Interactive effect of 2 dietary calcium and phytase levels on broilers challenged with subclinical necrotic enteritis: part 1—broiler performance, gut lesions and pH, bacterial counts, and apparent ileal digestibility

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ABSTRACT This study investigated the hypothesis that high dietary calcium (**Ca**) would stimulate necrotic enteritis (**NE**) and reduce performance, gut health, and nutrient digestibility, and if increased, phytase would reduce NE. Ross 308 male broilers (n = 768) were randomly distributed to 8 treatments in a factorial arrangement. Factors were NE challenge (no or yes), phytase level (500 or 1,500 FTU/kg using 500 FTU/kg matrix values), and Ca level (0.6 or 1.0% starter, 0.5 or 0.9% grower, 0.4 or 0.8% finisher) with the same level of available P (0.40 S, 0.35 G, and 0.35 F). There were 48 pens, 16 birds per pen and 6 replications. Half of the birds were challenged with *Eimeria spp* on day 9 and 10^8 CFU per mL of *Clostridium perfringens* strain EHE-NE18 on day 14 and 15. Gain was higher in birds fed high phytase on day 14 ($P < 0.01$), day 21 ($P < 0.01$), day 28 ($P < 0.01$), and day 35 ($P < 0.01$). Birds fed high phytase had greater livability on day 21 ($P < 0.01$). Ca was more digestible in high-Ca diets on day 16, and an NE × Ca interaction ($P < 0.05$) showed this effect to be more pronounced in unchallenged than in challenged birds. A challenge × Ca interaction for apparent ileal digestibility (**AID**) of crude protein (**CP**) ($P < 0.05$) indicated lower AID of CP in challenged birds fed high Ca. The challenge decreased AID of Ca ($P < 0.01$). Ca level had no impact on *C. perfringens* count, but it decreased *Lactobacillus* ($P < 0.05$) and *Bifidobacteria* ($P < 0.05$) populations in the ceca. High dietary Ca decreased feed conversion ratio. Overall (42 D), the highest WG was observed in unchallenged birds fed high Ca and high phytase with the lowest WG observed in NE-challenged birds fed low Ca and low phytase. The results suggest that full matrix values for high doses of phytase may be appropriate during NE challenge.

Key words: broiler, dietary calcium, phytase, necrotic enteritis, performance

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INTRODUCTION

Calcium (**Ca**) is an important constituent of bone, and the movement of Ca^+ ions across cytoplasmic membranes is a signal for many cellular processes (Steinhorst and Kudla, 2014). Ca is vital to many biological processes including blood coagulation, eggshell formation, transmission of nerve impulses to the muscle, muscle contraction, and immune function and is involved in energy and fat metabolism (Pilvi et al., 2008; Kozyreva et al., 2009; Vasin et al., 2010; Akbari Moghaddam Kakhki et al., 2018; Toyoda et al., 2018). Because limestone is cheap and readily available and often used as a flow conditioner in premixes and soybean meal, the industry tends to produce diets with higher levels of Ca than formulated. In such cases, there may be interference in Ca digestion and absorption by phytic acid leading to P deficiency even in excessively high-Ca diets (Cowieson and Bedford, 2009).

Ca is a divalent cation that forms chelates with polyanaionic phytate molecules to form mineral-phytate complexes. Phytate (myoinositol hexaphosphate or IP_6) is predominantly present in chicken diet at a concentration of about 10 g kg^{-1}. The extent to which Ca is chelated by phytate is partly determined by the level of Ca in the diet and its solubility (Li et al., 2016). A soluble Ca source is more likely to form a complex with phytate and thus reduce the efficacy of the exogenous phytase. Another factor that is crucial to Ca-phytate

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formation is gut pH. The acidic pH in the foregut of the chicken increases the solubility and susceptibility to degradation of phytate. However, Ca, especially as limestone, has a high acid-binding capacity and tends to increase digesta pH (Angel et al., 2002; Selle et al., 2009).

In addition, high-Ca diets such as those prescribed by the NRC. (1994) might have a deleterious effect on the efficacy of phytase as the activity is greater at low pH (Li et al., 2017; Sommerfeld et al., 2018). Furthermore, incremental dietary Ca may suppress phytase activity by precipitating the phytic acid or coordinating with phytate in solution and thus making it incapable for entering the active site of the enzyme (Qian et al., 1997). Another crucial factor in the activity of phytase is the inclusion level of Ca or Ca-P ratio. The current consensus is that a narrower Ca-P ratio is beneficial to birds fed phytase-supplemented diets (Qian et al., 1997). Tamim et al. (2004) and Wilkinson et al. (2014) have reported substantial hydrolysis of phytate by exogenous phytases in the intestine of broilers fed restricted level of dietary Ca.

The effects of high dietary Ca on the growth of pathogenic bacteria such as *Clostridium perfringens* has not been thoroughly studied; however, Ca has been implicated in the pathogenesis of necrotic enteritis (**NE**) (Williams, 2005). Also, NetB toxin produced by *C. perfringens* type A is reported to depend on Ca for full activity (Keyburn et al., 2010). According to those authors, the NetB toxin forms pores in the enterocyte membrane, which results in an influx of ions into the cytoplasm. An influx of Ca ions into the cytoplasm can lead to deregulation of mitochondrial activity and reduction in adinosinetriphosphate levels and the production of reactive oxygen species (Kennedy et al., 2009) and eventually leads to osmotic cell lysis.

A recent study was conducted to determine the influence of Ca source (highly soluble calcified seaweed or limestone), phytase supplementation (0 vs. 1,000 FTU/kg), and dietary levels of Ca (0.6 vs. 0.9%) on the performance of broiler chickens (Paiva et al., 2013). In that study, birds were naturally inoculated with NE by being placed on used litter from a previous flock that exhibited clinical signs of NE. That study indicated that higher dietary Ca (0.9 vs. 0.6%) had a negative effect on mortality associated with NE and on bird performance. To more fully investigate how Ca and phytase levels might impact the onset of NE, this study was conducted to examine the effects of 2 levels of dietary Ca (0.6 vs. 1.0%) and phytase (500 vs. 1,500 FTU/kg) on performance and digestibility of unchallenged and challenged broilers.

MATERIALS AND METHODS

Birds, Management, and Diet Composition

All experimental procedures were reviewed and approved by the University of New England's Animal Ethics Committee (authority no. AEC 18-031). A total of 768 of the chicks were weighed (average weight of 42 g) and randomly allocated to 48-floor pens with 6 replicates per treatment and 16 birds each. Softwood shavings were used as bedding material of about 8-cm deep in each pen. Each pen was fitted with a single tube feeder (32 cm diameter) and 4 nipple drinkers. The lighting and temperature program during the experimental period followed the Ross 308 guidelines (Aviagen, 2014). Mortality was recorded daily, and cumulative pen weight and feed intake (**FI**) were recorded on day 7, 14, 21, 28, 35, and 42. However, day 14 and 35 are not reported in this article.

Four diets were formulated for each phase in accordance with Ross 308 nutrient specifications. Treatments were arranged in a 2 × 2 × 2 factorial arrangement. Factors were NE challenge (no or yes); phytase: 500 or 1,500 FTU/kg (Quantum Blue; AB Vista, Malborough, UK); and Ca: 0.6% or 1.0% in starter (S, 0–14 D), 0.5 or 0.9% in grower (G, 14–28 D), and 0.4 or 0.8% in finisher (F, 28–42 D). Diets were formulated to a constant level of available phosphorus (**P**) (0.40% in S, 0.35% in G, and 0.35% in F) irrespective of the Ca level. Thus, diets with high dietary Ca had a wider Ca-P of 2.5, and those with lower dietary Ca had a narrower ratio of 1.5. The phytase was formulated into the diets using a 500-FTU/kg matrix value recommended by the manufacturer. The starter diets were offered in a crumbled form while the grower and finisher diets were mixed and pelleted at 65°C. The diets are shown in Table 1.

Challenge

The NE challenge was performed in accordance with reported procedures (Stanley et al., 2014; Rodgers et al., 2015). Half of the birds (384) were orally gavaged with 5,000 oocysts of field strains of *Eimeria acervulina* and *Eimeria maxima* and 2,500 oocytes of *Eimeria brunetti* (Eimeria Pty Ltd.) on day 9 and 10^8 CFU per mL of *C. perfringens* strain EHE-NE18 (known to express NetB toxin, CSIRO) on day 14 and 15.

Performance

The birds and feed were weighed on a pen basis weekly. FI, gain, and feed conversion ratio (**FCR**) (feed:gain) were calculated weekly except for intake and FCR on day 21. The birds were monitored for mortality twice daily, and a postmortem examination was conducted on dead birds throughout the study period. FI and FCR were corrected for mortality by adding the weight of the dead birds back to the live birds within each period. Livability was calculated for each period as the number of live birds/the number of birds starting birds × 100.

Gastrointestinal pH

Immediately after euthanasia on day 16 and 29, the gizzard, ileum, and ceca were removed intact from 2 birds per pen. The pH was measured and recorded in duplicate. In brief, a digital pH meter (Mettler-Toledo, UK) with a spear tip piercing pH electrode (Sensorex,

Table 1. Ingredient and nutrient composition of basal diets (g/kg), as-fed.

	Starter (0—14 D)				Grower (14–28 D)				Finisher (28–42 D)			
Ingredients	0.6% Ca + low phytase	0.6% Ca + high phtase	1.0% Ca + low phytase	1.0% Ca + high phytase	0.6% Ca + low phytase	0.6% Ca + high phytase	1.0% Ca + low phytase	1.0% Ca + high phytase	0.6% Ca + low phytase	0.6% Ca + high phytase	1.0% Ca + low phytase	1.0% Ca + high phytase
Wheat	620	620	597	597	708	708	687	687	723	723	702	702
SBM	296	296	302	302	185	185	189	189	166	166	170	170
Canola expeller cold press	50	50	50	50	70	70	70	70	70	70	70	70
Canola oil	12	12	18	18	14	14	21	21	23	23	30	30
Limestone	6	6	16	16	5	5	15	15	3	3	14	14
MDC phosphate[1]	4.19	4.19	4.26	4.26	2.42	2.42	2.5	2.5	0.64	0.64	0.71	0.71
Xylanase[2]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Phytase[3]	0.1	0.3	0.1	0.3	0.1	0.3	0.1	0.3	0.1	0.3	0.1	0.3
Salt	1.41	1.41	1.43	1.43	0.83	0.83	0.85	0.85	0.82	0.82	0.84	0.84
Na bicarbonate	2	2	2	2	2	2	2	2	2	2	2	2
TiO_2	–	–	–	–	5	5	5	5	5	5	5	5
Vitamins[4]	1	11	1	1	0.7	0.7	0.7	0.7	0.5	0.5	0.5	0.5
Trace minerals[5]	1.2	1.2	1.2	1.2	1	1	1	1	1	1	1	1
Choline Cl 60	0.64	0.64	0.67	0.67	0.74	0.74	0.77	0.77	0.58	0.58	0.6	0.6
L-lysine HCl	2.34	2.34	2.24	2.24	2.81	2.81	2.75	2.75	2.61	2.61	2.55	2.55
DL-methionine	1.99	1.99	2	2	1.58	1.58	1.6	1.6	4.89	4.89	4.9	4.9
L-threonine	0.54	0.54	0.53	0.53	0.57	0.57	0.58	0.58	0.51	0.51	0.51	0.51
Nutrients												
MEn, kcal/kg	3,000	3,000	3,000	3,000	3,100	3,100	3,100	3,100	3,100	3,100	3,100	3,100
Crude protein, %	24.4	24.4	24.3	24.3	20.9	20.9	20.8	20.8	20.3	20.3	20.3	20.3
SID, %												
Arginine	1.40	1.40	1.41	1.41	1.14	1.14	1.14	1.14	1.09	1.09	1.09	1.09
Lysine	1.24	1.24	1.24	1.24	1.05	1.05	1.05	1.05	0.99	0.99	0.99	0.99
Methionine	0.5	0.5	0.5	0.5	0.43	0.43	0.43	0.43	0.75	0.75	0.75	0.75
d M + C	0.89	0.89	0.88	0.88	0.80	0.80	0.80	0.80	1.11	1.11	1.11	1.11
Tryptophan	0.27	0.27	0.27	0.27	0.23	0.23	0.23	0.23	0.22	0.22	0.22	0.22
Isoleucine	0.88	0.88	0.88	0.88	0.73	0.73	0.73	0.73	0.70	0.70	0.70	0.70
Threonine	0.79	0.79	0.79	0.79	0.68	0.68	0.68	0.68	0.65	0.65	0.65	0.65
Valine	0.98	0.98	0.98	0.98	0.84	0.84	0.84	0.84	0.81	0.81	0.81	0.81
Calcium, %	0.6	0.6	1	1	0.51	0.51	0.91	0.91	0.43	0.43	0.83	0.83
Available P, %	0.4	0.4	0.4	0.4	0.36	0.36	0.36	0.36	0.32	0.32	0.32	0.32
Sodium, %	0.18	0.18	0.18	0.18	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
Choline, pmm	1,700	1,700	1,700	1,700	859	859	885	885	731	731	757	757
Analyzed, DM basis												
Calcium, %	0.51	0.52	0.77	0.84	0.40	0.40	0.82	0.92	0.38	0.33	0.76	0.79
Total P, %	0.59	0.60	0.59	0.61	0.54	0.54	0.58	0.59	0.51	0.52	0.52	0.51

Abbreviation: DM, dry matter; MDC, mono-dicalcium phosphate; MEn, metabolizable energy corrected to zero nitrogen retention; SBM, soybean meal; SID, standardized ileal digestible.

[1]MDC phosphate contains 21% P and 16% Ca, Kynofos 21, sourced from BEC, Brisbane, QLD.

[2]Xylanase was Econase XP 25, providing 160,000 birch xylanase units per gram inclusion, AB Vista Feed Ingredients, UK, no matrix values applied.

[3]Phytase was Quantum Blue 5G, AB Vista Feed Ingredients, UK, to provide 500 FTU/kg (0.1% phytase) or 1,500 FTU/kg (0.3% phytase). Matrix values used Ca, P, Na, ME, CP, arginine, lysine, methionine, methionine + cystine, tryptophan, isoleucine, threonine, and valine of 1,650, 1,500, 350%, 520,000 kcal/kg, 4,210, 130, 170, 39, 390, 190, 255, 330, and 230%, respectively, (amino acids expressed as SID).

[4]Vitamin premix per kg diet: vitamin A, 12 MIU; vitamin D, 5 MIU; vitamin E, 75 mg; vitamin K, 3 mg; nicotinic acid, 55 mg; pantothenic acid, 13 mg; folic acid, 2 mg; riboflavin, 8 mg; cyanocobalamin, 0.016 mg; biotin, 0.25 mg; pyridoxine, 5 mg; thiamine, 3 mg; antioxidant, 50 mg.

[5]Trace mineral concentrate supplied per kilogram of diet: Cu (sulfate), 16 mg; Fe (sulfate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulfate and oxide), 120 mg; Zn (sulfate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

Garden Grove, CA) was directly inserted into the digesta in the lumen of the proximal gizzard (proventricular opening), ileum, and ceca of the same bird, while avoiding direct contact of the pH electrode with the gut wall. Once the 2 readings per sample were recorded, the probe was rinsed with ultra-pure water (ICW 3,000 water purifier for ion chromatograph; Millipore, Burlington, MA). The mean of the 2 readings per site of the tract was then calculated and recorded. The digesta of the ileum and ceca from the 2 birds were sampled to determine the digestibility of nutrients and bacterial quantification, respectively.

Intestinal Lesions

The entire length of the small intestine (duodenum, jejunum, and ileum) of the sampled birds was scored for lesions as described by Keyburn et al. (2006) as follows: 0, no lesions; 1, thin-walled and friable intestines; 2, focal necrosis or ulceration (1–5 foci); 3, focal necrosis (6–15 foci); 4, focal necrosis (16 or more foci); 5, patches of necrosis 2- to 3-cm long; and 6, diffuse necrosis typical of acute field cases. Two experienced personnel, with no knowledge of treatment arrangements across the pens, were involved in the scoring process. The average of the scores was computed, and the pen was the experimental unit for lesion scoring.

Chemical Analyses and Digestibility Calculations

The diets and ileal digesta samples (from 2 birds on day 16) were analyzed for nitrogen, carbon, Ca, P, Mg, K, and Zn contents. The nitrogen and carbon content of the diets and digesta samples was determined on a 0.25-g sample using a combustion analyzer (Leco model FP-2000 N analyzer; Leco Corp., St. Joseph, MI) with EDTA as a calibration standard, with crude protein (**CP**) being calculated by multiplying percentage N by a correction factor (6.25). Mineral content was determined using inductively coupled plasma-optical emission spectroscopy after digestion in concentrated HNO_3. The spectrophotometric method described by Short et al. (1996) was followed to measure TiO_2 concentration in the diet and ileal digesta samples. The apparent ileal digestibility (**AID**) of CP and minerals was calculated using the indigestible marker and the following formula:

$$\text{Apparent ileal digestibility (\%)} = \{1 - [\text{TiO}_2\text{diet(\%)} / \text{TiO}_2\text{digesta(\%)}] \times [\text{digesta nutrient(\%)} / \text{diet nutrient(\%)}]\} \times 100$$

Extraction of Cecal Bacterial DNA

The DNA of cecal content collected on day 16 was extracted using a PowerFecal QIAcube HT Kit (Qiagen, Inc., Doncaster, Australia), with slight modification. In brief, approximately 100 mg of frozen cecal contents was weighed in a 2-mL Eppendorf tube containing 300 mg of glass beads (0.1 mm) and then 500 μL of prewarmed PW1 was pipetted to samples with prior disrupted cells by Tissuelyser II (QIAGEN, Venlo, The Netherlands) for 5 min at frequency 30/S. The samples were then incubated at 90°C for 15 min before centrifuging at 20,000 *g* for 1 min. An aliquot of 400 μL of supernatant was mixed with 150 μL of Buffer C3. The mixture was incubated for 5 min at 4°C before centrifuging at 20,000 *g* for 1 min. The supernatant was transferred into a loading block (S-block) containing 20 μL of Proteinase K and incubated for 10 min at room temperature, and the extraction was performed following the manufacture's instruction using a QIACube HT instrument (QIAGEN). The extracted cecal DNA was stored at −20°C until required.

Cecal Bacterial Enumeration

The quantitative real-time PCR of *Bifidobacterium* spp., *Lactobacillus* spp., *Bacillus* spp., *Ruminococcus* spp., *Bacteroides* spp., total anaerobic bacteria, and *Clostridium* spp. was achieved by using a qPCR assay. The extracted cecal DNA was diluted 20 times in sterile water. The Rotorgene 6000 real-time PCR machine (Corbett, Sydney, Australia) was used for qPCR assay of the desired bacteria from the extracted cecal DNA. PCR was performed in duplicate for each sample in 10 μL of reaction where PCR was repeated when the difference between the threshold cycle (CT) values of the duplicates was >0.5. For PCR reactions, an SYBR Green-containing Mix (SensiMix SYBR No-Rox; Bioline, Sydney, Australia) was applied. The reaction in a volume of 10 μL contained 5 μL of 2 × SensiMix, 300 mmol of each primer, and 2 μL of DNA template. Table 2 shows the specific 16S rRNA primers used for the quantification of different groups of bacteria. PCR was performed in a Rotorgene 6500 real-time PCR machine, and a threshold cycle average from the duplicate samples was used for data analysis. Serial dilutions of linearized plasmid DNA (pCRR 4-TOPO Vector; Life Technologies, Carlsbad, CA) inserted with respective bacterial amplicons were used to construct a standard curve. The concentrations of the plasmid DNA were measured using NanoDrop ND-8000 (Thermo Fisher Scientific, Waltham, MA) before the serial dilutions. The number of target DNA copies was calculated from the mass of DNA taking into account the size of the amplicon insert in the plasmid. Bacteria numbers were expressed as $\log_{10}$ (genomic DNA copy number)/g digesta.

Statistical Analysis of Data

The data were evaluated as a fixed-effect model using the effects described in the statistical model, as follows:

$$Y_{ijkl} = \mu + NE_i + P_j + Ca_k + (NE * P)_{ij} + (NE * Ca)_{ik} + (P * Ca)_{jk} + (NE * P * Ca)_{ijk} + e_{ijkl}$$

Table 2. Sequence of primers used for the qPCR analysis of selected microbial populations in cecal digesta samples.

Target group or organism	Primer sequence (5′-3′)	Amplicon length (bp)	Annealing T^0 (C°)	Reference
Bacillus spp.	F-GCA ACG AGC GCA ACC CTTGA R-TCA TCC CCA CCT TCC TCC GGT	92	63	Zhang et al. (2015)
Bacteroides spp.	F-GAG AGG AAG GTC CCC CAC R-CGC TAC TTG GCT GGT TCA G	106	63	Layton et al. (2006)
Bifidobacterium spp.	F-GCG TCC GCT GTG GGC R-CTT CTC CGG CAT GGT GTT G	106	63	Requena et al. (2002)
Lactobacillus spp.	F-CAC CGC TAC ACA TGG AG R-AGC AGT AGG GAA TCT TCC A	186	63	Wise and Siragusa (2007)
Ruminococcus spp.	F-GGC GGC YTR CTG GGC TTT R- CCA GGT GGA TWA CTT ATT GTG TTA A	157	63	Ramirez-Farias et al. (2008)
Total bacteria	F- CGG YCC AGA CTC CTA CGG G R- TTA CCG CGG CTG CTG GCA C	204	63	Lee et al. (1996)
C. perfringens	F- CCG CCT TCA CAT AAA GCT TGG R- TCA GGC CAT TTC ATT TTT CC Probe-5'-FAM-TCA TCA TTC AAC CAA AGG AGC AAT CC-TAMRA-3	105	58	Wise and Siragusa (2005)

where Y_{ij} is the response expected in independent variables, μ = overall mean, NE_i = fixed effect of NE (i = challenged or not challenged), P_j = the fixed effect of phytase (j = low or high), Ca_k = the fixed effect of Ca (k = low or high), $(NE*P)_{ij}$ = interaction between NE and phytase, $(NE*Ca)_{ik}$ = interaction between NE and Ca, $(P*Ca)_{jk}$ = interaction between phytase and Ca, $(NE*P*Ca)_{ijk}$ = is the 3-way interaction, and e_{ij} is the random residual error ~ $N\,(0, s2e)$.

The data were analyzed as a completely randomized design with a 2 × 2 × 2 factorial arrangement of treatments using the Minitab 19 Statistical Software (Minitab LLC, State College, PA) to assess the main effects (NE challenge [no or yes]; phytase: 500 or 1,500 FTU/kg; and Ca: low or high and 2 or 3-way interactions). Tukey's mean separation test was used to make pairwise comparisons between treatment means ($P < 0.05$). The Box-Cos transformation of the Minitab 19 statistical software was used to test and confirm the normality of all the data before analysis.

RESULTS

Broiler Performance

At day 7 (data not shown), a 2-way phytase × Ca interaction was detected for FCR ($P < 0.05$) where the lowest FCR was recorded in birds fed high Ca and high phytase. The results for growth performance are as shown in Tables 3–5. The challenge as a main effect across phytase and Ca levels reduced WG at day 14 ($P < 0.01$), day 21 ($P < 0.001$), and day 28 ($P < 0.001$) as shown in Tables 3 and 4. The challenge as a main effect increased FCR at day 14 ($P < 0.05$),

Table 3. Effect of necrotic enteritis, phytase, and calcium on the performance of broilers from 0 to 14 and 0 to 21 D.

				0–14 D				0–21 D	
Effects				Gain, g	FCR	Intake, g	Livability, %	Gain, g	Livability, %
	NE	Phy	Ca						
Main effects									
NE	−			414[a]	1.179[b]	488	99	905[a]	98
	+			396[b]	1.202[a]	476	100	687[b]	99
Phy		500		394[b]	1.197	471[b]	99	775[b]	97[b]
		1,500		416[a]	1.184	493[a]	100	817[a]	99[a]
Ca			Low	403	1.205[b]	486	100	800	99
			High	407	1.176[a]	478	100	792	98
SEM				13.60	0.02	16.65	2.08	66.90	2.08
P value									
NE				0.009	0.049	0.122	0.144	0.001	0.529
Phy				0.002	0.243	0.009	0.144	0.005	0.018
Ca				0.568	0.013	0.355	0.144	0.605	0.070
NE × Phy				0.071	0.744	0.095	0.453	0.696	0.529
NE × Ca				0.540	0.411	0.267	0.453	0.242	0.983
Phy × Ca				0.735	0.617	0.997	0.453	0.360	0.529
NE × Phy × Ca				0.770	0.064	0.388	0.980	0.396	0.983

[a,b]Means in the same column within a main effect, 2-way interaction, or treatment with different lowercase superscript letters are different ($P < 0.05$). Phytase (Quantum Blue 5). Two- or 3-way interactions separated by Tukey.

Abbreviations: Ca, calcium; FCR, feed conversion ratio; NE, necrotic enteritis; qPCR, quantitative real-time polymerase chain reaction; phy, phytase.

Table 4. Effect of necrotic enteritis, phytase, and calcium on the performance of broilers from 0 to 28 D.

Effects				Gain g	FCR	Intake, g	Livability, %
	NE	Phy	Ca				
Main effects							
NE	−			1,588[a]	1.473[b]	2,338[b]	97
	+			1,311[b]	1.599[a]	2,083[a]	97
Phy		500		1,416[b]	1.536	2,164[b]	96[b]
		1,500		1,483[a]	1.526	2,256[a]	99[a]
Ca			Low	1,457	1.556[a]	2,259[a]	98
			High	1,442	1.507[b]	2,161[b]	97
SEM				89.63	0.48	93.46	2.08
P value							
NE				0.001	0.001	0.001	1.000
Phy				0.006	0.543	0.019	0.046
Ca				0.507	0.005	0.012	0.302
NE × Phy				0.662	0.433	0.981	1.000
NE × Ca				0.684	0.358	0.680	1.000
Phy × Ca				0.333	0.481	0.625	0.302
NE × Phy × Ca				0.420	0.683	0.265	1.000

[a,b]Means in the same column within a main effect, 2-way interaction, or treatment with different lowercase superscript letters are different ($P < 0.05$). Phytase (Quantum Blue 5). Two- or 3-way interactions separated by Tukey.

Abbreviations: Ca, calcium; FCR, feed conversion ratio; NE, necrotic enteritis; phy, phytase.

day 28 ($P < 0.001$), and day 35 (data not shown) and decreased FI at day 28 ($P < 0.001$). The WG was higher in birds fed high phytase relative to those fed low phytase level on day 14 by 23 g ($P < 0.01$), day 21 by 41 g ($P < 0.01$), and day 28 by 66 g ($P < 0.01$), and day 35 (data not shown) by 100 g ($P < 0.01$). The high dietary phytase (superdosing) as a main effect increased FI at day 14 ($P < 0.01$) and day 28 ($P < 0.05$). Birds fed the high phytase had greater livability than those fed low phytase at day 21 ($P < 0.05$) and day 28 ($P < 0.05$). There was no Ca level main effect on the gain at every time point ($P > 0.05$). High Ca as a main effect improved FCR at day 14 ($P < 0.05$), day 28 ($P < 0.01$), and day 42 ($P < 0.001$) and decreased FI at day 28 ($P < 0.05$). At day 42 (Table 5), a 3-way challenge, phytase and Ca interaction was also observed for gain ($P < 0.05$); the unchallenged birds that received high phytase and high Ca diet had higher ($P < 0.05$) WG than those challenged, regardless of dietary treatment. A 3-way challenge, phytase and Ca interaction was detected for FI on day 42 ($P < 0.05$), wherein the challenge group birds fed high phytase and low Ca

Table 5. Effect of necrotic enteritis, phytase, and calcium on the performance of broilers from 0 to 42 D.

Effects				Gain, g	FCR	Intake, g	Livability, %
	NE	Phy	Ca				
Treatment means	NE	Phy	Ca				
1.	−	500	Low	3,151[a,b,c]	1.423	4,485[a]	98
2.	−	1,500	Low	3,191[a,b,c]	1.438	4,586[a]	97
3.	−	500	High	3,194[a,b]	1.277	4,084[a,b,c]	92
4.	−	1,500	High	3,288[a]	1.332	4,381[a]	97
5.	+	500	Low	2,743[d]	1.403	3,852[b,c]	97
6.	+	1,500	Low	2,985[b,c,d]	1.429	4,268[a,b]	96
7.	+	500	High	2,911[c,d]	1.299	3,782[b,c]	91
8.	+	1,500	High	2,811[d]	1.286	3,609[c]	94
Main effects							
Ca			Low	3,018	1.423[a]	4,298	97[a]
			High	3,051	1.299[b]	3,964	93[b]
SEM				155.92	0.04	214.29	3.84
P value							
NE				0.001	0.445	0.001	0.344
Phy				0.125	0.231	0.058	0.344
Ca				0.449	0.001	0.001	0.034
NE × Phy				0.963	0.417	0.641	0.781
NE × Ca				0.411	0.931	0.712	0.713
Phy × Ca				0.112	0.985	0.240	0.121
NE × Phy × Ca				0.031	0.244	0.022	0.781

[a-c]Means in the same column within a main effect, 2-way interaction, or treatment with different lowercase superscript letters are different ($P < 0.05$). Phytase (Quantum Blue 5). Two- or 3-way interactions separated by Tukey.

Abbreviations: Ca, calcium; FCR, feed conversion ratio; NE, necrotic enteritis; phy, phytase.

Table 6. Effect of necrotic enteritis, phytase, and calcium on intestinal pH, day 16 after hatch.

Effects				Crop	Duodenum	Jejunum	Ileum	Ceca
	NE	Phy	Ca					
2-way interactions								
Phy × Ca								
		500	Low	5.14	5.68[a]	5.71	5.91	6.00
		500	High	5.14	5.59[b]	5.83	6.02	6.03
		1,500	Low	5.09	5.56[b]	5.61	6.04	5.92
		1,500	High	5.25	5.80[a]	5.82	6.12	6.36
Main effects								
NE	−			4.90[b]	5.83	5.93[a]	6.04	6.08
	+			5.41[a]	5.49	5.54[b]	6.01	6.08
Ca			Low	5.11	5.62	5.66[b]	5.97	5.96[b]
			High	5.20	5.69	5.82[a]	6.07	6.20[a]
SEM				0.26	0.19	0.15	0.29	0.18
P value								
NE				0.001	0.001	0.001	0.865	0.961
Phy				0.779	0.553	0.467	0.491	0.208
Ca				0.434	0.341	0.030	0.561	0.023
NE × Phy				0.416	0.613	0.996	0.875	0.925
NE × Ca				0.066	0.938	0.121	0.976	0.303
Phy × Ca				0.450	0.030	0.553	0.937	0.051
NE × Phy × Ca				0.628	0.468	0.478	0.551	0.704

[a,b]Means in the same column within a main effect, 2-way interaction, or treatment with different lowercase superscript letters are different ($P < 0.05$). Phytase (Quantum Blue 5). Two- or 3-way interactions separated by Tukey.

Abbreviations: Ca, calcium; NE, necrotic enteritis; phy, phytase.

Reference pH values: crop, 5.5; proventriculus/gizzard, 2.5 to 3.5; duodenum, 5 to 6; jejunum, 6.5 to 7.0; ileum, 7.0 to 7.5; ceca 7.0 to 8.0. Morgan et al., 2014; Pang and Applegate, 2007; Ravindran, 2013.

recorded the highest FI. However, in the unchallenged group, those fed low phytase and high Ca recorded the least FI.

Gut pH

As shown in Table 6, on day 16, a phytase × Ca interaction was detected for duodenal pH ($P < 0.05$). Duodenal pH was higher in birds fed low Ca and 500 FTU/kg phytase and high Ca and 1,500 FTU/kg phytase than in those on the other dietary treatments. In birds fed the higher phytase dose, caeca pH was higher in birds fed the higher level of Ca. The challenged birds had lower jejunal pH ($P < 0.001$) but higher crop pH than the unchallenged birds. Birds fed high-Ca diets had higher jejunal ($P < 0.05$) and cecal ($P < 0.05$) pH. On day 29 (Table 7), challenged birds had lower crop and caeca pH ($P < 0.001$) but higher ileal pH

Table 7. Effect of necrotic enteritis, phytase, and calcium on intestinal pH, day 29 after hatch.

Effects				Crop	Gizzard	Duodenum	Jejunum	Ileum	Ceca
	NE	Phy	Ca						
Main effects									
NE	−			5.18[a]	2.71	5.71	5.83	5.97[b]	5.93[a]
	+			4.74[b]	2.52	5.83	5.87	6.64[a]	5.69[b]
Phy		500		4.83[b]	2.64	5.74	5.80[b]	6.18	5.80
		1,500		5.08[a]	2.59	5.80	5.90[a]	6.43	5.81
Ca			Low	5.01	2.35[b]	5.79	5.85	6.08[b]	5.65[b]
			High	4.91	2.88[a]	5.75	5.85	6.53[a]	5.97[a]
SEM				0.24	0.23	0.37	0.10	0.14	0.17
P value									
NE				0.001	0.115	0.113	0.386	0.001	0.004
Phy				0.040	0.691	0.412	0.043	0.110	0.867
Ca				0.355	0.001	0.526	0.921	0.006	0.001
NE × Phy				0.752	0.432	0.988	0.231	0.628	0.649
NE × Ca				0.681	0.232	0.663	0.818	0.094	0.406
Phy × Ca				0.402	0.148	0.224	0.605	0.480	0.586
NE × Phy × Ca				0.542	0.361	0.890	0.699	0.829	0.561

[a,b]Means in the same column within a main effect, 2-way interaction, or treatment with different superscript lowercase letters are different ($P < 0.05$). Phytase (Quantum Blue 5). Two- or 3-way interactions separated by Tukey.

Abbreviations: Ca, calcium; NE, necrotic enteritis; phy, phytase.

Reference pH values: crop, 5.5; proventriculus/gizzard, 2.5 to 3.5; duodenum, 5 to 6; jejunum, 6.5 to 7.0; ileum, 7.0 to 7.5; ceca 7.0 to 8.0. Morgan et al., 2014; Pang and Applegate, 2007; Ravindran, 2013.

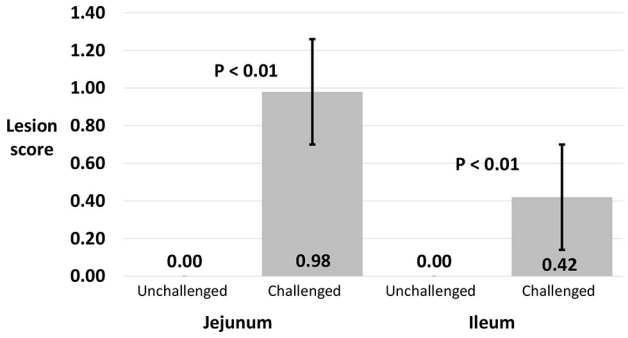

Figure 1. Effect of necrotic enteritis challenge on lesion scores in jejunum and ileum on day 16.

than unchallenged birds. Birds fed high phytase dose had higher crop ($P < 0.05$) and jejunal ($P < 0.05$) pH. A higher pH in the gizzard ($P < 0.001$), ileum ($P < 0.01$), and ceca ($P < 0.001$) was observed in birds fed high Ca on day 29.

Lesion Score, Day 16 After Hatch

Challenged birds presented higher ($P > 0.05$) lesion scores in the jejunum and ileum. No interactions or effects of dietary phytase or Ca were observed on intestinal lesions (Figure 1). No treatment effect was observed for lesions in the duodenum.

Digestibility, Day 16 After Hatch

A challenge × Ca interaction was observed for AID of CP ($P < 0.05$), C ($P < 0.01$), K ($P < 0.05$), and Zn ($P < 0.05$), where the AIDs were higher in the unchallenged birds than in the challenged birds (Table 8). In the challenged birds, the CP, C, K, and Zn AID were lower in birds fed high Ca. The challenge as a main effect decreased the AID of Ca ($P < 0.01$) and P ($P < 0.001$), but it increased the AID of Mg ($P < 0.05$). High dietary Ca as a main effect decreased the AID of P ($P < 0.001$) and Mg ($P < 0.05$).

Bacterial Enumeration, Day 16 After Hatch

The challenge decreased the counts of *Ruminococcus* spp. and total bacteria in the ceca ($P < 0.05$) as shown in Table 9. High dietary Ca decreased ($P < 0.05$) *Lactobacillus* spp. and *Bifidobacteria* spp. ($P < 0.05$) counts.

DISCUSSION

Performance

This study investigated interactions between dietary Ca level, phytase level, and infection with NE. Overall, to 42 D, the results were equivocal with a three-way interaction observed for WG and FI. The highest WG and FI occurred in nonchallenged birds fed the high-Ca and high-phytase diet. The lowest BWG and FI were observed in challenged birds fed the low-Ca and low-phytase diet. This suggests that Ca level in itself has no direct or lasting effect on performance of birds challenged with NE. However, unchallenged birds fed high Ca benefitted from a high level of phytase. A previous report by Sebastian et al. (1996) in healthy birds showed increasing Ca levels to reduce the benefits of phytase inclusion on body weight from 0 to 14 D. In

Table 8. Effect of necrotic enteritis, phytase, and calcium on apparent nutrient digestibility of nutrients, day 16 after hatch.

Effects				CP, %	C, %	Ca, %	P, %	Mg, %	K, %	Zn, mg/kg
	NE	Phy	Ca							
Two-way interactions										
NE*Ca										
	−		Low	0.77^{a,b}	0.55^{a}	0.72	0.79	0.24	0.87^{a}	0.06^{a}
	−		High	0.79^{a}	0.64^{a}	0.79	0.64	0.22	0.85^{a,b}	0.03^{a}
	+		Low	0.62^{b}	0.33^{b}	0.63	0.69	0.40	0.77^{b}	0.14^{a}
	+		High	0.41^{c}	0.18^{c}	0.61	0.44	0.23	0.63^{c}	−0.27^{b}
Main effects										
NE	−			0.78	0.59	0.75^{a}	0.72^{a}	0.23^{b}	0.86	0.04
	+			0.52	0.22	0.62^{b}	0.56^{b}	0.31^{a}	0.70	−0.07
Ca			Low	0.69	0.43	0.67	0.74^{a}	0.32^{a}	0.82	0.10
			High	0.60	0.38	0.70	0.54^{b}	0.22^{b}	0.74	−0.12
SEM				0.17	0.21	0.09	0.10	0.07	0.08	0.13
P value										
NE				0.001	0.001	0.003	0.001	0.034	0.001	0.133
Phy				0.310	0.518	0.525	0.369	0.364	0.681	0.370
Ca				0.029	0.301	0.524	0.001	0.018	0.003	0.005
NE × Phy				0.404	0.563	0.857	0.423	0.231	0.664	0.584
NE × Ca				0.011	0.006	0.310	0.170	0.053	0.014	0.011
Phy × Ca				0.680	0.433	0.303	0.861	0.782	0.319	0.236
NE × Phy × Ca				0.408	0.998	0.991	0.828	0.260	0.845	0.448

[a-c]Means in the same column within a main effect, 2-way interaction, or treatment with different superscript lowercase letters are different ($P < 0.05$). Phytase (Quantum Blue 5). Two- or 3-way interactions separated by Tukey.

Abbreviations: C, carbon; Ca, calcium; CP, crude protein; K, potassium; Mg, magnesium; NE, necrotic enteritis; P, phosphorus; phy, phytase; Zn, zinc.

Table 9. Effect of necrotic enteritis, phytase, and calcium on bacterial quantification as $\log_{10}$ genomic DNA copies per gram of cecal contents from broilers, day 16 after hatch.

Effects				*Lactobacillus* spp.	*Ruminococcus* spp.	*Bacteroides* spp.	*Bacillus* spp.	*Bifidobacteria* spp.	Total bacteria	*Clostridium perfringens*
	NE	Phy	Ca							
Main effects										
NE	−			9.81	8.87[a]	8.63	8.18	10.38	10.80[a]	0.00[b]
	+			9.68	8.68[b]	8.38	8.31	10.30	10.66[b]	8.99[a]
Ca			Low	9.85[a]	8.78	8.23	8.29	10.45[a]	10.81[a]	4.45
			High	9.64[b]	8.77	8.78	8.20	10.23[b]	10.65[b]	4.54
SEM				0.21	0.26	0.65	0.21	0.14	0.13	2.15
P value										
NE				0.143	0.031	0.361	0.126	0.441	0.022	0.001
Phy				0.977	0.743	0.349	0.812	0.406	0.901	0.127
Ca				0.026	0.924	0.051	0.283	0.028	0.011	0.437
NE × Phy				0.876	0.656	0.223	0.700	0.942	0.592	0.127
NE × Ca				0.696	0.286	0.990	0.920	0.496	0.387	0.437
Phy × Ca				0.569	0.374	0.974	0.482	0.781	0.551	0.306
NE × Phy × Ca				0.738	0.072	0.179	0.678	0.412	0.481	0.306

[a,b]Means in the same column within a main effect, 2-way interaction, or treatment with different superscript lowercase letters are different ($P < 0.05$). Phytase (Quantum Blue 5). Two- or 3-way interactions separated by Tukey.

Abbreviations: Ca, calcium; NE, necrotic enteritis; phy, phytase.

that study, the optimum FI, body weight, and FCR were observed in birds fed 0.6% dietary Ca plus phytase (600 FTU/kg) compared to their counterparts fed 1.0% Ca with phytase (500 FTU/kg). However, the phytase by Ca interaction was no longer apparent by day 21. Sommerfeld et al. (2018) reported that birds fed a wide Ca-P with 1,500 FTU/kg phytase had increased FCR relative to those on the narrower ratio at the same level of phytase, thus supporting the findings of the present study. Faridi et al. (2015) suggested that the suppressive growth effect of high dietary Ca could be reduced by supplementing diets with phytase, also agreeing with the results of the present study. High phytase with high Ca appeared to only be detrimental to growth under challenged conditions concurring with the hypothesis of this study.

The low WG and reduced phytase effects observed in the challenged birds fed diets with a wide Ca-P ratio may be due to formation of insoluble Ca-phytate complexes that are less accessible to phytase. As phytase loses its ability to hydrolyze phytate, the phytate may react with dietary and endogenous proteins such as pepsin and pepsinogen and minerals, thereby reducing the activity of these enzymes (Woyengo et al., 2010; Yu et al., 2012). Not only are the activities of these gastric secretions reduced, but their losses may also increase. The interaction of phytate with proteolytic enzymes in the upper gastrointestinal (**GI**) tract may increase secretion of HCl. This might lead to endogenous losses with higher secretion of pancreatic bicarbonate to neutralize the acid digesta. A reduction in phytase activity might reduce amino acid digestibility with the formation of phytate-gastric proteolytic enzyme complexes (Rutherfurd et al., 2004; Walk and Olukosi, 2019). This would reduce amino acid absorption and increase endogenous losses at the same time. Also, an increase in gastric secretions may lead to an increased mucin production further increasing endogenous losses. The digestive inefficiencies resulting from the aforementioned interactions would be expected to result in the accumulation of undigested nutrients in the lower gut and providing nutrients for overgrowth of *C. perfringens* in challenged birds. Mucin has been shown to serve as a nutrient source for opportunistic *C. perfringens* (Collier et al., 2003). The low FI in challenged birds, fed high Ca and high phytase, has been reported previously (Brennan et al., 2001; Cravens et al., 2013; M'Sadeq et al., 2015). An additional report showed FI to increase by 29% in birds fed diets with low Ca concentration (Wilkinson et al., 2014). This concurs with the day-28 results of the present study.

Birds fed the high dose of phytase (1,500 vs. 500 FTU/kg) had higher WG (day 0–14; day 0–21; day 0–28) in the present study in agreement with numerous reports that superdosing phytase improves growth (Pillai et al., 2006; Amerah et al., 2014; Beeson et al., 2017; Scholey et al., 2018; Walk et al., 2018). The benefits derived from phytase supplementation at levels above recommended doses are due to increased digestibility of amino acids, energy, Ca, and trace minerals, from inactivation of phytate antinutritional properties (Selle et al., 2000; Camden et al., 2001; Shirley and Edwards, 2003; Bournazel et al., 2018).

pH

Maintaining optimum pH across the gut is important to maximize nutrient bioavailability and maintain homeostastis of intestinal microbiota. Changes in gut pH may be caused by diets or disease. Ca has acid-binding capacity, and excessive limestone levels may raise gut pH (Paiva et al., 2014; Walk, 2016). The increase in crop pH on day 16 observed in challenged birds in the present study might be due to low FI. Hinton et al. (2000) reported feed withdrawal to increase pH of crop contents in broilers due to a reduced population of lactic acid bacteria. Fonseca et al. (2010) observed high counts of *Lactobacillus* in the crop to be associated with a

reduction in crop pH and reduction of *Salmonella*. In the present study, high dietary Ca increased gizzard pH on day 28 likely due to the buffering action of limestone. This observation is consistent with the report by Guinotte et al. (1995). Increased gizzard, ileal, and cecal pH recorded in the present study are an indication that high dietary Ca (as limestone) serves as antiacid in these regions of the GI tract (Walk et al., 2012). A higher pH in the gizzard may favor binding between positively charged Ca and negatively charged phytate. At a lower pH levels, IP6 becomes less negatively charged and loses its ability to chelate positively charged ions. An increasing pH in the crop reduces the ability of phytase to hydrolyze phytate. This results in formation of Ca-phytate precipitates. High cecal pH will favor growth of *Clostridia* (Williams, 2005). *C. perfringens* are acid sensitive, and acidic conditions are known to suppress their growth (Skřivanová et al., 2005; Namkung et al., 2011; Roy et al., 2018).

Lesions

Lesion scores were observed in birds challenged with NE in this experiment, indicating it was a successful challenge. This observation is a common occurrence in many NE studies (Jia et al., 2009; Cravens et al., 2013; Amerah and Ravindran, 2015). Toxin secretions of *C. perfringens* compromise intestinal ion transporters and the tight junction barrier which may lead to the loss of intestinal epithelial homeostasis and cause destruction in the epithelial cells (Field, 2003). In the case of impaired intestinal ion transport, there is hypersecretion and malabsorption through direct or indirect interaction of toxins with individual intestinal ion transporters by decreasing their activity and presence in the apical membrane of the epithelial cells (Awad et al., 2014). Again, with the loss of intestinal barrier function, antigens residing in the lumen gain access to the immune cells in the lamina propria and aggravate inflammation (Berkes et al., 2003).

Digestibility

Destruction of the intestinal mucosa due to NE and high dietary Ca can pose a challenge to the digestibility of nutrients. In the present study, the challenge birds fed high dietary Ca had lower CP, C, K, and Zn digestibility. The reduction in the digestibility of these nutrients might have resulted from Ca increasing the outcome of the NE infection by providing a favorable condition for the activity of *C perfringens*. A change in the architecture of the epithelium of the intestine due to NE compromises nutrient digestion and absorption (Persia et al., 2006; Rochell et al., 2016). The disruption of the integrity of the intestinal mucosa due to the challenge might have also led to a reduction in the AID of P and CP. Shafey and McDonald (1991) have reported the effect of high Ca concentration in reducing the digestibility of amino acid in broiler chickens. The low P and Mg digestibility can be explained by the fact that a high concentration of Ca relative to available P or Mg increased the chances of these minerals being precipitated. A decrease in P digestibility with either high Ca concentration or increasing Ca-P ratio is documented (Tamim et al., 2004; Olukosi and Fru-Nji, 2014).

Bacteria Counts

The challenge in this study generally decreased the population of beneficial bacteria, with butyrate-producing *Ruminococcus* and total anaerobic bacteria being significantly affected. Even though Ca as a main effect did not make any difference in the count of *C. perfringens*, high Ca significantly increased the counts of *Bacteroides*, which is another potential pathogenic bacterium (Wexler, 2007). Bacteroides are normal intestinal flora but can evolve into a pathogenic form, and the amounts could increase when the gut is pathologically changed or impaired (Phong et al., 2010). *Lactobacillus*, *Bifidobacteria*, and total bacteria were decreased by high-Ca diets. These bacteria are short-chain fatty acid–producing bacteria which also secret bacteriocins at an acidic pH to suppress the growth of pathogens in the GI tract. The ability of some species of *Lactobacilli* to inhibit the growth of pathogens is known (Dec et al., 2014). It stands to reason that a low count of these bacteria might be due to high Ca and pH, thereby favoring acid-sensitive pathogenic bacteria. A report suggests that under the favorable condition in the ceca, both anaerobic bacteria and *Lactobacillus* tended to outnumber the population of *C. perfringens* (Olnood et al., 2015).

CONCLUSION

This study addressed the hypothesis that high Ca would decrease the performance of birds during challenge with NE and increasing phytase from 500 FTU/kg to 1,500 FTU/kg would improve the situation. The results showed that high dietary Ca decreased FCR both early in the study (immediately after challenge) and overall from 0 to 42 D. Immediately after challenge on day 16, high Ca levels increased crop pH, yet no Ca × NE challenge interactions were observed for performance. Overall to 42 D, the highest WG was observed in unchallenged birds fed high Ca and high phytase. The lowest WG was observed in NE-challenged birds fed low Ca and low phytase. High phytase improved WG and FI early in the study, but by 42 D, FI was diminished in the high-phytase group challenged with NE and fed high Ca. All feeds formulated in the present study used the manufacturers recommended matrix values for 500 FTU/kg phytase. The results suggest that using full matrix values for high doses of phytase (>500 FTU/kg) may be appropriate during NE challenge.

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