Effects of phytase and coccidial vaccine on growth performance, nutrient digestibility, bone mineralization, and intestinal gene expression of broilers

Hanyi Shi,* Jinquan Wang,* Po-Yun Teng ,* Yuguo Hou Tompkins,* Brian Jordan,† and Woo Kyun Kim *,[1]

*Department of Poultry Science, University of Georgia, Athens 30602, Georgia; and †Departments of Population Health and Poultry Science, Poultry Diagnostic and Research Center, Athens, 30602, Georgia

ABSTRACT A study was conducted to evaluate effects of phytase and coccidial vaccine on growth performance, bone mineralization, nutrient digestibility, and intestinal gene expression of broiler chickens. The experiment was conducted in a 2 × 4 completely randomized factorial arrangement with 6 replicates per treatment and 10 birds each. Applications of coccidiosis vaccine and different dietary treatments were the 2 main factors in the current study. The dietary treatments included 1) a positive control (**PC**; 0.90% Ca and 0.45% available P: **avP**); 2) a negative control (**NC**; 0.75% Ca and 0.30% AvP); 3) NC + 500 FTU/kg of phytase (**NC + 500PHY**); and 4) NC + 1500 FTU/kg of phytase (**NC + 1500PHY**). Data were analyzed using SAS by 2-way ANOVA via GLM procedure. The statistical significance was set at $P \leq 0.05$, and means were further separated using Tukey's Test. The results indicated that vaccination had no effect on growth performance except for feed intake from 0 to 14 d but negatively ($P < 0.05$) regulated bone ash and Ca digestibility. Birds fed with the Ca and P-reduced diet (NC) showed a lower BWG and bone ash compared to birds fed with the normal diet (PC), but supplementing phytase mitigated the negative effects on those birds. Broilers fed the NC diet had higher ($P < 0.05$) total Ca and P digestibility, and phytate degradation; supplementing phytase further increased P digestibility and phytate degradation of the broilers. A significant interaction ($P < 0.05$) between phytase and vaccination was observed, suggesting the vaccinated birds fed the PC diet and the unvaccinated birds fed the NC + 1500PHY increased calcium-sensing receptor gene expression compared with the unvaccinated birds fed the PC diet. In conclusion, in spite of coccidiosis vaccine, supplementing phytase at 1,500 FTU/kg alleviated the negative effects on growth performance, bone mineralization, and apparent ileal digestibility of P and phytate.

Key words: bone ash, coccidial vaccine, gene expression, nutrient digestibility, phytase

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INTRODUCTION

Coccidiosis caused by *Eimeria* spp. is one of the most common diseases in the poultry industry and costs around 14 billion United States dollars, including costs during production and for prophylaxis and treatment (Blake et al., 2020; Teng et al., 2021a, 2021b). It causes extensive damage to the intestine of birds, leading to performance reduction (Williams, 2002) and malabsorption of nutrients (Persia et al., 2006). Vaccination has been used as one of the primary methods for coccidiosis prevention for chicken (Kadykalo et al., 2018). Coccidial vaccines can mitigate negative effects of coccidiosis by enhancing immunity of the birds with low dose exposure of *Eimeria* oocysts (Chapman et al., 2002; Chapman, 2014). However, they may lead to sub-clinical infection and potential growth reduction in early period (2 wk following vaccination), which is often associated with mild intestinal inflammation and oxidative stress as a result of damage to bird intestinal epithelium (Li et al., 2005; Cervantes, 2015), resulting in nutrient malabsorption, enhanced immune response and decrease in the expression of brush-border membrane nutrient transporters (Paris and Wong, 2013; Su et al., 2014; Lee et al., 2022). Moreover, the coccidial vaccination is reported to induce the incidence of bacterial enteritis (Williams, 2002). The inflammation and oxidative stress, which are essential to trigger both innate and adaptive immunity, caused by coccidial vaccine can potentially affect broiler growth, nutrient utilization, and bone development.

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[1]Corresponding author: wkkim@uga.edu

Bone mineralization is an indicator of body abnormalities which could affect the performance and health of broilers. Studies have shown that birds undergoing *Eimeria* infection reduced absorption of calcium (**Ca**) and phosphorous (**P**) (Mansoori et al., 2010; Shaw et al., 2012) and adversely affected the bone mineralization (Watson et al., 2005; Sakkas et al., 2018; Oikeh et al., 2019). Our recent study indicated that oxidative stress caused by *Eimeria* infection could lead to inhibition of bone mineralization and osteogenesis, especially in the high challenge group of broilers (Tompkins et al., 2022). However, unlike the known negative effects caused by *Eimeria* infection on broilers, the impact of coccidial vaccination to bone mineralization of birds is still not well understood. Therefore, there is a need to investigate the relationship between vaccination and bone mineralization due to the wide-use of coccidial vaccines in the modern poultry industry as well as animal welfare issues, which triggers the researchers to take bone health into considerations on market age broiler chickens. Thus, one of the objectives of the study was to evaluate bone mineralization of broilers under vaccination.

The amounts of phytate represent between 60% and 80% of the total P in plant seeds that are used to feed monogastric animals such as poultry and swine which do not have hydrolytic enzymes to digest phytate in feed (Turner et al., 2007; Wang et al., 2019a). Phytate can chelate essential minerals, including Ca and P, thereby limiting the availability of macro-minerals in the feedstuffs to broilers. The unabsorbed nutrients also increase the excretion of mineral wastes to the environment (Shafey et al., 1991; Maenz et al., 1999; Wang and Kim, 2021). Application of phytase can initiate the release of phosphorous from phytate, thus making it available for absorption (Boling et al., 2000; Zwart, 2006). Phytase supplementation has been shown to improve growth performance (Cowieson et al., 2006; Shang et al., 2015), bone mineralization (Emami et al., 2013; Wang et al., 2019a; Wang and Kim, 2021), and nutrient utilization (Selle and Ravindran, 2007; Ravindran et al., 2008; Cowieson et al., 2017). In addition to these improvements in unvaccinated broilers, phytase supplementation may be beneficial in broilers with coccidiosis vaccination. Watson et al. (2005) reported that phytase improved growth performance and tibia ash concentration in *E. acervuline*-challenged chicks. Other studies also indicated that supplementing phytase improved broiler performance (Shaw et al., 2012) and bone ash in coccidial vaccinated broilers (Walk et al., 2011b). Adedokun and Adeola (2016) reported that phytase supplementation increased nitrogen (N) and P digestibility in birds challenged with 25 × coccidial vaccine. Furthermore, there is a report indicating that phytase can mitigate the negative impact of coccidiosis on bone quality (Kiarie et al., 2019). Walk et al. (2011a) claimed that dietary enzyme supplementation did not alleviate reduction in growth performance or P utilization of vaccinated broilers. Another study also reported that supplementing phytase in the diet of vaccinated broilers improved apparent ileal digestibility of amino acids, but did not improve performance (Lehman, 2011). Shaw et al. (2011) reported that coccidiosis led to reductions in performance, absorption of Ca and P, and bone strength; however, phytase supplementation did not mitigate the adverse effects of coccidiosis on phosphorous utilization.

Although the effects of enzyme supplementation or coccidial vaccination have been reported, published data are inconsistent and inconclusive regarding phytase effects under a low dosage vaccination and the interaction between different doses of phytase and coccidial vaccination. Thus, the objective of the study was to evaluate the effects of phytase and coccidial vaccination on growth performance, apparent ileal digestibility, bone mineralization, and gene expression of intestinal mineral transporters and tight junction proteins in broiler chickens.

MATERIALS AND METHODS

Birds, Housing, and Treatments

The study was approved by the Institutional Animal Care and Use Committee at University of Georgia and conducted at the Poultry Research Center at University of Georgia (Athens, GA). A total of 480 one-day old male Cobb 500 broilers (8 trts × 6 reps × 10 birds/cage) were obtained from a Cobb Vantress Hatchery (Cleveland, GA) and were randomly selected, weighed, and placed in battery brooders (Gettysburg, OH; Dimension for each cage: length × width × height, 80.5 × 37.5 × 25 cm) by nonvaccinated or vaccinated groups. The experiment was conducted in a 2 × 4 completely randomized factorial arrangement with dietary treatments and coccidia vaccination as the main factors. The four diet treatments included 1) a positive control (**PC**; 0.90% Ca and 0.45% available P); 2) a negative control (**NC**; 0.75% Ca and 0.30% available P: **avP**); 3) NC + 500 FTU/kg of phytase (**NC + 500PHY**; Axtra PHY, Danisco UK Ltd, Marlborough, UK); and 4) NC + 1500 FTU/kg of phytase (**NC + 1500PHY**). On arrival, half of the birds were sprayed with a commercially approved coccidial vaccine (Coccivac-B52, Merck Animal Health, Kenilworth, NJ) according to the manufacturer's recommendations. Paper pads were placed on the bottom of the cages to allow birds getting access to their feces for successful *Eimeria* recycling. Diets for this experiment were fed in mash form and formulated on a corn-soybean meal basis to meet Cobb 500 nutrient requirements (Cobb500, 2018), with the exception of Ca and avP in the Ca and P-reduced diets (Table 1). All diets were mixed with 0.3% chromic oxide (Cr_2O_3; Sigma Aldrich, St. Louis, MO) as an indigestible marker for calculating the apparent ileal digestibility. Unvaccinated birds were placed in batteries that were separated from vaccinated birds in the same environmentally controlled room. Throughout the 21-d trial period, precautions were taken to reduce cross-contamination via handling the birds, feed, water, and feces in the nonvaccinated groups before handling those in the vaccinated birds. Feed and water were provided ad libitum, and the

Table 1. Composition and nutrient content of basal diets (as dry basis).[1]

Treatments	PC	NC
Ingredient, %		
Corn, Grain	57.77	57.77
Soybean Meal	35.09	35.09
Soybean Oil	2.13	2.13
Dical. Phos.	1.59	0.78
Limestone	1.16	1.08
product space/Sand	0.70	1.57
Cr_2O_3	0.30	0.30
Common Salt	0.35	0.35
DL-Methionine	0.31	0.31
Vitamin Premix[2]	0.25	0.25
L-Lysine HCl	0.20	0.20
Thr	0.09	0.09
Mineral Premix[3]	0.08	0.08
Calculated nutrients		
ME (kcal/kg)	3010.00	3010.00
Crude protein (%)	21.25	21.25
Crude fiber (%)	2.15	2.15
Calcium[4] (%)	0.90 (0.94)	0.75 (0.78)
Total P (%)[4]	0.71 (0.72)	0.56 (0.54)
avP (%)[4]	0.45 (0.38)	0.30 (0.23)
Phytate P (%)[4]	0.26 (0.34)	0.26 (0.31)

[1]Diets were fed in mash form from d 0 to 21. PC = positive control diet; NC = negative control diet. Negative control diets were supplemented with 0, 500 and 1500 FTU/kg of phytase (Axtra PHY, Danisco UK Ltd, Marlborough, UK). avP = available phosphorus.

[2]Provided per kg of DSM Vitamin premix: Vit. A 2,204,586 IU, Vit. D3 200,000 ICU, Vit. E 2,000 IU, Vit. B12 2 mg, Biotin 20 mg, Menadione 200 mg, Thiamine 400 mg, Riboflavin 800 mg, d-Pantothenic Acid 2,000 mg, Vit. B6 400 mg, Niacin 8,000 mg, Folic Acid 100 mg, Choline 34,720 mg.

[3]Provided per kg of Mineral premix: Ca 0.72 g, Mn 3.04 g, Zn 2.43 g, Mg 0.61 g, Fe 0.59 g, Cu 22.68 g, I 22.68 g, Se 9.07 g.

[4]The analyzed Ca and P values in the diet were showed in the parenthesis.

environmental temperature program followed the recommendation of Cobb Broiler Management Guide (Cobb500, 2018; Vantress, 2017).

Growth Performance and Sample Collection

Body weight (**BW**) and feed intake (**FI**) were recorded by cage on d 0, 7, 14, and 21. Body weight gain (**BWG**) and feed conversion ratio (**FCR**) were measured for each week and cumulatively (d 0−21). On day 21, all birds were sacrificed by cervical dislocation for sample collection. The ileal digesta were collected from 5 birds per cage from Meckel's diverticulum to about 1 inch anterior to ileocecal junction and pooled within the cage and dried in 75°C oven. The left tibia bones from 2 of these 5 birds were collected for bone ash analysis, and the middle part of the ileum and the ceca tonsil from 1 of the 2 bone ash birds were collected for gene expression analyses.

Nutrient Digestibility

Dried feed and ileal digesta samples were ground to measure Ca, P, and phytate contents. Calcium and phosphorous were analyzed at the Chemical Laboratories at University of Missouri-Columbia. Phytate content was analyzed following the method by Latta and Eskin (1980), and chromic oxide concentration was measured in duplicate according to Adhikari et al. (2020). For chromic oxide analysis, 1 g of feed sample or 0.3 g of digesta sample was weighed and ashed in a nickel crucible at 600°C overnight to remove organic materials, then fused the sample with 5.8 g of fusion mixture (190 g KNO_3 to 100 g Na_2CO_3) and 5.6 g NaOH, and burned at 600°C for additional 2 h. The fusion mix was dissolved in water, and H_2O_2 oxidized chromite to chromate. The concentration of chromate was determined at 400 nm by a spectrophotometer (Spectramax M5, Molecular Devices, San Jose, CA). The apparent ileal digestibility of Ca, P, and phytate were calculated using the following equation (Cowieson and Adeola, 2005):

$$\text{AID } (\%) = [1 - (Cr_i/Cr_0) \times (N_0/N_i)] \times 100$$

where Cr_i represents the concentration of chromium in diet (%); Cr_0 represents the concentration of chromium in the ileal digesta (%); N_i represents the concentration of P, Ca, or phytate in diets (%); and N_0 represents the concentration of Ca, P, or phytate in the ileal digesta (%).

Bone ash Analysis

On day 21, the left tibias were collected and kept at a −20°C freezer until bone ash analysis. Bone ash parameters were measured according to the methods described by Zhang and Coon (1997) and Kim et al. (2004). Briefly, all bones were weighed before and after suspended in water at room temperature. The bone volume was calculated with the assumption that the specific gravity of water is 1 g/cm^3 at room temperature. To determine the fat-free dry matter, bones were dried in an oven at 100°C for 24 h and refluxed with hexane (Fisher 138 Scientific International Inc., MA) in a Soxhlet apparatus for 48 h at 70°C. Then the fat-free bones were dried at 100°C for additional 24 h and reweighed. After burning in a furnace at 600°C overnight, the ash weight for all bones were measured. Bone ash concentrations were calculated by dividing the ash weight of each bone by its volume, and ash percentages were calculated by dividing the ash weight of each bone by its fat-free dry weight according to Zhang and Coon (1997).

Real-Time PCR Analysis

On day 21, the middle part of the ileum and the ceca tonsil were collected by wrapping with tin foil, frozen in liquid nitrogen immediately, and stored in a −80°C freezer for further analyses. The RNA was extracted after homogenization in QiAzol lysis reagents (Qiagen, Valencia, CA) according to the manufacturer's instruction. The RNA purity and quantity measurements were accomplished by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Then, the cDNA was reverse-transcribed by high-capacity cDNA synthesis kits (Applied Biosystems, Foster City, CA). For real-time PCR reaction, it was measured in

Table 2. List of primers for qPCR.

Gene[1]	Accession number	Forward primer	Reverse primer
Housekeeping gene			
GAPDH	NM_204305.1	CCTCTCTGGCAAAGTCCAAG	GGTCACGCTCCTGGAAGATA
HMBS	XM_004947916.3	GGCTGGGAGAATCGCATAGG	TCCTGCAGGGCAGATACCAT
Ca^{2+} and Pi transporters			
PMCA1	NM_001168002	TTAATGCCCGGAAAATTCAC	TCCACCAAACTGCACGATAA
NCX1	NM_001079473	TCACTGCAGTCGTGTTTGTG	AAGAAAACGTTCACGGCATT
CASR	XM_416491	CTGCTTCGAGTGTGTGGACT	GATGCAGGATGTGTGGTTCT
$CALB1_{28}$	NM_205513	AAGCAGATTGAAGACTCAAAGC	CTGGCCAGTTCAGTAAGCTC
PiT1	XM_015297502	TATCCTCCTCATTTCGGCGG	CTCTTCTCCATCAGCGGACT
NaPiⅢb	NM_204474	AAAGTGACGTGGACCATG	GAGACCGATGGCAAGATCAG
Tight junction proteins			
CLDN1	NM_001013611.2	TGGAGGATGACCAGGTGAAGA	CGAGCCACTCTGTTGCCATA
OCLN	XM_025144248.1	ACGGCAGCACCTACCTCAA	GGCGAAGAAGCAGATGAG
JAM2	XM_025149444.1	AGCCTCAAATGGGATTGGATT	CATCAACTTGCATTCGCTTCA
Mucin			
MUC2	JX_284122.1	ATGCGATGTTAACACAGGACTC	GTGGAGCACAGCAGACTTTG

[1]GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMBS, hydroxymethylbilane synthase; PMCA1, plasma membrane calcium-transporting ATPase 1; NCX1, sodium/calcium exchanger 1; CASR, extracellular calcium sensing receptor; $CALB1_{28}$, calbindin 1, 28kD isoform; PiT1, phosphate transporter 1; NaPiⅢb, sodium-dependent phosphate transport protein 2B; CLDN1, claudin 1; OCLN, occludin; JAM2, junctional adhesion molecule 2; MUC2, mucin 2.

duplicate with SYBR Green Master mix (Bio-Rad Laboratories, Hercules, CA) by a Step One thermocycler (Applied Biosystem, Foster City, CA) using the following conditions for all genes: 95°C for 10 min followed by 40 cycles at 95°C for 15 s, then annealing temperature for 20 s, and extension at 72°C for 15 s. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH, forward primer: CCTCTCTGGCAAAGTCCAAG; reverse primers: GGTCACGCTCCTGGAAGATA) and hydroxymethylbilane synthase (HMBS, forward primer: GGCTGGGAGAATCGCATAGG; reverse primer: TCCTGCAGGGCAGATACCAT) were used as housekeeping genes. The target gene expression was analyzed using the $2^{-\triangle\triangle Ct}$ method according to Livak and Schmittgen (2001). Primers for housekeeping genes and target genes are listed in Table 2.

Statistical Analyses

All data were analyzed by 2-way ANOVA using the GLM model for a completely randomized design of SAS software (SAS Institute Inc., Cary, NC). Cage served as the experimental unit of this study. The statistical model included diet, vaccination, and their interaction. The Tukey's honestly significant difference test was used to separate means with significance levels. Statistical significance was set at $P \leq 0.05$.

RESULTS

Growth Performance

The effects of vaccination and phytase on growth performance are presented in Tables 3 and 4. Dietary

Table 3. Growth performance during d 0 to 7, d 8 to 14, and d 0 tp 14.

		D 0-7				D 8-14				D 0-14		
Treatment[1]		BW (g)	BWG (g)	FI (g)	FCR (g/g)	BW (g)	BWG (g)	FI (g)	FCR (g/g)	BWG (g)	FI (g)	FCR (g/g)
UNVAC	PC	174	131	133	1.018	474	300	400	1.333	431	543	1.260
	NC	161	117	135	1.160	445	282	385	1.362	402	515	1.282
	NC+500 PHY	170	127	141	1.112	472	303	401	1.322	429	541	1.260
	NC+1500 PHY	168	125	141	1.127	454	285	374	1.314	411	515	1.256
VAC	PC	167	124	140	1.133	465	296	387	1.308	422	530	1.256
	NC	161	118	132	1.113	433	272	382	1.412	390	514	1.318
	NC+500 PHY	165	122	138	1.130	450	283	359	1.274	406	497	1.226
	NC+1500 PHY	168	125	134	1.075	471	303	363	1.200	428	497	1.160
SEM		1.065	1.061	1.845	0.016	3.434	3.224	4.383	0.015	3.433	4.853	0.010
UNVAC		168	125	137	1.104	462	293	391	1.332	419	530[a]	1.264
VAC		165	122	136	1.113	454	288	373	1.304	411	510[b]	1.244
PC		170[a]	127[a]	136	1.076	470[a]	298	394	1.322[ab]	427[a]	537	1.258[ab]
NC		161[b]	118[b]	134	1.137	439[b]	277	383	1.389[a]	396[b]	514	1.302[a]
NC+500 PHY		168[ab]	124[ab]	139	1.121	462[a]	294	382	1.300[ab]	419[a]	521	1.245[ab]
NC+1500 PHY		168[ab]	125[ab]	137	1.101	463[a]	294	368	1.257[b]	419[a]	506	1.208[b]
						P-value						
VAC*PHY		0.4948	0.4265	0.6459	0.2287	0.1233	0.1967	0.3937	0.1758	0.1222	0.3851	0.0598
VAC		0.1695	0.1525	0.6634	0.7839	0.2772	0.5085	0.0515	0.1838	0.2693	0.0406	0.153
PHY		0.0092	0.0107	0.7801	0.565	0.0046	0.087	0.2308	0.0085	0.005	0.1382	0.0048

[a,b]Means within a column with different superscripts are significantly different ($P < 0.05$).

[1]UNVAC, unvaccination; VAC, vaccination; PC, positive control; NC, negative control (reduced 0.15% Ca and P); 500 PHY, 500 FTU/kg of phytase; 1500 PHY, 1500 FTU/kg of phytase. BW, body weight; BWG' body weight gain; FI, feed intake; FCR, feed conversion ratio.

Table 4. Growth performance during d 15 to 21 and d 0 to 21

Treatment[1]		D 15–21 BW (g)	D 15–21 BWG (g)	D 15–21 FI (g)	D 15–21 FCR (g/g)	D 0–21 BWG (g)	D 0–21 FI (g)	D 0–21 FCR (g/g)
UNVAC	PC	905[a]	431	678[ab]	1.573[ab]	862[a]	1220[a]	1.415
	NC	838[c]	393	650[b]	1.656[a]	795[c]	1165[abc]	1.464
	NC + 500 PHY	874[abc]	402	649[b]	1.615[ab]	831[abc]	1185[abc]	1.427
	NC + 1500 PHY	881[ab]	432	640[b]	1.483[b]	838[ab]	1154[abc]	1.377
VAC	PC	850[bc]	398	622[b]	1.565[ab]	807[bc]	1144[bc]	1.420
	NC	852[bc]	419	646[b]	1.550[ab]	809[bc]	1160[abc]	1.433
	NC + 500 PHY	838[c]	399	632[b]	1.592[ab]	795[c]	1127[c]	1.420
	NC + 1500 PHY	889[ab]	427	717[a]	1.678[a]	846[ab]	1213[ab]	1.432
SEM		5.614	4.037	7.709	0.018	5.623	9.398	0.009
UNVAC		876	416	654	1.579	833	1182	1.419
VAC		858	411	654	1.596	814	1161	1.426
PC		878	415[ab]	650	1.569	835	1182	1.418
NC		846	407[ab]	648	1.598	803	1162	1.447
NC + 500 PHY		856	400[b]	640	1.603	813	1156	1.423
NC + 1500 PHY		885	430[a]	678	1.581	842	1184	1.404
					P-value			
VAC*PHY		0.0422	0.0539	0.0144	0.0249	0.0403	0.0473	0.362
VAC		0.0877	0.5826	0.9958	0.6777	0.0859	0.2685	0.7431
PHY		0.0227	0.0341	0.2458	0.8637	0.0227	0.6145	0.3447

[a,b,c]Means within a column with different superscripts are significantly different ($P < 0.05$).

[1]UNVAC, unvaccination; VAC, vaccination; PC, positive control; NC, negative control (reduced 0.15% Ca and P); 500 PHY, 500 FTU/kg of phytase; 1500 PHY, 1500 FTU/kg of phytase. BW, body weight; BWG' body weight gain; FI, feed intake; FCR, feed conversion ratio.

treatment significantly regulated growth performance of birds, whereas vaccination did not show impacts on growth performance, except FI from d 0 to 14 (Table 3). Birds fed the diet with reduction of 0.15% of Ca and available P (NC) had a lower ($P < 0.05$) BWG during d 0 to 7 and d 0 to 14 compared to the PC group. Supplementation of phytase at 500 or 1,500 FTU/kg on birds fed with Ca and P-reduced diet (NC + 500PHY or NC + 1500PHY) was able to improve BW or BWG close to the level as the PC. During d 8 to 14 and d 0 to 14, the birds fed with NC + 1500PHY diet improved ($P < 0.01$; by 9.5%) FCR compared to the NC group.

Interactions between 2 factors for BW, BWG, FI, and FCR were observed in this study during d 15 to 21 and d 0 to 21 (Table 4). The unvaccinated birds fed the NC diet reduced BW during d 15 to 21 and BWG during d 0 to 21 compared to the unvaccinated PC birds ($P < 0.05$), whereas the unvaccinated groups fed NC + 500PHY or NC + 1500PHY diet had improved BWG and were able to reach the same level of growth performance as the unvaccinated PC birds. The vaccinated PC, NC, and NC + 500PHY groups showed lower ($P < 0.05$) BW during d 15 to 21 and BWG during d 0 to 21 than the unvaccinated PC birds. However, supplementation of phytase at 1,500 FTU/kg (NC + 1500PHY) to vaccinated birds improved BW and BWG to the same level as the unvaccinated PC group. During day 15 to 21, the vaccinated birds fed the NC + 1500PHY diet increased ($P < 0.05$) FI compared to the other groups except the unvaccinated PC group. In addition, the unvaccinated birds fed the NC diet and the vaccinated birds fed the NC + 1500PHY diet showed higher ($P < 0.05$) FCR than the unvaccinated NC + 1500PHY birds during d 15 to 21. During 0 to 21 d, the vaccinated birds fed the PC or the NC + 500PHY diet had lower ($P < 0.05$) FI than the unvaccinated PC group, whereas supplementing 1,500 FTU/kg of phytase (NC + 1500PHY) under the vaccination improved the birds' FI to the same level of the unvaccinated PC group.

Apparent Ileal Digestibility of Ca and P and Ileal Phytate Degradation

On day 21, a significant interaction ($P = 0.027$) was observed between coccidial vaccination and phytase supplementation on ileal phytate degradation (Table 5).

Table 5. Effect of phytase supplementation and coccidial vaccine on ileal nutrient digestibility of broiler chickens at day 21.

Treatment[1]		Ca (%)	P (%)	Phytate (%)
UNVAC	PC	58.37	59.22	23.51[f]
	NC	68.53	64.92	44.84[d]
	NC + 500 PHY	66.1	71.23	59.09[c]
	NC + 1500 PHY	65.66	78.02	72.39[b]
VAC	PC	55.71	59.78	35.05[e]
	NC	62.48	63.73	45.43[d]
	NC + 500 PHY	62	71.93	77.95[ab]
	NC + 1500 PHY	57.45	74.65	82.31[a]
SEM		0.983	1.1	3.102
UNVAC		64.50[a]	68.5	50.18
VAC		59.41[b]	67.52	60.18
PC		57.04[b]	59.50[d]	29.28
NC		65.23[a]	64.27[c]	45.16
NC+500 PHY		64.05[a]	71.58[b]	68.52
NC+1500 PHY		61.55[ab]	76.34[a]	77.35
			P-value	
VAC*PHY		0.658	0.533	0.027
VAC		0.003	0.463	<.0001
PHY		0.005	<.0001	<.0001

[a,b,c,d,e,f]Means within a column with different superscripts are significantly different ($P < 0.05$).

[1]UNVAC, unvaccination; VAC, vaccination; PC, positive control; NC, negative control (reduced 0.15% Ca and P); 500 PHY, 500 FTU/kg of phytase; 1500 PHY, 1500 FTU/kg of phytase.

Table 6. Calculated total digested amount of Ca, P and phytate P via feed nutrient percentage, apparent ileal digestibility and feed intake from day 0 to day 21.

Treatment[1]		Digested Ca (g)	Digested P (g)	Digested phytate P (g)
UNVAC	PC	669	521	97
	NC	621	407	159
	NC + 500 PHY	621	458	216
	NC + 1500 PHY	605	495	256
VAC	PC	599	493	136
	NC	566	400	162
	NC + 500 PHY	550	438	269
	NC + 1500 PHY	558	499	306
SEM		9.228	7.737	10.802
UNVAC		629[a]	473	183[b]
VAC		568[b]	457	218[a]
PC		634	507[a]	116[d]
NC		591	403[c]	160[c]
NC + 500 PHY		585	448[b]	243[b]
NC + 1500 PHY		581	497[a]	281[a]
		P-value		
VAC*PHY		0.9424	0.6977	0.1302
VAC		0.0005	0.2109	<.0001
PHY		0.093	<.0001	<.0001

[a,b,c,d]Means within a column with different superscripts are significantly different ($P < 0.05$).

[1]UNVAC, unvaccination; VAC, vaccination; PC, positive control; NC, negative control (reduced 0.15% Ca and P); 500 PHY, 500 FTU/kg of phytase; 1500 PHY, 1500 FTU/kg of phytase.

The birds fed the NC diet showed higher phytate degradation compared to PC birds, and supplementing 500 or 1,500 FTU/kg phytase (NC + 500PHY or NC + 1500PHY) further increased their phytate degradation, in spite of coccidial vaccination. In addition, the phytate degradation of PC, NC + 500PHY, and NC + 1500PHY groups was significantly elevated by vaccination, and the vaccinated NC + 1500PHY group showed the highest phytate degradation level compared to all other groups. However, the phytate degradation of birds in the NC group was not affected by vaccination.

At day 21, no interactions were found between coccidial vaccination and dietary treatments for Ca and P digestibility. The apparent ileal digestibility of Ca was significantly decreased ($P = 0.003$) by vaccination. Dietary treatments significantly regulated AID of Ca and P in broilers. Reducing 0.15% of Ca and P (NC) increased ($P < 0.01$) Ca and P digestibility compared to the PC, and supplementing phytase at 500 FTU/kg or 1,500 FTU/kg (NC + 500PHY or NC + 1500PHY) further increased P digestibility, but did not improve the Ca digestibility.

Total digested Ca, P, and Phytate P

There was no interaction between vaccination and phytase supplementation from day 0 to 21 (Table 6). Vaccination significantly reduced total digested Ca amount ($P = 0.0005$) but increased total digested phytate P amount ($P < 0.0001$) although vaccination did not improve phytate P digestibility (%) of birds in the NC group. For total digested P amount, the NC group showed a negative effect compared to the PC, and supplementing phytase at 500 FTU/kg (NC + 500PHY) was able to improve it; additionally, birds fed diet supplementing phytase at 1,500 FTU/kg (NC + 1500PHY) mitigated the negative effect of the NC diet and showed the same digested P level as the PC group. Moreover, the result showed that the birds fed the NC diet increased total digested phytate P amount compared to the PC group, and supplementing phytase at 500 or 1,500 FTU/kg further improved the digested phytate P amount on birds ($P < 0.0001$).

Bone Ash

There were no interactions between phytase supplementation and vaccination for bone ash parameters from d 0 to 21. Phytase supplementation significantly improved bone ash parameters of birds fed the NC diet, whereas vaccination showed negative impact on these parameters (Table 7). There was an effect of phytase supplementation on bone ash parameters, that, the birds fed NC diet showed lower ($P < 0.05$) ash weight, ash percentage and ash concentration compared to the PC group during d 0 to 21, and supplementing phytase at 500 or 1,500 FTU/kg was able to improve these bone parameters to the same levels as the PC group. In addition, the vaccinated group had lower ($P < 0.05$) ash weight, ash percentage and ash concentration than the unvaccinated group during d 0 to 21.

Gene Expression of Nutrient Transporters and Tight Junction Proteins

An interaction between phytase and vaccination was observed on mRNA expression of nutrient transporters (Table 8). The unvaccinated NC + 1500PHY and the vaccinated PC group showed upregulated CASR gene expression compared to the unvaccinated PC birds ($P < 0.05$). However, there were no significant differences in NCX1, $CALB1_{28}$, PiT1, NaPiIIb, and PMCA1 gene

Table 7. Effect of phytase supplementation and coccidial vaccine on bone ash during d 0 to 21.

Treatment[1]		Volume (cm^3)	FFDW (g)	Ash weight (g)	Ash percentage (%)	Ash concentration (g/cm^3)
UNVAC	PC	8.550	4.1264	2.2321	54.08	0.261
	NC	8.635	3.8922	2.0215	51.91	0.234
	NC+500 PHY	9.363	4.3163	2.3046	53.56	0.247
	NC+1500 PHY	8.479	4.0608	2.2013	54.22	0.260
VAC	PC	8.827	4.0954	2.1671	52.90	0.246
	NC	8.615	3.8416	1.9781	51.51	0.230
	NC+500 PHY	8.624	3.8502	2.0042	52.11	0.234
	NC+1500 PHY	8.412	3.9832	2.1332	53.58	0.253
SEM		0.100	0.050	0.028	0.227	0.002
UNVAC		8.735	4.0984	2.1923[a]	53.50[a]	0.251[a]
VAC		8.620	3.9426	2.0706[b]	52.53[b]	0.241[b]
PC		8.689	4.1109	2.1996[a]	53.49[a]	0.253[ab]
NC		8.624	3.8646	1.9978[b]	51.69[b]	0.232[c]
NC + 500 PHY		8.960	4.0620	2.1407[ab]	52.77[ab]	0.240[bc]
NC + 1500 PHY		8.445	4.0220	2.1673[ab]	53.90[a]	0.256[a]
				P-value		
VAC*PHY		0.3513	0.3642	0.2516	0.7611	0.6591
VAC		0.4983	0.1180	0.0222	0.0215	0.0142
PHY		0.2959	0.3172	0.0379	0.0015	0.0001

[a,b,c]Means within a column with different superscripts are significantly different ($P < 0.05$).

[1]UNVAC, unvaccination; VAC, vaccination; PC, positive control; NC, negative control (reduced 0.15% Ca and P); 500 PHY, 500 FTU/kg of phytase; 1,500 PHY, 1,500 FTU/kg of phytase; FFDW, fat free dry weight.

expression among the treatments. Coccidial vaccination upregulated ($P < 0.05$) MUC2 expression in the ileum (Table 9). However, no other significant differences were found in gene expression of tight junction proteins in the ileum.

DISCUSSION

Prevention of coccidiosis in poultry has relied on dietary anticoccidials and vaccination administrations. However, under No Antibiotics Ever or Antimicrobial-Free production scheme, the poultry industry has to use more vaccination administration for coccidiosis control (Soutter et al., 2020). Previous reports demonstrated that coccidial vaccines negatively affected FI and BWG, especially when broilers are at a young age (Matthews and Southern, 2000; Watson et al., 2005; Yi et al., 2005; Parker et al., 2007; Lehman et al., 2009; Lee et al., 2011; Shaw et al., 2011; Luquetti et al., 2016; Wang et al., 2019b). In the current study, a vaccination effect was observed at day 14 with vaccinated broilers having significantly lower (4%) FI. However, vaccination only caused numerical decrease of BWG and FI throughout the study. A similar finding was reported that during 21 d, there was no difference on body weight or feed consumption between unvaccinated group and vaccinated group (Suarez et al., 2021). Decreased feed intake at day

Table 8. Effects of phytase supplementation and coccidial vaccine on the expression of Ca^{2+} and Pi transporters in the ileum at day 21.

Treatment[1]		NCX1	CASR	$CALB1_{28}$	PiT1	NaPiIIb	PMCA1
UNVAC	PC	1.000	1.000[b]	1.000	1.000	1.000	1.000
	NC	0.826	3.138[ab]	0.850	0.816	0.770	1.257
	NC + 500 PHY	1.125	3.186[ab]	0.726	1.047	1.201	0.988
	NC + 1500 PHY	1.045	3.753[a]	1.154	1.041	0.942	1.215
VAC	PC	0.978	3.726[a]	1.100	1.041	1.165	1.282
	NC	1.096	3.326[ab]	1.286	1.114	0.830	1.159
	NC + 500 PHY	0.881	2.404[ab]	0.882	1.128	0.649	0.985
	NC + 1500 PHY	1.026	2.016[ab]	1.071	1.223	1.253	1.085
SEM		0.0449	0.2293	0.1011	0.0414	0.1262	0.0626
UNVAC		0.994	2.831	0.941	0.973	0.967	1.120
VAC		0.995	2.868	1.085	1.127	0.974	1.128
PC		0.989	2.487	1.050	1.021	1.090	1.141
NC		0.961	3.232	1.068	0.965	0.800	1.208
NC + 500 PHY		0.992	2.760	0.811	1.091	0.900	0.986
NC + 1500 PHY		1.035	2.885	1.113	1.132	1.098	1.150
			P-value				
VAC*PHY		0.3000	0.0038	0.8536	0.6921	0.6892	0.6683
VAC		0.9645	0.8125	0.4799	0.0755	0.9878	0.9219
PHY		0.9514	0.5330	0.7490	0.4987	0.8347	0.6832

[a,b]Means within a column with different superscripts are significantly different ($P < 0.05$). NCX1, sodium/calcium exchanger 1; CASR, calcium-sensing receptor; $CALB1_{28}$, calbindin 1, 28kD isoform; PiT1, phosphate transporter 1; NaPiIIb, sodium-dependent phosphate transport protein 2B; PMCA1, plasma membrane calcium-transporting ATPase 1.

[1]UNVAC, unvaccination; VAC, vaccination; PC, positive control; NC, negative control (reduced 0.15% Ca and P); 500 PHY, 500 FTU/kg of phytase; 1,500 PHY, 1,500 FTU/kg of phytase.

Table 9. Effects of phytase supplementation and coccidial vaccine on gene expression of tight junction proteins and mucin in the ileum at day 21.

Treatment[1]		CLDN1	JAM2	OCLDN	MUC2
UNVAC	PC	1.000	1.000	1.000	1.000
	NC	1.523	0.971	1.229	1.633
	NC+500 PHY	1.968	1.004	0.960	1.040
	NC+1500 PHY	1.330	1.090	1.102	1.006
VAC	PC	1.358	1.062	1.229	1.727
	NC	1.238	0.883	1.192	1.610
	NC+500 PHY	1.127	0.937	1.258	1.861
	NC+1500 PHY	1.242	0.901	1.234	1.600
SEM		0.0716	0.0524	0.0387	0.089
UNVAC		1.401	1.017	1.078	1.183[b]
VAC		1.247	0.946	1.227	1.700[a]
PC		1.179	1.031	1.115	1.364
NC		1.394	0.927	1.210	1.622
NC+500 PHY		1.488	0.967	1.109	1.488
NC+1500 PHY		1.286	0.995	1.168	1.330
		P-value			
VAC*PHY		0.0545	0.8836	0.4622	0.2501
VAC		0.1296	0.5335	0.0512	0.0022
PHY		0.3548	0.9243	0.7617	0.5273

[a,b]Means within a column with different superscripts are significantly different ($P < 0.05$). CLDN1, claudin 1; JAM2, junctional adhesion molecule 2; OCLDN, occluding; MUC2, mucin 2.

[1]UNVAC, unvaccination; VAC, vaccination; PC, positive control; NC, negative control (reduced 0.15% Ca and P); 500 PHY, 500 FTU/kg of phytase; 1500 PHY, 1500 FTU/kg of phytase.

14 is likely due to vaccinated broilers being exposed to the first coccidia cycling, which allows the difference to be more noticeable (Suarez et al., 2021). An explanation of no difference from vaccination during 21 d may also be related to the low dosage (1X) of live oocyte vaccine that we used in this study, compared to others where higher dosages of vaccines were used.

Additionally, interactions between phytase supplementation and vaccination were observed for BWG and FI during 0 to 21 d in the current study, where reducing 0.15% Ca and P in diet compromised growth performance and bone mineralization in broilers, but supplementing phytase at 500 or 1,500 FTU/kg mitigated the negative effects. These responses were likely attributed to the release of P from phytate by phytase, mainly enhancing P digestibility, total digested P amount, and total digested phytate P. However, no improvement of Ca digestibility or total digested Ca amount was observed by phytase supplementation. Similar results were observed by Adedokun and Adeola (2016) that phytase supplementation (1,000 and 5,000 FTU/kg) improved P digestibility but not Ca digestibility. Moreover, the current study showed that phytase supplementation improved bone mineralization of birds fed with a Ca and P-reduced diet by improving P utilization and eventually compromised the negative effect for growth performance. The birds fed phytase at 1,500 FTU/kg had better effects on growth performance, P digestibility, and bone quality compared to those fed phytase at 500 FTU/kg. Hamdi et al. (2018) reported that dietary phytase at 1,000 FTU/kg had positive effects on growth performance and bone mineralization, whereas lower doses of phytase supplementation did not improve both parameters. Furthermore, phytase supplementation alone or in combination with other enzymes improved Ca and P availability by hydrolyzing phytate and increasing bone ash (Onyango et al., 2005; Yan et al., 2006; Francesch and Geraert, 2009; Walk, 2009; Wang, et al., 2019a; Wang and Kim, 2021). In the present study, bone mineralization was significantly affected by vaccination, dietary Ca and P content, and phytase supplementation. Tibia ash is the most sensitive indicator of mineral absorption in broilers, and reductions in dietary Ca and P in the NC diets reduced ($P \leq 0.05$) the percentage and concentration of tibia ash regardless of vaccination status, which has been reported previously in healthy broilers (Dilger et al., 2004; Onyango et al., 2005; Walk et al., 2011b). Moreover, vaccination reduced ash weight, ash percentage, and ash concentration compared with the non-vaccinated groups. Similar findings were reported that coccidial vaccinated birds lowered ash weight or ash percentage (Lehman, 2011; Suarez et al., 2021). Interestingly, vaccination lowered Ca digestibility and total digested Ca, but increased phytate degradation and total digested phytate P. One explanation may be that the narrower Ca:P ratio from lower Ca digestibility and higher phytate P utilization due to vaccination, was related to bone mineralization compromise in the current study. Other studies showed that *E. acervulina* challenge reduced tibia ash percentage in chicks, which is in agreement with our findings (Ward et al., 1993; Watson et al., 2005). In contrast, there was no effect on tibia ash of broilers exposed to a live coccidia oocyst vaccine according to Walk et al. (2011b). Low dietary Ca and P significantly reduced bone ash regardless of vaccination but only influenced growth performance of the unvaccinated groups during 21 d, suggesting that tibia ash is more sensitive to dietary mineral levels than overall growth performance, especially if the chickens are not infected with parasites (Walk et al., 2011b). However, regardless of vaccination, broilers fed PC, NC, or NC + 500 FTU/kg phytase showed a similar BWG from day 15 to 21, whereas the broilers fed 1,500 FTU/kg phytase had the highest BWG. It is known that birds are less responsive to nutrient changes or feed additives during later growth periods (Olukosi et al., 2017). Similarly, no difference was found on growth performance during *Eimeria* challenge (from day 12 to 20) between broilers fed a low-crude protein diet and a regular protein diet (Teng et al., 2021a), even though there were significant differences in growth performance between the regular protein and low-crude protein groups under a non-challenge condition, indicating that *Eimeria* challenge may modulate nutrient requirement and utilization. The current results further confirmed that bone ash is more sensitive than growth performance in terms of dietary Ca and P change. The influence of coccidiosis on growth performance and mineral absorption may be related to *Eimeria* species and infection severity. A previous study reported that increasing the severity of *Eimeria* infection would result in a linear reduction of growth performance and certain mineral digestibility (Teng et al., 2020). Addition to the infection severity, the success of recycling *Eimeria* cycles

is also an important factor when applying coccidial vaccine due to the complex life cycle and intricate host immune response to *Eimeria* (Yun et al., 2000). In the current study, paper pads were placed on the bottoms of the cages to ensure birds' access to their excreta from the day of hatch. This might have helped *Eimeria* cycle and created proper infection for the current study.

Reducing Ca and P in diets significantly increased apparent ileal digestibility of Ca, P, and phytate degradation in the present study. The results were in agreement with previous studies, where broilers fed with Ca or P reduced diets had a higher Ca (Sebastian et al., 1996) or P (Walk et al., 2012) digestibility or phytate degradation (Mohammed et al., 1991). In addition, phytase supplementation further improved P digestibility and phytate degradation in the present study. Phytase supplementation at 500 FTU/kg in the NC diet resulted in the considerable increase for phytate degradation from 45.3% to 73.3% and for P digestibility from 64.3% to 71.6%. The increased P digestibility and phytate degradation indicated that phytase supplementation successfully degraded phytate and released more available P in the feed ingredient. However, digestibility of Ca did not result in any significant improvement in phytase supplementation groups (both 500 and 1,500 FTU/kg) compared to the Ca and P-reduced (NC) group. By calculating total digested amount of Ca, P and phytate P based on FI, and analyzed feed nutrient content and the apparent ileal digestibility (Table 6), we found that 1) coccidial vaccination decreased Ca digestibility and total digested Ca amount but increased the amount of total digested phytate P, which was consistent with the digestibility trend; 2) reducing Ca and P in the diet (NC) lowered the amount of total digested P even though it increased total digested phytate P, which is mainly due to reduction of feed intake by reducing Ca and P in the diet; and 3) phytase supplementation elevated the amounts of total digested P and phytate P, but not digested Ca. Similar results were found that Ca digestibility was not influenced by phytase supplementation (Sebastian et al., 1996; Powell et al., 2011; Walk et al., 2012). In the present study, when 500 FTU/kg of phytase was supplemented to the Ca and P-reduced diet, phytate degradation was increased by 14.25% units (44.84−59.09%) in the unvaccinated group, which was even further increased by 32.52% units (45.43−77.95%) in the vaccinated group (Table 5). This difference indicated that there may be more advantages on phytase effect in coccidial vaccinated birds than the unvaccinated. Masey (2014) reported that a standard phytase dose of 500 FTU/kg is expected to release 0.15% P (0.12% digestible P for poultry) and achieves 50 to 70% of the maximum phytate destruction. Additionally, it is speculated that high phytase doses may achieve more phytate destruction. Angel et al. (2001) reported the equivalent effect of 0.09% nonphytate P for 500 FTU/kg of phytase when using monocalcium phosphate as the standard. Mitchell and Edwards Jr (1996) also reported 600 FTU/kg of phytase is equivalent to 0.20% inorganic P from di-calcium phosphate. It suggests that regardless of vaccination status, phytase is able to enhance P digestibility and phytate degradation in the intestine. However, in the present study, improvement was not seen in Ca digestibility when 500 or 1500 FTU/kg phytase was added to the Ca and P-reduced diet, which was also observed by Walk et al. (2012) and Hamdi et al. (2018). A possible explanation is that the Ca content in NC diet was still adequate for birds' growth and bone development. The optimal Ca:avP ratio for growth is around 2:1. In the current study, we reduced 0.15% of Ca and avP in the NC diet compared to PC normal diet, leading to a wider Ca:P ratio; thus, the Ca level in the diet might had been adequate without stimulating Ca digestibility in the intestine. Additionally, it was observed that higher phytate degradation and total digested phytate P amount were observed in birds fed the Ca and P reduced diet, as well as under vaccination. Similar results were found that degradation of phytate in the digestive tract was increased when broiler chickens were provided with diets having low Ca and P contents, and phytase supplementation further elevated the phytate degradation (Zeller et al., 2015; Sommerfeld et al., 2018; Künzel et al., 2019). This may be explained by a substantial endogenous phytase activity originating from the epithelial tissue or the microbiota resident in the digestive tract (Künzel, et al., 2019; Sommerfeld, et al., 2019). In contrast, previous studies have found negative effects of additional Ca and P to poultry diets on phytate degradation (Tamim et al., 2004; Shastak et al., 2014), while supplementation of microbial phytases increased P availability and reduced the complex formation between phytate and susceptible minerals (Lei and Porres, 2007). It was unexpected that coccidial vaccination decreased the ileal Ca digestion, while it increased phytate P degradation in the current study. Meanwhile, it is notable that the total P digested by birds was not affected by vaccination, but vaccination still compromised bone mineralization. The current results suggest that the minerals released from phytate may interact with other cations like Ca to modulate their digestion and absorption. Moreover, it was reported that bone mineralization was reduced by *Eimeria* infection that caused inflammation and oxidative stress, linking to modification of both bone resorption and formation activities through increasing osteoclast activity and reducing osteoblast activity (Tompkins et al., 2022). Further studies are necessary to corroborate this hypothesis on coccidial vaccination, phytate degradation, mineral utilization, and their interaction on bone development.

In the current study, phytase supplementation and vaccine upregulated the expression of CASR gene in the ileum, whereas a tendency for a decrease in CASR gene expression with phytase supplementation in vaccinated birds was also observed. Proszkowiec-Weglarz et al. (2019) reported that CASR plays a key role in regulating calcium homeostasis of chickens and exists in Ca^{2+} regulatory tissues such as parathyroid, kidney, and intestine. Through CASR, parathyroid chief cells can maintain Ca^{2+} concentrations in plasma by modulating release of

parathyroid hormone into the circulation, and its levels are influenced by plasma vitamin D_3 and Ca (Zanu et al., 2020). The characterization of CASR in intestinal epithelial cells indicates that CASR may mediate Ca^{2+} absorption (Gama, et al., 1997). In the present study, unvaccinated birds fed the PC diet showed the lowest CASR expression compared to other groups. It is speculated that NC diet with reduced Ca and P might have probably resulted in low Ca content in the intestine and blood, hence resulting in elevated calcium-sensing receptor expression which is likely a response to optimize Ca absorption.

Once Ca gets into the cell, it bounds to the cytoplasmic chaperone Calbindin-28 (**$CALB1_{28}$**) and is translocated from the brush border to the basolateral membrane in the intestinal (Nemere et al., 1991). After that, Ca is delivered to the basolateral membrane pumps, such as the plasma membrane calcium-transporting ATPase 1 (**PMCA1**), that is the main transporter to be expressed in broiler intestines (Quinn et al., 2007). In contrast, sodium/calcium exchanger 1 (**NCX1**) expression gets increased as a response to Ca deficient diets in the intestines (Centeno et al., 2004; Hoenderop and Nilius, 2005). Phosphate transporter 1 (**PiT1**) is located on the intestine, kidney, and parathyroid glands, and it has regulatory functions in response to dietary Pi concentrations according to (Giral et al., 2009). Sodium-dependent phosphate co-transporter types IIb (**NaPiIIb**) is a major Na-dependent Pi transporter in the jejunum regulated by vitamin D and P levels in feed (Katai et al., 1999) and plays a major role in P absorption from the intestine. In the present study, dietary treatments showed no effect on mRNA expression of Ca or P transporters ($CALB1_{28}$, PMCA1, NCX1, PiT1, and NaPiIIb), which may indicate that these transporters are more regulated by vitamin D and plasma Ca and P levels instead of Ca and P contents in the intestine. Further study needs to be conducted to understand the interactions among vitamin D, Ca, and P in broilers, especially when birds are under diseases such as *Eimeria* infections.

Coccidiosis influences transcellular translocation by impairing intestinal epithelial cells as well as paracellular translocation by breaking the tight junctions between enterocytes (Teng et al., 2021a, 2021b). The tight junction proteins are located at the apical side of epithelial cells, acting as transmembrane structure of the intestinal junctional complex as well as blocking the paracellular pathway between epithelial cells, in order to regulate intestinal permeability (Ulluwishewa et al., 2011). This complex includes several types of proteins, such as CLDN, OCLDN, JAM, and ZO families (Awad et al., 2017) reported that graded *E. maxima* challenge increased gene expression of CLDN1 and JAM2 but decreased OCLDN expression. Mucin 2 gene can create important protective mucosal layer between intestinal epithelium and the lumen of the gut of chickens (Horn et al., 2009). It is regarded as the first line of defense guards against attacks from microorganisms and is integral to the innate immune system (Jiang et al., 2013). Our finding suggested that coccidial vaccination significantly increased gene expression of MUC2, providing potential protection against pathogens in the intestine. It was surprising that vaccinated birds increased MUC2 mRNA levels because other studies have found reduced MUC2 expression in the intestine during *Eimeria* infection (Tan et al., 2014; Chen et al., 2015; Teng et al., 2021b). This discrepancy may be due to infection severity by high dosages of live *Eimeria* challenge vs. low levels of coccidia vaccination. No difference was found on gene expression of Ca and P transporters or tight junction proteins except CASR, which was consistent with the growth performance of chickens that no difference between unvaccinated group and vaccinated group during 21 d, suggesting that low dose of coccidia vaccine (1X) did not significantly affect intestinal integrity nor compromise FI and BW of broilers. It is logical that broiler gut integrity and most of the nutrient transporters were not altered by vaccination, because the coccidial vaccination was only used as a standard dosage, which is not intended to trigger any clinical or subclinical symptoms. However, the bone mineralization was compromised for vaccinated birds. The decreased Ca digestibility may be partially contributed to the reduction in bone ash. Meanwhile, the nutrients may be redirected to the immune system when birds are vaccinated. In the current study, the upregulation of ileal MUC2 gene expression suggests that there was more mucus produced in vaccinated birds, which also implied coccidial vaccination effect is more related to mucus production and immune regulation than nutrient absorption and gut integrity. It has been well-documented that immune regulation is costly for host (Klasing, 2007). Additionally, bone ash is also reported more sensitive than growth performance especially in Ca and P deficient conditions (Li et al., 2015; Wang and Kim, 2021). Thus, the reduced bone mineralization could be related to the decrease of Ca digestion and immune regulation.

In conclusion, the present study showed that coccidial vaccination and Ca and P reduced diet inhibited growth performance of broilers, while supplementing 1,500 FTU/kg of phytase mitigated the negative effects in vaccinated birds fed a marginally low Ca and P diet. The vaccination resulted in a decrease in bone ash, but supplementing phytase at 500 or 1,500 FTU/kg in Ca and P-reduced diet compensated the reduction of bone ash in the vaccinated birds. Furthermore, phytase showed improvement on P and phytate digestibility. However, no significant regulation of Ca digestibility was observed by phytase supplementation. Both phytase and vaccination showed a tendency to influence the absorption of minerals and bone mineralization, but not much impact on the expression of Ca and P transporters nor tight junction proteins. The results suggest that Ca and P (in this case, 0.15%) levels in the broiler diet can be reduced by supplementing 1,500 FTU/kg of phytase, with or without coccidial vaccination Application of phytase in the Ca and P-reduced diet will reduce the supplementation of inorganic Ca and P without compromising growth performance of broiler chickens but save feed cost in the starter and grower diets.

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DISCLOSURES

The authors declare no conflicts of interest.

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