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

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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.
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). Phytase can chelate essential minerals, including Ca and P, thereby limiting the availability of macro-minerals in the feed-stuffs 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). Adegokun 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 + 500 PHY; Axtra PHY, Danisco UK Ltd, Marlborough, UK); and 4) NC + 1500 FTU/kg of phytase (NC + 1500 PHY). 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% chronic oxide (Cr2O3; 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
analyses. The 2 bone ash birds were collected for gene expression in the middle part of the ileum and the ceca tonsil from 1 of these 5 birds were collected for bone ash analysis, and the left tibia bones from 2 inch anterior to ileocecal junction and pooled within the cage and dried in 75°C oven. The left tibia bones from 2 birds per cage from Meckel diverticulum to about 1 inch anterior to ileocecal junction and pooled within the ileal digesta were collected from 5 birds per 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.

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

| Ingredient, % | PC | NC |
|---------------|----|----|
| Corn, Grain   | 57.77 | 57.77 |
| Soybean Meal  | 35.09 | 35.09 |
| Soybean Oil   | 2.13 | 2.13 |
| Dical. Phos.  | 1.59 | 1.59 |
| Limestone     | 1.16 | 1.16 |
| product space/Sand | 0.70 | 0.70 |
| Ce3O8 | 0.30 | 0.30 |
| Common Salt   | 0.35 | 0.35 |
| DL-Methionine | 0.31 | 0.31 |
| Vitamin Premix | 0.25 | 0.25 |
| L-Lysine HCl  | 0.20 | 0.20 |
| Thr            | 0.09 | 0.09 |
| Mineral Premix| 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 (%) | 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) |

Glucose was prepared from an anhydrous-glucose solution (Daejon Bio, San Jose, CA). The apparent ileal digestibility of Ca, P, and phytate were calculated using the following equation (Cowieson and Adeola, 2005):

\[ \text{AID} (%) = \left[ 1 - \frac{(\text{Cr}1/\text{Cr}0) \times (\text{N}0/\text{N}1)}{100} \right] \]

where \( \text{Cr}1 \) represents the concentration of chromium in diet (%); \( \text{Cr}0 \) represents the concentration of chromium in the ileal digesta (%); \( \text{N}1 \) represents the concentration of P, Ca, or phytate in diets (%); and \( \text{N}0 \) 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/cm³ at room temperature. To determine the fat-free dry matter, bones were dried in an oven at 100°C for 24 h and refueled 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 QiAozol 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...
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 $2^{-\Delta \Delta C_{t}}$ 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

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Table 2. List of primers for qPCR.

| Gene                  | Accession number | Forward primer | Reverse primer          |
|-----------------------|------------------|----------------|-------------------------|
| Housekeeping gene     |                  |                |                         |
| GAPDH                 | NM_204305.1      | CTCCTCTGGCAAAGTCCAAG | GGTCAAGCTCCTGGAGAGATA   |
| HMBS                  | XM_00497916.3    | GCCTGAGGAGATCGCATAGG | TCCCTGAGGAGGATCAGGACT   |
| Ca²⁺ and Pi transporters |              |                |                         |
| PMCA1                 | NM_001168002.2   | TTAATGCTCGGAAATTTTCAC | TCCCAACAAACTGCAAGATAA   |
| NCX1                  | NM_001079473.1   | TCAGTGCCTGCTGTTTGGT | AAGAAAAGCTGCTGGAGCAT    |
| CASR                  | XM_416491        | CGCTCTCGGAGGAGCATAGG | GATGCAAGTGTTGTTGCTTCT   |
| CALB128               | NM_205513        | AAGCGATTTGAGACTCAAGC | CTGGCAGATTTGCAAGACTC    |
| Pit1                  | XM_01290786.2    | TATCTCTCATTGGTCGCGG | TCCTTCTCTCATCAGGGAGCAT  |
| NaPiIb                | NM_204474        | AAGTGCAGTGGGACCATAG  | GAGACAGTTAGGGACAGATG    |
| Tight junction proteins |                |                |                         |
| CLDN1                 | NM_001013611.2   | TGGAGGAGATGCACTGTTGGAAGA | CGACACACTCCTGGTGGCATATA   |
| OCLN                  | XM_0251414248.1  | AGCGAGCACTACTACATCAA | GGGGAAGAGACGAGATGAG     |
| JAM2                  | XM_025149444.1   | AGGCTCAAATGGGTGTGGATT | CATCAGATTGCGATTCTGCACT   |
| Mucin                 | JX_284122.1      | ATGGGACATTTACACAGGATCT | GTGGACAGCAGCAGACTTTTG    |

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

| Treatment | BW (g) | BWG (g) | FI (g) | FCR (g/kg) | FCR (g/kg) | FCR (g/kg) |
|-----------|--------|---------|--------|------------|------------|------------|
| UNVAC PC  | 174    | 131     | 133    | 1.018      | 4.74       | 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| 168    | 125     | 141    | 1.127      | 454        | 285        | 374        | 1.314      | 415        | 515        | 1.256      |
| NC+1500 PHY| 168    | 125     | 141    | 1.127      | 454        | 285        | 374        | 1.314      | 415        | 515        | 1.256      |
| VAC PC    | 167    | 124     | 140    | 1.133      | 465        | 296        | 387        | 1.308      | 423        | 530        | 1.256      |
| NC        | 161    | 118     | 134    | 1.137      | 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     | 138    | 1.135      | 471        | 303        | 363        | 1.200      | 428        | 497        | 1.160      |

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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.

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Note: Means within a column with different superscripts are significantly different ($P < 0.05$).
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 4. Growth performance during d 15 to 21 and d 0 to 21**

| Treatment | BW (g) | BWG (g) | FI (g) | FCR (g/g) | BWG (g) | FI (g) | FCR (g/g) |
|-----------|--------|---------|--------|-----------|---------|--------|-----------|
| UNVAC PC  | 905\textsuperscript{a} | 431     | 678\textsuperscript{ab} | 1.573\textsuperscript{ab} | 862\textsuperscript{a} | 1220\textsuperscript{b} | 1.415       |
| NC        | 838\textsuperscript{b} | 393     | 650\textsuperscript{b} | 1.656\textsuperscript{b} | 795     | 1165\textsuperscript{b} | 1.464       |
| NC + 500 PHY | 874\textsuperscript{abc} | 402     | 649\textsuperscript{b} | 1.615\textsuperscript{b} | 831\textsuperscript{abc} | 1185\textsuperscript{b} | 1.427       |
| NC + 1500 PHY | 881\textsuperscript{abc} | 432     | 640\textsuperscript{b} | 1.483\textsuperscript{b} | 838\textsuperscript{abc} | 1154\textsuperscript{b} | 1.377       |
| VAC PC    | 856\textsuperscript{bc} | 398     | 622\textsuperscript{b} | 1.565\textsuperscript{b} | 807\textsuperscript{bc} | 1144\textsuperscript{b} | 1.420       |
| NC        | 852\textsuperscript{bc} | 419     | 646\textsuperscript{b} | 1.550\textsuperscript{b} | 809\textsuperscript{bc} | 1160\textsuperscript{b} | 1.433       |
| NC + 500 PHY | 838\textsuperscript{c} | 399     | 632\textsuperscript{b} | 1.592\textsuperscript{b} | 795     | 1127     | 1.420       |
| NC + 1500 PHY | 889\textsuperscript{c} | 427     | 717\textsuperscript{a} | 1.678\textsuperscript{a} | 846\textsuperscript{c} | 1213\textsuperscript{c} | 1.432       |
| SEM       | 5.614  | 4.037   | 7.799  | 0.018     | 5.023   | 9.398    | 0.000       |

\( ^{a,b,c} \)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.

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

| Treatment | Ca (%) | P (%) | Phytate (%) |
|-----------|--------|-------|-------------|
| UNVAC PC  | 58.37  | 59.22 | 23.51        |
| NC        | 68.53  | 64.92 | 44.84        |
| NC + 500 PHY | 66.1  | 71.23 | 59.09        |
| NC + 1500 PHY | 65.66 | 78.02 | 57.29        |
| VAC PC    | 55.71  | 59.78 | 35.05        |
| NC        | 62.48  | 63.73 | 45.43        |
| NC + 500 PHY | 62    | 71.93 | 77.95        |
| NC + 1500 PHY | 57.45 | 74.65 | 82.31        |
| SEM       | 0.983  | 1.1   | 3.102        |

\( ^{a,b,c,d,e,f} \)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.
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.

### 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 | Digested Ca (g) | Digested P (g) | Digested phytate P (g) |
|-----------|-----------------|---------------|-----------------------|
| UNVAC PC  | 669             | 521           | 97                    |
| UNVAC NC  | 621             | 407           | 159                   |
| UNVAC NC + 500 PHY | 621         | 458           | 216                   |
| UNVAC NC + 1500 PHY | 605         | 495           | 256                   |
| VAC PC    | 599             | 493           | 136                   |
| VAC NC    | 566             | 400           | 162                   |
| VAC NC + 500 PHY | 550         | 438           | 269                   |
| VAC NC + 1500 PHY | 558         | 499           | 306                   |
| SEM       | 9.228           | 7.373         | 10.802                |
| UNVAC     | 629$^a$         | 473           | 183$^b$               |
| VAC       | 568$^b$         | 457           | 218$^a$               |
| PC        | 634             | 507$^a$       | 116$^a$               |
| NC        | 591             | 403$^b$       | 160$^c$               |
| NC + 500 PHY | 585          | 448$^c$       | 243$^b$               |
| NC + 1500 PHY | 581          | 497$^a$       | 281$^a$               |

- $^a,b,c,d$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.

### 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 further improved the digested phytate P amount on birds ($P < 0.0001$).

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

| Treatment  | Volume (cm³) | FFDW (g) | Ash weight (g) | Ash percentage (%) | Ash concentration (g/cm³) |
|------------|--------------|----------|----------------|--------------------|--------------------------|
| UNVAC PC   | 8.550        | 4.1264   | 2.2321         | 54.08              | 0.261                    |
| NC         | 8.645        | 3.8922   | 2.0215         | 51.91              | 0.234                    |
| NC + 500 PHY | 9.363   | 4.1363   | 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   | 3.8592   | 2.0402         | 52.11              | 0.234                    |
| NC + 1500 PHY | 8.412  | 3.9832   | 2.1332         | 55.58              | 0.253                    |
| SEM        | 0.00          | 0.050    | 0.028          | 0.227              | 0.002                    |
| UNVAC      | 8.735        | 4.0984   | 2.1923         | 53.50              | 0.251                    |
| VAC        | 8.620        | 3.9426   | 2.0706         | 52.53              | 0.241                    |
| PC         | 8.689        | 4.1109   | 2.1996         | 53.49              | 0.253                    |
| NC         | 8.624        | 3.8646   | 1.9793         | 51.69              | 0.232                    |
| NC + 500 PHY | 8.560   | 4.0620   | 2.1407         | 52.77              | 0.240                    |
| NC + 1500 PHY | 8.445  | 4.0220   | 2.1673         | 53.90              | 0.256                    |

| SEM         | 0.100        | 0.050    | 0.028          | 0.227              | 0.002                    |

$^a,b,c$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.

**Table 8.** Effects of phytase supplementation and coccidial vaccine on the expression of Ca²⁺ and Pi transporters in the ileum at day 21.

| Treatment  | NCX1   | CASR   | CALB1$_{28}$ | PiT1   | NaPi$_{2B}$ | PMCA1 |
|------------|--------|--------|--------------|--------|-------------|-------|
| UNVAC PC   | 1.000  | 1.000  | 1.000        | 1.000  | 1.000       | 1.000 |
| NC         | 0.958  | 0.940  | 1.050        | 1.021  | 1.105       | 1.282 |
| NC + 500 PHY | 1.125 | 3.186  | 1.050        | 1.105  | 1.105       | 1.282 |
| NC + 1500 PHY | 1.075 | 3.186  | 1.050        | 1.105  | 1.105       | 1.282 |
| VAC PC     | 1.090  | 3.000  | 1.090        | 1.105  | 1.105       | 1.282 |
| NC         | 1.090  | 3.000  | 1.090        | 1.105  | 1.105       | 1.282 |
| NC + 500 PHY | 1.090 | 3.000  | 1.090        | 1.105  | 1.105       | 1.282 |
| NC + 1500 PHY | 1.090 | 3.000  | 1.090        | 1.105  | 1.105       | 1.282 |

| SEM        | 0.00419| 0.2293 | 0.0111       | 0.0414 | 0.1262      | 0.0626 |
| UNVAC      | 0.994  | 2.831  | 0.941        | 0.973  | 0.974       | 1.120 |
| VAC        | 0.994  | 2.831  | 0.941        | 0.973  | 0.974       | 1.120 |
| PC         | 0.994  | 2.831  | 0.941        | 0.973  | 0.974       | 1.120 |
| NC         | 0.994  | 2.831  | 0.941        | 0.973  | 0.974       | 1.120 |
| NC + 500 PHY | 0.994 | 2.831  | 0.941        | 0.973  | 0.974       | 1.120 |
| NC + 1500 PHY | 0.994 | 2.831  | 0.941        | 0.973  | 0.974       | 1.120 |

| P-value    | 0.00419| 0.2293 | 0.0111       | 0.0414 | 0.1262      | 0.0626 |

$^a,b,c$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.
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). Moreover, vaccination status, which has been reported previously 

### 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 | 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|
| VAC       | 1.247 | 0.946| 1.227 | 1.700|
| 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|
| PHY       | 0.3548| 0.9243| 0.7617| 0.5273|
| 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|

*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.
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 chicks 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-Wegl arz 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 D₃ and Ca (Zanu et al., 2020). The characterization of CASR in intestinal epithelial cells indicates that CASR may mediate Ca²⁺ 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 (CALB₁₂₈) 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 (CALB₁₂₈, 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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REFERENCES

Adedokun, S., and O. Adeola. 2016. The response in jejunal and ileal nutrient and energy digestibility and the expression of markers of intestinal inflammation in broiler chickens to coccidial vaccine challenge and phytase supplementation. Can. J. Anim. Sci. 97:268–267.

Adhikari, R., D. White, J. House, and W. Kim. 2020. Effects of additional dosage of vitamin D3, vitamin D2, and 25-hydroxyvitamin D3 on calcium and phosphorus utilization, egg quality and bone mineralization in laying hens. Poult. Sci. 99:364–373.

Angel, R., A. Dhandu, T. Applegate, and M. Christman. 2001. Phosphorus sparing effect of phytase, 25-hydroxycholecalciferol, and citric acid when fed to broiler chicks. Poult. Sci. 80:133–134.

Awad, W. A., C. Hess, and M. Hess. 2017. Enteric pathogens and their toxin-induced disruption of the intestinal barrier through alteration of tight junctions in chickens. Toxins 9:60.

Blake, D. P., J. Knox, B. Deaceck, B. Huntington, T. Rathinam, V. Ravipati, S. Ayoade, W. Gilbert, A. O. Adebambo, and I. D. Jatau. 2020. Re-calculating the cost of coccidiosis in chickens. Vet. Res. 51:1–14.

Boling, S., M. Douglas, R. Shirley, C. M. Parsons, and K. W. Koelkebeck. 2000. The effects of various dietary levels of phytase and available phosphorus on performance of laying hens. Poult. Sci. 79:535–538.

Centeno, V. A., G. E. D. de Barboza, A. M. Marchionatti, A. E. Alisio, M. E. Dalloro, R. Nasif, and N. G. T. de Talamoni. 2004. Dietary calcium deficiency increases Ca2+ uptake and Ca2+ extrusion mechanisms in chick enterocytes. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 139:133–141.

Cervantes, H. M. 2015. Antibiotic-free poultry production: is it sustainable? J. Appl. Poult. Res. 24:91–97.

Chapman, H. 2014. Milestones in avian coccidiosis research: a review. Poult. Sci. 93:501–511.

Chapman, H., T. Cherry, H. Danforth, G. Richards, M. Shirley, and R. Williams. 2002. Sustainable coccidiosis control in poultry production: the role of live vaccines. Int. J. Parasitol. 32:617–629.

Chen, J., G. Tellez, J. D. Richards, and J. Escobar. 2015. Identification of potential biomarkers for gut barrier failure in broiler chickens. Front. Vet. Sci. 2:14.

Cobb500. 2018. Cobb Broiler Management Guide. Cobb-Vantress, Siloam Springs, AR.

Cowieson, A., T. Acanovic, and M. Bedford. 2006. Supplementation of corn–soy-based diets with an Escherichia coli-derived phytase: effects on broiler chick performance and the digestibility of amino acids and metabolizability of minerals and energy. Poult. Sci. 85:1389–1397.

Cowieson, A., and O. Adeola. 2005. Carbohydrases, protease, and phytase have an additive beneficial effect in nutritionally marginal diets for broiler chicks. Poult. Sci. 84:1860–1867.

Cowieson, A., J.-P. Ruckebusch, J. Sorbara, J. Wilson, P. Guggenbuhl, and F. Roos. 2017. A systematic view on the effect of phytase on ileal amino acid digestibility in broilers. Anim. Feed Sci. Technol. 225:182–194.

Dilger, R., E. Onyango, J. Sands, and O. Adeola. 2004. Evaluation of microbial phytase in broiler diets. Poult. Sci. 83:962–970.

Emami, N. K., Z. S. Naeimi, and C. Ruiz-Feria. 2013. Growth performance, digestibility, immune response and intestinal morphology of male broilers fed phosphorus deficient diets supplemented with microbial phytase and organic acids. Livest. Sci. 157:506–513.

Francesch, M., and P. Geraert. 2009. Enzyme complex containing carboxyhydrazes and phytase improves growth performance and bone mineralization of broilers fed reduced nutrient corn-soybean-based diets. Poult. Sci. 88:1915–1924.

Gama, L., L. M. Baxendale-Cox, and G. E. Breitwieser. 1997. Ca2+ + sensing receptors in intestinal epithelium. Am. J. Physiol. Cell Physiol. 273:C1168–C1175.

Giral, H., Y. Caldas, E. Sutherland, P. Wilson, S. Breusegem, N. Barry, J. Blaine, T. Jiang, and X. X. Wang. 2009. Regulation of rat intestinal Na-dependent phosphate transporters by dietary phosphate. Am. J. Physiol. Renal Physiol. 297:F1466–F1475.

Hamulik, M., J. L,Perez, M.-J. Letourneau-Montinny, H. Franco-Rossello, R. Aligue, and D. Solé-Oriol. 2018. The effects of microbial phytases and dietary calcium and phosphorus levels on the productive performance and bone mineralization of broilers. Anim. Feed Sci. Techn. 243:41–51.

Hoenderop, J. G., and B. Nilius. 2005. Calcium absorption across epithelia. Physiol. Rev. 85:373–422.

Horn, N., S. Donkin, T. Applegate, and O. Adeola. 2009. Intestinal mucin dynamics: response of broiler chicks and White Pekin ducklings to dietary threonine. Poult. Sci. 88:1906–1914.

Jiang, Z. T. J. Applegate, and A. C. Losse. 2013. Cloning, annotation and developmental expression of the chicken intestinal MUC2 gene. PLoS One 8:53781.

Kadykalo, S., T. Roberts, M. Thompson, J. Wilson, M. Lang, and O. Espeisse. 2018. The value of anticoccidials for sustainable global poultry production. Int. J. Antimicrob. Agents 51:304–310.

Katai, K., H. Tanaka, S. Tatsumi, Y. Fukumaga, K. Genjida, K. Morita, N. Kuboyama, T. Suzuki, T. Akiba, and K.-i. Miyamoto. 1999. Nicotinamide inhibits sodium-dependent phosphate cotransport activity in rat small intestine. Nephrol. Dial. Transplant. 14:1195–1201.

Klassing, K. C. 2007. Nutrition and the immune system. Br. Poult. Sci. 48:525–537.

Kiarie, E. G., H. Leung, R. Akbari Moghadam Kakhki, R. Patterson, and J. R. Barta. 2019. Utility of feed enzymes and yeast derivatives in ameliorating deleterious effects of coccidiosis on intestinal health and function in broiler chickens. Front. Vet. Sci. 6:473.

Kim, W., L. Donalson, P. Herrera, C. Woodward, L. Kubena, D. Nisbet, and S. Riche. 2004. Research note: Effects of different bone preparation methods (fresh, dry, and fat-free dry) on bone parameters and the correlations between bone breaking strength and the other bone parameters. Poult. Sci. 83:1663–1666.

Künzel, S., D. Borda-Molina, R. Kraft, V. Sommerfeld, I. Kühn, A. Camarinha-Silva, and M. Rodehutscord. 2019. Impact of coccidiostat and phytase supplementation on gut microbiota composition and phytate degradation in broiler chickens. Anim. Microbiome 1:1–14.

Latta, M., and M. Eskin. 1980. A simple and rapid colorimetric method for phytate determination. J. Agric. Food Chem. 28:1313–1315.

Lee, J., E. Eckert, K. Ameiss, S. Stevens, P. Anderson, S. Anderson, A. Barri, A. McElroy, H. Danforth, and D. Caldwell. 2011. The effect of dietary protein level on performance characteristics of coccidiosis vaccinated and nonvaccinated broilers following mixed-species Eimeria challenge. Poult. Sci. 90:1916–1925.

Lee, Y., M. Lu, and H. S. Lillehoj. 2022. Coccidiosis: Recent progress in host immunity and alternatives to antibiotic strategies. Vaccines 10(5):215.

Lehman, R. E., Moran Jr, and J. H. Hess. 2009. Response of coccidiostat- versus vaccination-protected broilers to gelatin inclusion in high and low crude protein diets. Poult. Sci. 88:984–993.

Lehman, R. N. 2011. The effect of dietary phytic acid concentration and phytase supplementation on performance, bone ash, and intestinal health of broilers vaccinated with a live coccidial oocyst vaccine. Virginia Tech.

Li, G. Q., S. Kann, S. M. Xiao, and F. Y. Xiang. 2005. Responses of chickens vaccinated with a live attenuated multi-valent ionophore/antibiotic Eimeria vacccinis and nonvaccinated controls following mixed-species Eimeria challenge. Poult. Sci. 94:1916–1925.

Lee, X. G., and J. M. Porres. 2007. Phytase and inositol phosphates in animal nutrition: dietary manipulation and phosphorus excretion by animals. Inositol Phosphates. Linking Agriculture and the Environment, CABI, Wallingford, UK, 133–194.

Li, W., R. Angel, S. W. Kim, E. Jimenez-Moreno, M. Proszkowiec-Weglarz, and P. W. Plumstead. 2015. Impact of dietary phytate phosphorus equivalance. Poult. Sci. 94:2228–2234.
Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25:402–408.

Luqueti, B. C., M. F. F. Alarcon, R. Lunedo, D. M. B. Campos, R. L. Furlan, and M. Macari. 2016. Effects of glutamine on performance and intestinal mucosa morphology of broiler chickens vaccinated against coccidiosis. Sci. Agric. 73:322–327.

Maenz, D. D., C. M. Engele-Schaan, R. W. Newkirk, and H. L. Classen. 1999. The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-susceptible forms of phytic acid in solution and in a slurry of canola meal. Anim. Feed Sci. Technol. 81:177–192.

Mansoori, B., M. Modirsanei, H. Nodeh, and S. Rahbari. 2010. The interactive effect of phytase and coccidia on the gross lesions as well as the absorption capacity of intestine in broilers fed with diets low in calcium and available phosphorous. Vet. Parasitol. 168:111–115.

Masey, O. 2014. ‘Neill HV, Bedford MR & Walker N’ (2015) Recent developments in feed enzyme technology. Pages 97-106 in Recent Advances in Animal Nutrition. PC Garnsworthy and J Wiseman, eds. Context Products, United Kingdom.

Matthews, J., and L. Southern. 2000. The effect of dietary betaine in Eimeria acervulina-infected chicks. Poult. Sci. 79:60–65.

Mitchell, R., and H. Edwards Jr. 1996. Effects of phytase and 1,25-dihydroxycholecalciferol on phytate utilization and the quantitative requirement for calcium and phosphorus in young broiler chicks. Poult. Sci. 75:95–110.

Mohammed, A., M. Gibney, and T. Taylor. 1991. The effects of dietary levels of inorganic phosphorus, calcium and cholecalciferol on the digestibility of phytate-P by the chick. Br. J. Nutr. 66:251–259.

Nemere, I., V. L. Leathers, B. S. Thompson, R. A. Luten, and A. W. Norman. 1991. Redistribution of calbindin-D28k in chick intestine in response to calcium transport. Endocrinology 129:2972–2984.

Olukosi, O. A., and S. A. Aredolokun. 2017. Species-dependent response to the influence of adaptation length during assay for metabolisable energy of cereal grains employing the difference method. Anim. Feed Sci. Technol. 231:111–118.

Onyango, E., M. Bedford, and O. Adeola. 2005. Efficacy of an evolved Escherichia coli phytase in diets of broiler chicks. Poult. Sci. 84:248–255.

Paris, N., and E. Wong. 2013. Expression of digestive enzymes and nutrient transporters in the intestine of Eimeria maxima-infected chickens. Poult. Sci. 92:1331–1335.

Park, J., E. Oviido-Rondón, B. A. Clack, S. Clemente-Hernandez, J. Osborne, J. Remus, H. Kettunen, H. Mäkilävuo, and E. Pierson. 2007. Enzymes as feed additive to aid in responses against Eimeria species in coccidia-vaccinated broilers fed corn-soybean meal diets with different protein levels. Poult. Sci. 86:643–653.

Persia, M., E. Young, P. Utterback, and C. Parsons. 2006. Effects of dietary ingredients and Eimeria acervulina infection on chick performance, apparent metabolizable energy, and amino acid digestibility. Poult. Sci. 85:48–55.

Powell, S., T. Bidner, and L. Southern. 2011. Phytase supplementation improved growth performance and bone characteristics in broilers fed varying levels of dietary calcium. Poult. Sci. 90:600–608.

Proszkowiec-Weglarz, M., L. L. Schreier, K. B. Miska, R. Angel, S. Kahl, and B. Russell. 2019. Effect of early neonatal development and delayed feeding post-hatch on jejunal and ileal calcium and phosphorus transporter genes expression in broiler chickens. Poult. Sci. 98:186–187.

Quinn, S. J., O. Kifor, I. Kifor, R. R. Butters Jr, and E. M. Brown. 2007. Role of the cytoskeleton in extracellular calcium-regulated PTH release. Biochem. Biophys. Res. Commun. 354:8–13.

Ravinlran, V., A. Cowieson, and P. Selle. 2008. Influence of dietary electrolyte balance and microbial phytase on growth performance, nutrient utilization, and excreta quality of broiler chickens. Poult. Sci. 87:677–688.

Sakkas, P., I. Öiêeh, D. P. Blake, M. J. Nolan, R. A. Bailey, A. Oxley, I. Rychlik, G. Lietz, and I. Kyriazakis. 2018. Does selection for growth rate in broilers affect their resistance and tolerance to Eimeria maxima? Vet. Parasitol. 258:88–98.

Sebastian, S., S. Touchburn, E. Chavez, and P. Lague. 1996. Efficiency of supplemental microbial phytase at different dietary calcium levels on growth performance and mineral utilization of broiler chicks. Poult. Sci. 75:1516–1523.

Shi, Y., and V. Ravindran. 2007. Microbial phytase in poultry nutrition. Anim. Feed Sci. Techn. 135:1–41.

Shi, T., M. McDonald, and J. Dingle. 1991. Effects of dietary calcium and available phosphorus concentration on digesta pH and on the availability of calcium, iron, magnesium and zinc from the intestinal contents of meat chickens. Br. Poult. Sci. 32:185–194.

Shang, Y., A. Rogiewicz, R. Patterson, B. Slominski, and W. Kim. 2015. The effect of phytase and fructooligosaccharide supplementation on growth performance, bone quality, and phosphorus utilization in broiler chickens. Poult. Sci. 94:955–964.

Shastak, Y., E. Zeller, M. Witzig, M. Schollenberger, and M. Rodehutscord. 2014. Effects of the composition of the basal diet on the evaluation of mineral phosphorus sources and interactions with phytate hydrolysis in broilers. Poult. Sci. 93:2548–2559.

Shaw, A., F. Van Ginkel, K. Macklin, and J. Blake. 2011. Effects of phytase supplementation in broiler diets on a natural Eimeria challenge in naive and vaccinated birds. Poult. Sci. 90:781–790.

Shaw, A. L., K. S. Macklin, and J. P. Blake. 2012. Phytase supplementation in a reduced calcium and phosphorus diet fed to broilers undergoing an Eimeria challenge. J. Poult. Sci. 49:178–182.

Sommerfeld, V., M. Schollenberger, I. Kühn, and M. Rodehutscord. 2018. Interactive effects of phosphorus, calcium, and phytase supplements on products of phytate degradation in the digestive tract of broiler chickens. Poult. Sci. 97:1177–1188.

Su, S., K. Miska, R. Fetterer, M. Jenkins, and E. Wong. 2014. Expression of digestive enzymes and nutrient transporters in Eimeria acervulina-challenged layers and broilers. Poult. Sci. 93:1217–1226.

Suarez, J., K. Knappe, and J. Carey. 2021. Evaluation of animal feed grade sodium bisulfate supplementation on performance, intestinal morphology and vitamin D status of broilers challenged with coccidiosis vaccine. J. Appl. Poult. Res. 30:1012–1027.

Tamim, N., R. Angel, and M. Christman. 2004. Influence of dietary calcium and phytase on phytate phosphorus hydrolysis in broiler chickens. Poult. Sci. 83:1358–1367.

Tang, J., T. J. Applegate, S. Liu, Y. Guo, and S. D. Eicher. 2014. Supplementation of dietary L-arginine attenuates intestinal mucosal disruption during a coccidial vaccine challenge in broiler chickens. Br. J. Nutr. 112:1098–1109.

Teng, P.-Y., J. Choi, S. Yadav, Y. Tompkins, and W. K. Kim. 2021a. Effects of low-crude protein diets supplemented with arginine, glutamine, threonine, and methionine on regulating nutrient absorption, intestinal health, and growth performance of Eimeria-infected chickens. Poult. Sci. 100:104427.

Teng, P.-Y., J. Choi, Y. Tompkins, H. Lillehoj, and W. Kim. 2021b. Impacts of increasing challenge with Eimeria maxima on the growth performance and gene expression of biomarkers associated with intestinal integrity and nutrient transporters. Vet. Res. 52:1–12.

Teng, P.-Y., S. Yadav, F. L. de Souza Castro, Y. H. Tompkins, and W. K. Kim. 2020. Influence of dietary L-arginine on regulating nutrient absorption, intestinal health, and growth performance of Eimeria-challenged chickens. Poult. Sci. 100:4203–4216.

Tompkins, Y., P.-Y. Teng, R. Pazdro, and W. K. Kim. 2022. Long bone mineral loss, bone microstructural changes and oxidative stress after Eimeria challenge in broilers. Front. Physiol. 1344.

Turner, B. L., A. E. Richardson, and E. J. Mullaney. 2007. Inositol phosphates: linking agriculture and the environment. CABI.
Ulluwishewa, D., R. C. Anderson, W. C. McNabb, P. J. Moughan, J. M. Wells, and N. C. Roy. 2011. Regulation of tight junction permeability by intestinal bacteria and dietary components. J. Nutr. 141:769–776.

Walk, C., M. Bedford, and A. McElroy. 2012. Influence of limestone and phytase on broiler performance, gastrointestinal pH, and apparent ileal nutrient digestibility. Poult. Sci. 91:1371–1378.

Walk, C., A. Cowieson, J. Remus, C. Novak, and A. McElroy. 2011. Effects of dietary enzymes on performance and intestinal goblet cell number of broilers exposed to a live coccidia oocyst vaccine. Poult. Sci. 90:91–98.

Walk, C., C. Wyatt, R. Upton, and A. McELROY. 2011. Effect of diet and phytase on the performance and tibia ash of broilers exposed to a live coccidia oocyst vaccine. J. Appl. Poult. Res. 20:153–161.

Walk, C. L. 2009. Effects of dietary enzyme supplementation on performance, bone ash, small intestinal morphology, and apparent ileal amino acid digestibility of broilers exposed to a live coccidia oocyst vaccine. Virginia Tech.

Wang, J., and W. Kim. 2021. Evaluation of a novel corn-expressed phytase on growth performance and bone mineralization in broilers fed different levels of dietary nonphytate phosphorus. J. Appl. Poult. Res. 30:100120.

Wang, J., R. Patterson, and W. Kim. 2019. Effects of extra-dosing phytase in combination with multi-carbohydrase on growth performance and bone mineralization using dual-energy x-ray absorptiometry in broilers. J. Appl. Poult. Res. 28:722–728.

Wang, X., E. D. Peebles, A. S. Kiess, K. G. Wamsley, and W. Zhai. 2019. Effects of coccidial vaccination and dietary antimicrobial alternatives on the growth performance, internal organ development, and intestinal morphology of Eimeria-challenged male broilers. Poult. Sci. 98:2054–2065.

Ward, T., K. Watkins, and L. Southern. 1993. Research Note: Interactive effects of sodium zeolite A and Eimeria acervulina infection on growth and tissue minerals in chicks. Poult. Sci. 72:2172–2175.

Watson, B., J. Matthews, L. Southern, and J. Shelton. 2005. The interactive effects of Eimeria acervulina infection and phytase for broiler chicks. Poult. Sci. 84:910–913.

Williams, R. 2002. Anticoccidial vaccines for broiler chickens: pathways to success. Avian Pathol 31:317–354.

Yan, F., J. Kersey, C. Fritts, and P. Waldroup. 2006. Effect of phytase supplementation on the calcium requirement of broiler chicks. Int. J. Poult. Sci. 5:112–120.

Yi, G., G. Allee, C. Knight, and J. Dibner. 2005. Impact of glutamine and oasis hatching supplement on growth performance, small intestinal morphology, and immune response of broilers vaccinated and challenged with Eimeria maxima. Poult. Sci. 84:283–293.

Yun, C., H. Lillehoj, and E. Lillehoj. 2000. Intestinal immune responses to coccidiosis. Dev. Comp. Immunol. 24:303–324.

Zanu, H. K., S. Kheravii, N. Morgan, M. Bedford, and R. Swick. 2020. Interactive effect of dietary calcium and phytase on broilers challenged with subclinical necrotic enteritis: part 2. Gut permeability, phytate ester concentrations, jejunal gene expression, and intestinal morphology. Poult. Sci. 99:4914–4928.

Zeller, E., M. Schollenberger, M. Witzig, Y. Shastak, I. Kühn, L. E. Hoelzle, and M. Rodehutscord. 2015. Interactions between supplemented mineral phosphorus and phytase on phytate hydrolysis and inositol phosphates in the small intestine of broilers. Poult. Sci. 94:1018–1029.

Zhu, B., and C. N. Coon. 1997. The relationship of various tibia bone measurements in hens. Poult. Sci. 76:1698–1701.

Zwart, S. 2006. Concerns in phytase use. Feed Tech 6:26–28.

Vantress, C. 2017. Broiler management guide. Accessed Aug. 2022. https://www.cobb-vantress.com/assets/5c7576a214/Broiler-guide-R1.pdf.