.35	0.578
P-value		0.327	0.254	0.024	0.822	0.964	0.377	0.614	0.188	0.198	0.254
Diet effect											
PC		186.89^{a}	153.36^{a}	466.87	1124.11	0.435	81.27^{a}	1153^{a}	1.053^{a}	56.58^{a}	0.679^{a}
NC		155.98^{b}	126.53^{b}	463.51	1013.28	0.405	63.00^{b}	910^{ab}	0.638^{bc}	35.98^{bc}	0.500^{bc}
NC + PHY		183.83^{a}	149.77^{a}	477.54	1116.68	0.436	80.03^{a}	1067^{ab}	0.902^{ab}	48.47^{ab}	0.594^{ab}
NC + 25OHD		154.06^{b}	125.25^{b}	450.13	975.38	0.421	64.66^{b}	786^{b}	0.515^{c}	30.56^{c}	0.410^{c}
NC + PHY + 25OHD		194.80^{a}	159.58^{a}	476.06	1152.63	0.437	84.98^{a}	999^{ab}	0.879^{ab}	46.78^{ab}	0.605^{ab}
P-value		<0.001	<0.001	0.209	0.066	0.123	<0.001	0.028	<0.001	<0.001	0.012

TV = tissue volume; BV = bone volume; TS = tissue surface; BS = bone surface; BMD = bone mineral density; BMC = bone mineral content; Po.N(cl) = number of closed pores; Po.V(cl) = volume of closed pores; Po.S(cl) = surface of closed pores; Po(cl) = closed porosity percentage.
[a-c]Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] $n = 6$ replicate cages × 1 bird/cage = 6.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

On 12 DPI (d 26), an interaction effect between *Eimeria* challenge and diets was observed concerning fat percentage ($P = 0.036$; Table 16). The *Eimeria* challenge led to a decrease in fat percentage for the NC + PHY group birds (12.40% vs. 15.42%; $P = 0.036$). No significant difference in fat percentage was observed among the unchallenged groups ($P > 0.05$). However, in the *Eimeria*-

Table 15
Effects of phytase and 25-Hydroxycholecalciferol and their combination on body composition of broilers infected with mixed *Eimeria* spp. on 6 days post inoculation (DPI) (d 20)[1].

Item[2]		BMD, g/cm^2	BMC, g	Bone area, cm^2	Fat percentage, %	Total tissue, g	Fat mass, g	Lean mass, g
Interaction								
UNCHA	PC	0.147	11.15	75.92	13.37	794.13	106.25	687.84
	NC	0.127	7.41	58.08	13.00	761.39	99.07	662.28
	NC + PHY	0.143	10.44	73.25	12.53	791.56	99.60	691.92
	NC + 25OHD	0.129	8.14	63.33	12.25	748.77	91.89	656.88
	NC + PHY + 25OHD	0.146	10.51	71.92	13.10	749.18	98.62	650.49
CHA	PC	0.149	9.91	66.50	13.00	735.09	95.59	639.49
	NC	0.130	7.79	59.92	11.98	750.70	89.74	660.96
	NC + PHY	0.144	10.06	69.75	11.02	732.06	81.61	650.41
	NC + 25OHD	0.132	7.79	58.83	11.42	744.69	85.73	658.96
	NC + PHY + 25OHD	0.151	10.44	69.08	12.22	730.02	89.24	640.81
SEM		0.0015	0.217	1.138	0.224	7.210	2.149	5.897
P-value		0.970	0.475	0.411	0.950	0.602	0.928	0.545
Challenge effect								
UNCHA		0.139	9.53	68.50^a	12.85^a	769.01^a	99.09^a	669.88
CHA		0.141	9.20	64.82^b	11.93^b	738.51^b	88.38^b	650.13
P-value		0.207	0.244	0.045	0.041	0.040	0.014	0.104
Diet effect								
PC		0.148^a	10.53^a	71.21^a	13.18	764.61	100.92	663.66
NC		0.129^b	7.60^b	59.00^b	12.49	756.04	94.40	661.62
NC + PHY		0.144^a	10.25^a	71.50^a	11.78	761.81	90.61	671.17
NC + 25OHD		0.131^b	7.97^b	61.08^b	11.83	746.73	88.81	657.92
NC + PHY + 25OHD		0.148^a	10.48^a	70.50^a	12.66	739.60	93.93	645.65
P-value		<0.001	<0.001	<0.001	0.234	0.793	0.434	0.740

BMD = bone mineral density; BMC = bone mineral content.
[a, b] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] $n = 6$ replicate cages × 2 birds/cage = 12.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

Table 16
Effects of phytase and 25-Hydroxycholecalciferol and their combination on body composition of broilers infected with mixed *Eimeria* spp. on 12 days post inoculation (DPI) (d 26)[1].

Item[2]		BMD, g/cm^2	BMC, g	Bone area, cm^2	Fat percentage, %	Total tissue, g	Fat mass, g	Lean mass, g
Interaction								
UNCHA	PC	0.154	19.18	124.75	14.72^{abc}	1306.69	192.40	1114.25
	NC	0.140	13.58	96.67	13.95^{abcd}	1275.43	178.38	1097.05
	NC + PHY	0.156	18.48	118.67	15.42^{ab}	1287.71	197.77	1089.98
	NC + 25OHD	0.144	15.43	107.58	14.17^{abcd}	1264.88	179.24	1085.64
	NC + PHY + 25OHD	0.155	18.27	118.33	15.27^{abc}	1269.08	195.16	1073.92
CHA	PC	0.148	17.31	116.83	15.80^a	1253.66	198.26	1055.36
	NC	0.133	13.02	98.17	13.50^{cd}	1221.07	165.07	1056.00
	NC + PHY	0.149	17.73	119.00	12.40^d	1224.21	152.48	1071.69
	NC + 25OHD	0.137	14.41	105.25	14.38^{abc}	1268.81	182.19	1086.62
	NC + PHY + 25OHD	0.160	19.54	121.75	13.82^{bcd}	1278.38	178.00	1100.34
SEM		0.0013	0.348	1.706	0.232	0.232	0.004	0.008
P-value		0.078	0.173	0.671	0.036	0.631	0.151	0.450
Challenge effect								
UNCHA		0.150^a	16.99	113.20	14.70	1280.76	188.59	1092.17
CHA		0.145^b	16.40	112.20	13.98	1249.23	175.20	1074.00
P-value		0.015	0.149	0.697	0.094	0.112	0.057	0.248
Diet effect								
PC		0.151^a	18.25^a	120.79^a	15.26	1280.17	195.33	1084.81
NC		0.136^b	13.30^b	97.42^b	13.73	1248.25	171.72	1076.53
NC + PHY		0.152^a	18.11^a	118.83^a	13.91	1255.96	175.12	1080.84
NC + 25OHD		0.141^b	14.92^b	106.42^b	14.28	1266.85	180.72	1086.13
NC + PHY + 25OHD		0.157^a	18.90^a	120.04^a	14.54	1273.73	186.58	1087.13
P-value		<0.001	<0.001	<0.001	0.184	0.841	0.219	0.992

BMD = bone mineral density; BMC = bone mineral content.
[a -d] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] $n = 6$ replicate cages × 2 birds/cage = 12.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

challenged groups, the NC, NC + PHY, and NC + PHY + 25OHD groups showed significantly reduced fat percentage compared to the *Eimeria*-challenged PC group ($P = 0.036$). Additionally, *Eimeria* challenge and diets demonstrated main effects on 12 DPI (d 26). *Eimeria*-challenged broilers exhibited decreased BMD compared to unchallenged birds (0.145 vs. 0.15 g/cm^2; $P = 0.015$). Like 6 DPI (d

Table 17
Effects of phytase and 25-Hydroxycholecalciferol and their combination on gene expression of duodenal-jejunal tight junction proteins of broilers infected with mixed *Eimeria* spp. on 6 days post inoculation (DPI) (d 20)[1].

Item[2]		*CLDN1*	*JAM2*	*OCLN*	*ZO1*	*MUC2*
Interaction						
UNCHA	PC	1.00	1.00^{c}	1.00^{abc}	1.00	1.00
	NC	1.04	1.51^{bc}	0.89^{bcd}	1.10	0.94
	NC + PHY	1.13	1.33^{c}	1.12^{ab}	1.07	1.18
	NC + 25OHD	1.29	1.96^{abc}	1.19^{a}	1.55	0.91
	NC + PHY + 25OHD	1.05	1.42^{c}	0.98^{abc}	1.14	0.92
CHA	PC	1.71	2.64^{a}	0.79^{cd}	1.33	0.66
	NC	2.41	1.55^{bc}	0.88^{bcd}	1.42	0.58
	NC + PHY	2.21	2.45^{ab}	0.69^{d}	1.12	0.84
	NC + 25OHD	1.80	1.63^{abc}	0.68^{d}	1.10	0.57
	NC + PHY + 25OHD	1.99	1.18^{c}	0.76^{cd}	1.28	0.84
SEM		0.102	0.121	0.033	0.048	0.032
P-value		0.527	0.020	0.050	0.070	0.217
Challenge effect						
UNCHA		1.10^{b}	1.44^{b}	1.03^{a}	1.17	0.99^{a}
CHA		2.05^{a}	1.89^{a}	0.77^{b}	1.26	0.70^{b}
P-value		<0.001	0.050	<0.001	0.425	<0.001
Diet effect						
PC		1.29	1.82	0.90	1.16	0.83^{ab}
NC		1.72	1.53	0.88	1.26	0.76^{b}
NC + PHY		1.62	1.84	0.92	1.09	1.03^{a}
NC + 25OHD		1.52	1.83	0.96	1.34	0.75^{b}
NC + PHY + 25OHD		1.52	1.30	0.87	1.21	0.88^{ab}
P-value		0.688	0.406	0.954	0.589	0.003

CLDN1 = claudin 1; *JAM2* = junctional adhesion molecule 2; *OCLN* = occludin; *ZO1* = zonula occludens 1; *MUC2* = mucin 2.
[a-d] Means within a column with different letter superscripts are significantly different ($P \leq 0.05$).
[1] $n = 6$ replicate cages × 1 bird/cage = 6.
[2] UNCHA = unchallenged group; CHA = *Eimeria* challenged group; PC = positive control; NC = negative control (reduced 0.20% Ca and avP); NC + PHY = NC + 1500 FTU/kg of phytase; NC + 25OHD = NC + 5000 IU/kg of 25OHD; NC + PHY + 25OHD = NC + 1500 FTU/kg of phytase + 5000 IU/kg of 25OHD.

20), on 12 DPI (d 26), birds fed with a reduced Ca and avP diet (NC) displayed lower BMD, BMC and bone area compared to PC birds (Table 16), whereas supplementing phytase alone (NC + PHY) or a combination of phytase and 25OHD (NC + PHY + 25OHD), but not supplementing 25OHD alone, improved BMD, BMC, and bone area to the same level as the PC group ($P < 0.05$).

3.7. Gene expression of tight junction proteins

The results of gene expression of duodenal-jejunal tight junction proteins on 6 DPI (d 20) are summarized in Table 17. Interaction effects between *Eimeria* challenge and diets were observed in junctional adhesion molecule 2 (*JAM2*; $P = 0.020$) and occludin (*OCLN*; $P = 0.050$). For *JAM2* gene expression, *Eimeria* challenge upregulated the mRNA expression of *JAM2* for the PC and NC + PHY groups ($P = 0.020$). There was no significant difference on *JAM2* mRNA expression among unchallenged groups ($P > 0.05$). However, in *Eimeria*-challenged groups, the NC and NC + PHY+25OHD groups showed downregulated expression of *JAM2* mRNA compared to the *Eimeria*-challenged PC group ($P = 0.020$). For *OCLN* mRNA expression, *Eimeria* challenge downregulated the mRNA expression of *OCLN* for the NC + PHY and NC + 25OHD groups ($P = 0.050$). In unchallenged groups, the NC + 25OHD group showed an upregulated mRNA expression of *OCLN* compared to the NC group (1.19-fold vs. 0.89-fold; $P = 0.050$). However, no significant difference on *OCLN* mRNA expression among *Eimeria*-challenged groups was observed ($P > 0.05$).

Additionally, *Eimeria* challenge and diets demonstrated main effects on 6 DPI (d 20). *Eimeria*-challenged birds showed an upregulated mRNA expression of claudin 1 (*CLDN1*; 2.05-fold vs. 1.1-fold; $P < 0.001$) and *JAM2* (1.89-fold vs. 1.44-fold; $P = 0.050$), and a downregulated expression of *OCLN* (0.77-fold vs. 1.03-fold; $P < 0.001$) and mucin 2 (*MUC2*; 0.7-fold vs. 0.99-fold; $P < 0.001$). Birds in the NC + PHY group showed upregulated expression of *MUC2* compared to the NC and NC + 25OHD groups ($P = 0.003$).

4. Discussion

In commercial production, enhancing the utilization of P in feed proves to be an effective way for mitigating P pollution. Extensive research has investigated the impact of phytase or 25OHD supplementation in chickens. However, 25OHD used together with phytase in *Eimeria*-challenged broilers has not been previously reported. The findings from the current study reaffirm the detrimental effects of coccidiosis on broilers and offer insights into how supplementing phytase and 25OHD in a Ca/P-reduced diet, either individually or in combination, influences the growth performance, gut integrity, bone development, and body composition of *Eimeria*-challenged birds. Coccidiosis decreased BWG and FI from 0 to 6 DPI and 0 to 12 DPI. It also increased the FCR of birds from 7 to 12 DPI and 0 to 12 DPI. Additionally, it decreased the daily FI of birds from 4 to 7 DPI (acute phase) and started to recover from 8 DPI (recovery phase) as shown in Table 5. Numerous studies have summarized the detrimental impact of *Eimeria* challenge on the growth performance of broilers, especially in acute phase, due to severe gut damage, impaired nutrient absorption, and a strong immune response diverting resources from growth (Yadav et al., 2020; Choi et al., 2023; Liu et al., 2024; Lopes et al., 2024). In the current study, reducing Ca and avP levels in the diet did not affect the FI or BWG of birds during 0 to 12 DPI, but negatively affected the FCR of birds during 7 to 12 DPI and overall period 0 to 12 DPI. However, supplementing phytase or 25OHD, or both, could alleviate the adverse effects of Ca and P reduction on FCR of birds. Similar results were found in other studies reporting that reducing Ca and P in diet during the grower phase (after d 14 to 28) did not affect the FI or BWG but increased the FCR of birds during d 0 to 28 (Delezie et al., 2015; Dersjant-Li et al., 2018). However, supplementing phytase, or

25OHD, or both improved the FCR to similar level of the PC group in the current study; these findings are align with previous studies on chicken (Angel et al., 2005; Ahmed et al., 2015; Taheri and Mirisakhani, 2020; Yavaş et al., 2020; Kermani et al., 2023) or swine (Li et al., 1998; Zhao et al., 2022). The results of the current study demonstrated that although Ca and avP were reduced in the diet, it appears that there were no detrimental effects associated with these reductions on growth performance during the recovery phase (7 to 12 DPI) and overall period, except the FCR. Notably, birds fed diets supplemented with both phytase and 25OHD showed better BWG or FI compared to the NC birds during 7 to 12 DPI and overall period. In the current study, dietary phytase or combination of phytase and 25OHD reduced the FCR of birds compared to the NC group, suggesting that phytase is the main contributor for reducing the FCR, while combination of phytase and 25OHD had a superposition effect on growth performance.

During the acute phase (0 to 6 DPI), interaction effects were found in BWG in the present study. Reducing Ca and P in the diet (NC) had negative effects on BWG in unchallenged birds but not in *Eimeria*-challenged birds. But, supplementing phytase or 25OHD alone but not combination improved the BWG of unchallenged birds to same levels of unchallenged PC birds; however, in the *Eimeria*-challenged groups, the phytase and 25OHD combination group had higher BWG compared to *Eimeria*-challenged PC group. The results demonstrated that reducing dietary Ca and P levels had more impact in unchallenged birds compared with *Eimeria*-challenged birds, and supplementation of phytase and 25OHD combination had more benefit effect in *Eimeria*-challenged birds. This may be attributed to the compromised gut health and integrity caused by *Eimeria* challenge, evidenced by the elevated levels of FITC-d, heightened intestinal lesion scores, and upregulated gene expression of tight junction proteins (*CLDN1* and *JAM2* in the present study), leading to reduced absorption and utilization of nutrients, such as Ca and P. The supplementation of the phytase and 25OHD combination may help to mitigate these effects by promoting gut health and improving nutrient absorption efficiency, thereby providing more benefits in *Eimeria*-challenged birds compared to unchallenged birds.

In this study, we assessed the impact of phytase and 25OHD supplementation on intestinal integrity by evaluating gut permeability, intestinal lesion scores, and mRNA expressions of tight junction proteins. Our results indicated dietary treatments did not affect the intestinal lesion scores of broilers. However, the 25OHD group exhibited an elevated level of FITC-d compared to both the phytase and phytase + 25OHD groups, with most of the increase observed in the *Eimeria*-challenged 25OHD group (135 ng/mL). Providing a higher level of vitamin D was linked to increased parasite replication and gastrointestinal tract (GIT) damage was reported by a previous study (Sakkas et al., 2019a). In the current study, the gene expression of *MUC2* was upregulated in the phytase group compared to the NC or 25OHD alone group, which was also reported by another study where the *MUC2* expression was upregulated by 3000 FTU/kg of phytase supplementation to a reduced Ca and P diet (Ajuwon et al., 2020). MUC2, secreted by goblet cells, adheres to the surface of intestinal villi, and serves as the primary physical barrier of the intestine (Kim and Ho, 2010). Stimulating goblet cells to secrete mucin can enhance the protective mucin layer, protecting the intestinal tract against invasion of pathogenic bacteria (Kim and Ho, 2010). In the current study, supplementing phytase in the NC diet might prove beneficial for the proliferation of intestinal goblet cells and the inhibition of bacterial translocation in broilers (Klinsoda et al., 2020; Li et al., 2022). The finding on the expression of *MUC2* are consistent with prior research that *Eimeria* challenge downregulates the expression of *MUC2* (Forder et al., 2012; Kitessa et al., 2014). It is plausible that as the intestine health is suppressed, *MUC2* expression decreases, potentially slowing the replenishment of the mucus layer.

The current study showed a significant upregulation in the gene expression of *CLDN1* and *JAM2* due to coccidiosis, which has been reported in other *Eimeria* challenge studies (Teng et al., 2021; Lin et al., 2022). Additionally, interaction effects of *Eimeria* challenge and dietary treatments were found in *JAM2* and *OCLN* gene expressions. The *JAM2* gene expression was upregulated in the challenged PC and NC + PHY groups compared to the unchallenged PC group. However, it was downregulated in the challenged NC + PHY + 25OHD group to the same level as the unchallenged PC group. This result indicates that the combination of phytase and 25OHD in *Eimeria*-infected birds likely provides a protective effect on the intestinal barrier, reducing the need for the body's emergency response to maintain tight junction integrity. The *Eimeria* challenge resulted in downregulation of *OCLN* gene expression in the current study, a finding consistent with reports by Teng et al. (2021) and Leung et al. (2019). This downregulation indicates increased gut permeability, as the tight junction protein *OCLN*, along with claudins and cadherins, is essential for proper intestinal epithelial barrier function (Al-Sadi et al., 2011). This was further supported by the FITC-d results in this study. Decreased expression of *OCLN* has been noted in human patients with intestinal permeability disorders, particularly in relation to macromolecules (Al-Sadi et al., 2011). Changes in tight junction proteins can lead to defects in intestinal integrity and barrier dysfunction, aligning with observations of increased gut permeability due to *Eimeria* challenge.

As expected, our observations showed that coccidiosis compromised the tibial bone ash parameters of broilers at 12 DPI, with *Eimeria*-challenged birds, exhibiting significantly lower initial bone weight, bone volume, FFBW and bone ash weight compared to unchallenged birds. The bone formation, as indicated by the MAR, was reduced by coccidiosis during 4 to 8 DPI. Furthermore, the bone microstructure was compromised by coccidiosis at both 6 DPI and 12 DPI, as evidenced by decreased total bone BV/TV, TV, TS, trabecular bone TV, and cortical bone TS. Consistent with our findings, Oikeh et al. (2019) noted a decrease in tibia ash content and ash percentage due to *Eimeria* challenge, with a more pronounced effect observed on 12 DPI compared to 6 DPI. Similarly, other studies have reported a reduction in bone ash by coccidiosis on 6 DPI or/and 12 DPI (Sakkas et al., 2018), as well as a notable decrease in femur microstructure parameters in broilers and pullets (Sharma et al., 2023; Lopes et al., 2024), suggesting that *Eimeria* challenge suppresses long bone development during the post-infection stage, with affected birds unable to catch up with their unchallenged counterparts even during the recovery phase.

Ca and P are essential for multiple physiological functions in the chicken, including growth performance and skeletal system. When the diet lacks adequate or has imbalanced levels of Ca and P, it can lead to potential issues such as malabsorption of these minerals, hindered bone mineralization and growth, and increased leg problems (Matuszewski et al., 2020). In the current study, decrease on bone ash, MAR, and bone microstructure parameters of broilers due to reduced levels of Ca and avP in the diet was observed. Similarly, previous studies have reported that a reduction in dietary Ca and P levels led to suppressed bone ash parameters compared to birds fed a nutritionally adequate diet, with improvements observed upon supplementation of phytase by d 21 (Shi et al., 2022, 2023). However, a recent study observed no difference in bone ash parameters between the Ca and P-reduced group (NC) and the basal diet group (PC) on both 6 DPI and 9 DPI (Shi et al., 2024). Olukosi and Fru-Nji (2014) reported that reducing the Ca and NPP level, increasing the Ca/NPP ratio, or exclusion of phytase of the diet not only decreased bone ash percentage, but also decreased Ca and P

concentration in tibia, therefore, the negative effect on bone ash is potentially extend to a reduction on the Ca and P content in the bone. The inconsistency may arise from the more pronounced reduction in Ca and P levels in this study compared to the previous one, resulting in significant differences between the NC and PC groups in bone ash parameters in the current study.

Interestingly, our investigation revealed that supplementing phytase alone or in combination with 25OHD, but not 25OHD supplementation alone, resulted in improvements in bone microstructure parameters comparable to those of the PC. This suggests that the primary enhancement of bone structural soundness stems from phytase supplementation rather than 25OHD. Ghasemi et al. (2019) reported a similar results that supplementation of 5 μg/kg of 1α–OH–D_3 in a reduced Ca and NPP diet did not improve the Ca and P content in tibia. In this study, we observed improved bone ash parameters and bone microstructure, as indicated by tibia FFBW, bone ash weight, bone ash percentage, bone ash concentration, as well as femur BV, BV/TV, BMC, and BMD by feeding treatments. These findings suggest that supplementing phytase at 1500 FTU/kg, either alone or combined with 25OHD, positively influences the mineralization and quality of broiler bones. Similar results have been reported in our previous studies (Shi et al., 2022, 2023, 2024). Notably, the supplementation of 25OHD to the NC diet improved the BWG, and FCR of birds to levels comparable to those of birds fed the PC diet during 7 to 12 DPI and 0 to 12 DPI, as well as improved the MAR during 4 to 8 DPI, but not the bone mineralization or bone microstructure during the post-infection stages in the current study. Birds in the 25OHD group exhibited elevated FITC-d levels compared to other groups, indicating increased gut permeability. Analysis of vitamin D_3 levels in each diet showed that the 25OHD group had the highest concentration (5752.6 IU/kg) among the dietary groups. The finding potentially explains the medium effect on bone development with 25OHD supplementation alone, as previous research has indicated that vitamin D_3 levels exceeding 5000 IU/kg in the diet may not have a linear effect on tibia bone mineralization in broilers (Sakkas et al., 2019b). Researchers have cautioned against supplementing vitamin D above the maximum level recommended for commercial practice, which is 5000 IU/kg (Whitehead et al., 2004). Whitehead et al. (2024) reported that supplementation with vitamin D was found to enhance bone formation and bone mineralization, though the improvement on bone mineralization was not as effective as phytase. Vitamin D is essential for Ca and P absorption in the intestines and boosts the activity of osteoblasts, the cells responsible for bone formation (Jones et al., 1998; Chen et al., 2020). However, the absence of vitamin D impact on bone microstructure suggests that other factors may also be involved in bone microstructure development. Future research on bone resorption parameters might shed light on this finding because bone remodeling involves both bone formation and resorption, which together influence bone microstructure (Goltzman, 2018). Although vitamin D is important for providing Ca and P for bone mineralization, the process also relies on the availability of these minerals in the diet and bloodstream and the balance between osteoblast and osteoclast activity (Rowe et al., 2018). This may explain the less pronounced effects on bone mineralization observed in the group fed the diet supplemented with vitamin D.

Body composition is a critical parameter in broiler production, closely associated with both meat yield and meat quality (Choi et al., 2023). *Eimeria* challenge resulted in reduced bone area, fat percentage, total tissue, and fat mass of broilers on 6 DPI, along with a decrease in BMD on 12 DPI in this study. These findings align with previous studies on *Eimeria* challenge (Fetterer et al., 2013; Sharma et al., 2022), indicating the detrimental effect of coccidiosis on broilers. Furthermore, the body composition outcomes from dietary effect mirrored those observed for bone ash and bone microstructure. Specifically, reducing Ca and P in the diet compromised BMD, BMC, and bone area of birds on both 6 and 12 DPI, while supplementing phytase, either alone or in combination with 25OHD, but not 25OHD alone, improved these parameters.

5. Conclusion

In summary, our results indicated that supplementing phytase, either alone or in combination with 25OHD, to diets low in Ca and P may mitigate the adverse effects of *Eimeria* infection on intestinal health and bone development. This is demonstrated by improvements in intestinal permeability, bone mineralization, bone microstructure, and body composition. While 25OHD supplementation on its own had a less significant impact on bone development compared to phytase, it did show some positive effects on growth performance, bone mineral apposition rate, bone ash concentration and bone ash percentage. In this study, the benefits of phytase supplementation were generally more pronounced than those associated with 25OHD supplementation; however, the combination of both induced more optimal effects.

CRediT author statement

Hanyi Shi: Conceptualization, Formal analysis, Investigation, Methodology, Writing – Original draft. **Venkata S.R. Choppa:** Investigation, Methodology, Writing – Review & Editing. **Deependra Paneru:** Investigation, Methodology, Writing – Review & Editing. **Woo K. Kim:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – Review & Editing.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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