The efficacy of 2 phytases on inositol phosphate degradation in different segments of the gastrointestinal tract, calcium and phosphorus digestibility, and bone quality of broilers

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ABSTRACT The anti-nutritional effects of dietary inositol phosphates (IP_6 through IP_3) have been recognized in broiler chickens; however, inositol hexaphosphate (IP_6) is more potent than the lower IP esters. The efficacies of 2 commercial phytases, a *Buttiauxella* sp. phytase (BSP) and a *Citrobacter braakii* phytase (CBP) at 500 and 1,000 FTU/kg, were studied on $IP_{6–3}$ concentrations in the crop, proventriculus + gizzard, and distal ileum digesta, and ileal IP_6 disappearance in broilers at day 22. Apparent ileal P and Ca digestibility, and bone quality at days 22 and 33 were also measured. Female Ross 308 broilers (n = 1,890; 30 birds × 7 diets × 9 replicates) were fed corn-soy-based crumbled diets. The 7 diets included a primary breeder recommendation-based positive control diet (PC); the PC marginally reduced in available P by 0.146% and Ca by 0.134% of the diet, (NC1) or moderately reduced by 0.174 and 0.159% of the diet, respectively (NC2). Other diets were the NC1 + BSP or CBP at 500 FTU/kg (NC1+500BSP and NC1+500CBP) and the NC2 + BSP or CBP at 1,000 FTU/kg (NC2+1,000BSP and NC2+1,000CBP). Each of the NC1 and NC2 had distal ileum IP_6 disappearance similar to that of PC, but each had lower P digestibility and the majority of measured bone quality parameters than the PC. The ileal IP_6 levels were decreased by 52.0 and 32.7% for NC1+500BSP and NC1+500CBP, respectively, relative to NC1 and by 73.6 and 50.9% for NC2+1,000BSP and NC2+1,000CBP, respectively, relative to NC2 ($P < 0.001$), with a similar effect for distal ileum IP_6 disappearance. Overall, phytase in the NC diets increased P digestibility, and femur breaking strength and cortical bone mineral density at days 22 and 33. Overall, each of the phytases at each dose degraded $IP_{6–3}$ across the gastrointestinal tract segments to increase P digestibility and the P and Ca utilization in bone. However, dietary BSP at 1,000 FTU/kg was most effective. Supplemental phytase degrades phytate to decrease the anti-nutritional effects in a dose- and phytase-dependent manner.

Key words: broiler chicken, gastrointestinal tract, phytate, phytase, bone

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INTRODUCTION

The ability of commercial phytases to increase nutrient digestibility, performance, and bone ash content of broilers has been well studied (Simons et al., 1990; Powell et al., 2011). However, the effects of supplemental phytase in available P- (**avP**) and Ca-deficient diets depend on the ability of the enzyme to degrade phytate (inositol hexaphosphate; **IP_6**) in the gastrointestinal tract (**GIT**). The efficacy of phytase to degrade phytate has been validated in in vitro (Menezes-Blackburn et al., 2015; Sommerfeld et al., 2017) and in vivo (Walk et al., 2014; Li et al., 2016; Beeson et al., 2017; Sommerfeld et al., 2018). Phytase degrades IP_6 into inositol penta-, tetra-, tri-, di-, and monophosphate (IP_5 through IP_1), releasing one phosphate molecule at a time in a stepwise manner (Greiner and Konietzny, 2011); however $IP_{6–3}$ are of greater importance because of their more pronounced anti-nutritional effects (Walk et al., 2014; Beeson et al., 2017). The presence of $IP_{6–3}$ in the GIT decreases nutrient absorption in monogastric animals (Persson et al., 1998). Commercially available phytases are from various bacterial or fungal sources. In vitro assessment of 7 commercial bacterial or fungal phytases showed large variations in biochemical and catalytic properties (Menezes-Blackburn et al., 2015).

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Substrate level, pH, temperature, and moisture content vary in each GIT segment of the chicken. Hence, the activity of a phytase in the different conditions of each GIT segment may contribute to its overall phytate-degrading efficacy. Increasing phytase dose increased in vivo IP_6 degradation in the gizzard and ileum (Li et al., 2016; Beeson et al., 2017). Only limited information is available on phytase source and dose effects on in vivo IP degradation in each GIT segment of broilers.

Phytase supplementation in avP- and Ca-reduced diets increased tibia breaking strength, weight, and ash content in broilers (Powell et al., 2011). Broiler bones contain both cortical and trabecular bone tissues. Cortical bone is compact and dense and trabecular is spongy and cancellous, and both bone types are important for load bearing and fracture resistance (Reich and Gefen, 2006). Cortical and trabecular bone densitometry (bone mineral density, cross-sectional area, and mineral content) can be individually analyzed using quantitative computed tomography (**QCT**; Korver et al., 2004). The efficacy of phytase supplementation in avP- and Ca-reduced diets to prevent bone loss to maintain bone ash, breaking strength, and mineral density across trabecular and cortical bone tissues is clear (Chung et al., 2013). However, there is no information on how source and dose of dietary phytase affect IP degradation in each segment of the GIT to affect cortical and trabecular bone densitometry in broilers. It was hypothesized that 2 phytase sources would differ in their efficacy, and a higher phytase dose would further degrade $IP_{6–3}$ than a lower dose to increase cortical and trabecular bone density in broilers. Therefore, the objective of this study was to assess the effects of 2 phytases at 500 or 1,000 FTU/kg on $IP_{6–3}$ degradation in different GIT segments; P and Ca digestibility; and bone strength, ash, and density in broilers.

MATERIALS AND METHODS

The protocol for this trial was approved by the Animal Care and Use Committee: Livestock of the University of Alberta and followed the Canadian Council on Animal Care guidelines (CCAC, 2009). Female Ross 308 broilers (n = 1,890) were obtained from a commercial hatchery at day 0 and housed with 30 chicks in each of 63 wire-floored pullet cages (59 × 53 × 44 cm for width, depth, and height, respectively; Specht Canada Inc., Stony Plain, AB, Canada) in an environmentally controlled facility. A total of 7 dietary treatments were each assigned to 9 replicate cages and were fed to the birds as crumbled starter (day 0 to 8), grower (day 9 to 22), and finisher (day 23 to 33) diets. After the diets were mixed and pelleted, the feed was sampled and analyzed for phytase activity (see details below). The phytase recovery was lower than expected, and so the diets were re-mixed with additional phytase and the diets re-tested. Birds were provided feed and water ad libitum throughout the trial. Standard broiler rearing management specified by the primary breeder (Aviagen, 2014) was maintained throughout the trial.

Treatments

The dietary treatments were a nutritionally adequate positive control diet (**PC**); the PC diet with avP and Ca marginally reduced by 0.146 and 0.134% of the diet, respectively (**NC1**), or moderately reduced by 0.174 and 0.159% of the diet, respectively (**NC2**). Other diets were the NC1 and NC2 diets supplemented with a *Buttiauxella* sp. phytase (**BSP**) (Axtra PHY, DuPont Animal Nutrition, Leiden, Netherlands) and a *Citrobacter braakii* phytase (**CBP**) at 500 FTU/kg to the NC1 diets (**NC1+500BSP** and **NC1+500CBP**, respectively) and at 1,000 FTU/kg to the NC2 diets **NC2+1,000BSP** and **NC2+1,000CBP**. Celite (Celite Corp., Lompar, CA) was included in all diets at 2% as an indigestible marker. In addition to avP and Ca reductions in the NC1 and NC2 diets relative to the PC diets, Na, AME, and digestible amino acids levels were also formulated accordingly in the negative control diets (Dersjant-Li et al., 2018). The Na level in the PC diet was reduced in the NC1 diet by 0.030% of the diet and in the NC2 diet by 0.041% of the diet across the 3 phases. The ME level in the NC1 diet was reduced by 2.50, 2.32, and 2.19% and in the NC2 diet by 2.73, 2.55, and 2.38% relative to the PC diet in the starter, grower, and finisher phases, respectively. In addition, the minimum specification for the 8 most limiting digestible amino acids (Met, Lys, Met plus Cys, Thr, Val, Ile, Arg, and Trp) in the PC diets were reduced, on average for these AA, by 0.116, 0.083, and 0.070% of the diet for the NC1 diets and by 0.223, 0.160, and 0.138% of the diet for the NC2 in the starter, grower, and finisher phases, respectively. The levels of digestible amino acids in each of PC were formulated to meet or exceed the primary breeder recommendation (Aviagen, 2014; Table 1). The reductions in Ca, avP, Na, ME, and amino acids in the NC1 and NC2 diets compared to the PC diet were based on the expected nutrient release by BSP at 500 or 1,000 FTU/kg in the respective diets. Dietary ingredient and calculated nutrient composition are shown in Table 1. Dietary Ca and total P levels in the diets were determined in triplicate using modified (Bello, 2018) AOAC methods 964.06 and 935.13, respectively (AOAC, 1990). Details of the P and Ca analyses were as described in Bello (2018). The 7 dietary treatments were randomly and evenly distributed across the 63 cages. Phytase activity in each of the diets at each phase was analyzed using Method 30024 of ISO (2009). One FTU is defined as the amount of enzyme that releases 1 μmol of inorganic orthophosphate from sodium phytate substrate at pH 5.5 and 37°C per minute. All members of the research team were blinded as to the identity of each phytase until analyses were complete.

Table 1. Ingredient and calculated nutrient composition of basal diets fed during starter, grower, and finisher phases.[1,2,3]

	Starter phase (day 0–8)			Grower phase (day 8–22)			Finisher phase (day 22–33)		
	PC	NC1	NC2	PC	NC1	NC2	PC	NC1	NC2
Ingredient (%)									
Yellow corn	50.0	52.7	52.9	54.5	56.9	56.9	58.4	60.7	60.6
Soybean meal	32.8	31.9	31.2	28.0	27.2	26.6	23.5	22.7	22.3
Canola meal	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50
Canola oil	3.41	1.50	1.19	4.19	2.36	2.07	5.05	3.26	3.00
Calcium carbonate	1.13	1.20	1.22	1.02	1.09	1.11	0.93	1.00	1.01
Dicalcium phosphate	1.72	0.92	0.77	1.52	0.73	0.58	1.35	0.56	0.41
Salt	0.32	0.25	0.22	0.33	0.25	0.22	0.33	0.25	0.23
L-lysine	0.16	0.16	0.15	0.16	0.16	0.16	0.17	0.17	0.16
DL-methionine	0.32	0.31	0.29	0.29	0.28	0.27	0.27	0.25	0.24
L-threonine	0.02	0.01	–	–	–	–	–	–	–
Phytase carrier: −/+[4,5]	–	1.00	2.00	–	1.00	2.00	–	1.00	2.00
Calculated nutrient values (as fed)									
AME, MJ/kg	12.6	12.3	12.2	13.0	12.9	12.7	13.4	13.1	13.1
Crude protein, %	23.6	23.4	23.2	21.5	21.4	21.2	19.6	19.4	19.3
Calcium, %	0.960	0.826	0.801	0.870	0.736	0.711	0.790	0.656	0.631
Total phosphorus, %	0.806	0.661	0.631	0.743	0.598	0.569	0.686	0.541	0.512
Available phosphorus, %	0.480	0.334	0.306	0.435	0.289	0.261	0.395	0.249	0.221
Phytate phosphorus, %	0.295	0.298	0.297	0.283	0.286	0.285	0.271	0.274	0.274
Dig. Lys, %[6]	1.28	1.26	1.24	1.15	1.13	1.12	1.03	1.01	1.00
Dig. Met, %	0.66	0.65	0.63	0.61	0.60	0.58	0.56	0.55	0.54
Dig. Met and Cys, %	0.95	0.93	0.91	0.87	0.85	0.84	0.80	0.79	0.77
Dig. Thr, %	0.86	0.84	0.83	0.77	0.76	0.76	0.70	0.70	0.69
Dig. Val, %	1.00	0.99	0.98	0.91	0.90	0.90	0.83	0.82	0.81
Dig. Ile, %	1.05	1.05	1.04	0.96	0.96	0.95	0.88	0.87	0.87
Dig. Arg, %	1.37	1.35	1.34	1.23	1.22	1.20	1.10	1.09	1.08
Dig. Trp, %	0.26	0.26	0.26	0.23	0.23	0.23	0.21	0.21	0.20

[1]Vitamin-mineral premix used at 0.50% in each diet to provide 1.65 mg I, 0.3 mg Se, 10,000 IU vitamin A, 4,000 IU vitamin D_3, 50 IU vitamin E, 4 mg vitamin K, 4 mg thiamin, 10 mg riboflavin, 65 mg niacin, 15 mg pantothenic acid, 5 mg pyridoxine, 2 mg folic acid, 0.02 mg cobalamins, 0.2 mg biotin, and 2.64 mg choline per kg feed.

[2]Monensin, Coban 90 (Elanco Animal Health, Indianapolis, IN) used in each diet at 0.05%.

[3]Celite (Celite Corp., Lompoc, CA) used per diet at 2%.

[4]BSP = *Buttiauxella* sp. phytase and CBP = *Citrobacter braakii* phytase.

[5]Finely ground yellow corn added without phytase in the negative controls 1 (NC1) and 2 (NC2) diets; with phytase A (BSP) in the NC1 + BSP at 500 FTU/kg (NC1+500BSP) and NC2 + BSP at 1,000 FTU/kg (NC2+1,000BSP) diets; and with phytase B (CBP) in the NC1 + CBP at 500 FTU/kg (NC1+500CBP) and NC2 + CBP at 1,000 FTU/kg (NC2+1,000CBP) diets.

[6]Digestible amino acid.

Bone and Digesta Sample Collection

At 8, 22, and 33 D of age, 14, 11, and 5 birds, respectively, from each cage were euthanized by cervical dislocation. The right femurs of 4 birds from each cage were excised and stored at −20°C. The digesta from the distal half of the ileum were collected and pooled within the cage (n = 63). At day 22 only, crop and proventriculus + gizzard digesta were collected, individually pooled within the cage (n = 189), and immediately frozen at −20°C for approximately 24 h. All samples were freeze-dried and ground (<1 mm) using a centrifugal mill (model ZM200; Retsch GmbH, Haan, Germany).

Bone Quality Analysis

At each of 8, 22, and 33 D, 2 femurs per cage (n = 378: 2 birds × 63 cages × 3 ages) were thawed and analyzed for bone breaking strength (**BBS**) using an Instron Universal Testing Instrument (Table Model 4411, Instron Corp., Canton, MA) with a 200 N load cell (Riczu et al., 2004). All fragments of the broken bones were oven-dried at 105°C for 7 D and ashed at 500°C for 48 h using a muffle furnace (Table Model 30,400; Barnstead Thermolyne Corp., Dubuque, IA) for determination of bone ash content (AOAC, 1990). At each of 22 and 33 D, 2 femurs per cage (n = 252: 2 birds × 63 cages × 2 ages) were also thawed and analyzed for bone densitometry at the mid-diaphysis (50% mid-point of bone length) and proximal metaphysis (30% of bone length from the proximal end) using QCT (Saunders-Blades et al., 2009). Bone cross-sectional areas (**BCSA** in mm^2) and mineral density (**BMD** in mg/mm^3) of total, cortical, and trabecular bone tissues were measured. Bone mineral content (**BMC** in mg/mm bone length) in each of total, cortical, and trabecular bone tissues was calculated by multiplying the respective BCSA by BMD to yield the amount of mineral (mg) contained in a volume of each of the bone tissues (Bello, 2018).

Digestibility Assays

Samples of starter, grower, and finisher diets, and distal ileum digesta collected at the end of each dietary phase were analyzed for ileal P and Ca levels

Table 2. Analyzed dietary calcium, total phosphorus, and phytase activities.[1]

	PC[3]	NC1[3]	NC1+ 500BSP[2,3]	NC1+ 500CBP[2,3]	NC2[3]	NC2+ 1,000BSP[2,3]	NC2+ 1,000CBP[2,3]
Calcium %							
Starter	0.750	0.823	0.950	0.895	0.794	0.983	0.798
Grower	0.977	0.840	0.834	0.753	0.825	0.801	0.766
Finisher	0.642	0.783	0.738	0.718	0.680	0.692	0.660
Total phosphorus %							
Starter	0.738	0.728	0.720	0.697	0.630	0.699	0.578
Grower	0.844	0.629	0.601	0.572	0.630	0.559	0.526
Finisher	0.637	0.606	0.557	0.548	0.492	0.513	0.493
Phytase activity FTU/kg							
Starter	<100	<100	858	1,221	<100	1,237	1,763
Grower	<100	<100	619	519	<100	1,078	1,140
Finisher	267	<100	606	771	<100	915	1,327

[1]Starter phase was from day 0 to 8, grower phase was from day 8 to 22, and finisher phase was from day 22 to 33.
[2]BSP = *Buttiauxella* sp. phytase and CBP = *Citrobacter braakii* phytase.
[3]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet respectively, (NC1) or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (BSP) or phytase B (CBP) at 500 FTU/kg (NC1+500BSP and NC1+500CBP); and the NC2 + BSP or CBP at 1,000 FTU/kg (NC2+1,000BSP and NC2+1,000CBP).

(Bello, 2018). The diets and the distal ileum samples were analyzed for acid insoluble ash (**AIA**, Scott and Boldaji, 1997). The apparent ileal digestibility of P (**AIDP**) and Ca (**AIDCa**) were determined based on the analyzed P and Ca levels and analyzed AIA in diet and digesta samples.

Inositol Phosphate Degradation Assays

The 7 grower diets and 189 digesta samples collected at 22 D of age (63 cages × 3 GIT segments) were analyzed for IP concentrations using high-performance liquid chromatography anion exchange with a post-column reagent for detection (Newkirk and Classen, 1998). The analyzed IP_6 concentration in the diet and distal ileum samples and the analyzed diet and distal ileum AIA were used to determine ileal IP_6 disappearance.

Calculations and Statistical Analyses

The calculation used for AIDP and AIDCa determination were done using the formula:

$$\begin{aligned}\text{AIDP or AIDCa} = 100 \\ -[100 \times (\%\,\text{AIA in diet}/\%\,\text{AIA in digesta}) \\ \times (\%\,\text{mineral in digesta}/\%\,\text{mineral in diet})].\end{aligned}$$

Similarly, IP_6 disappearance was calculated using the formula:

$$\begin{aligned}100 - [100 \times (\%\,\text{AIA in diet}/\%\,\text{AIA in ileal digesta}) \\ \times (\%\,\text{ileal IP}_6/\%\,\text{diet IP}_6)].\end{aligned}$$

The cage was the experimental unit, with 9 cages assigned to each of the 7 dietary treatments. A completely randomized design was used in the placement of chicks in cages and in the assignment of cages to diets. Crop, proventriculus + gizzard, and ileal IP_{6-3} concentrations and ileal IP_6 disappearance at day 22, and BBS, bone ash content, and total, cortical, and trabecular BCSA, BMD, and BMC at days 22 and 33 were each analyzed for diet effect using Proc. Mixed of SAS 9.3 (SAS Institute, 2013). AIDP and AIDCa were each analyzed for diet × age interaction also with Proc Mixed of SAS 9.3 (SAS Institute, 2013). For the BBS and bone ash content, and total, cortical, and trabecular BCSA, BMD, and BMC analyses, BW was used as a covariate. Differences between diet means were separated using the least significant difference test when $P \leq 0.05$.

RESULTS

The analyzed Ca and total P levels, and phytase activities in each diet at each phase are shown in Table 2. The analyzed Ca and P levels in the 7 starter, grower, and finisher diets ranged from 78 to 123% and from 92 to 114%, respectively, relative to the formulated dietary mineral levels. The analyzed phytase activities in the 500 and 1,000 FTU/kg BSP-supplemented diets were 172 and 124%, 124 and 108%, and 121 and 92% during the starter, grower, and finisher dietary phases, respectively, compared to targeted dose. The analyzed phytase activities in the 500 and 1,000 FTU/kg CBP-supplemented diets were each 244 and 176%, 104 and 114%, and 154 and 133% during the starter, grower, and finisher dietary phases, respectively. The concentrations of IP_{6-3} in each diet are shown in Table 3.

Bone Quality Traits

At 22 D of age, the NC1 and NC2 diets decreased mid-diaphysis and proximal-metaphysis cortical BMD relative to the PC group. At 500 FTU/kg, only BSP

Table 3. Concentrations of IP_{6-3} as a percentage of grower broiler diets (dry matter basis).[1]

	IP_6	IP_5	IP_4	IP_3
Diets[2,3]				
PC	1.02	0.11	0.02	0.06
NC1	1.07	0.13	0.04	0.06
NC1+500BSP[2]	1.00	0.16	0.05	0.06
NC1+500CBP[2]	1.05	0.12	0.04	0.06
NC2	1.07	0.14	0.04	0.06
NC2+1,000BSP[2]	1.00	0.17	0.06	0.06
NC2+1,000CBP[2]	1.02	0.13	0.04	0.06

[1]IP_{6-3} (IP_6 = myo-inositol hexa-phosphates, IP_5 = myo-inositol penta-phosphates, IP_4 = myo-inositol tetra-phosphates, and IP_3 = myo-inositol tri-phosphates) means calculated from triplicate analyses of each diet.

[2]BSP = *Buttiauxella* sp. phytase and CBP = *Citrobacter braakii* phytase.

[3]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet, respectively (NC1), or by 0.174 and 0.159% of the diet, respectively (NC2); the NC1 + phytase A (BSP) or phytase B (CBP) at 500 FTU/kg (NC1+500BSP and NC1+500CBP); and the NC2 + BSP or CBP at 1,000 FTU/kg (NC2+1,000BSP and NC2+1,000CBP).

alleviated the adverse effects of NC1, but at 1,000 FTU/kg, both BSP and CBP alleviated the adverse effects of NC2 ($P \leq 0.001$; Table 4). Diet effects on each of day 22 mid-diaphysis total and cortical BMC were similar to the diet effects on day 22 mid-diaphysis cortical BMD ($P \leq 0.001$). The day 22 mid-diaphysis total and trabecular BSCA each tended to be decreased ($P = 0.055$ and 0.092, respectively) by NC1 and NC2 relative to PC; BSP and CBP at 500 and 1,000 FTU/kg tended to alleviate the adverse effect of the NC1 and NC2 diets, respectively. Relative to the PC, the day 22 proximal metaphysis trabecular BMD and BMC were each maintained by the NC1 diet but were decreased by the NC2 diet and at 1,000 FTU/kg, only BSP alleviated the adverse effect of NC2 ($P = 0.010$). In comparison to PC, the day 22 proximal metaphysis total BMC was decreased by both NC1 and NC2 and were alleviated by BSP at 500 and 1,000 FTU/kg, respectively ($P < 0.001$). Also relative to the PC, the day 22 proximal metaphysis cortical BMC was decreased by both NC1 and NC2, and supplementation of either phytase alleviated the adverse effect ($P = 0.011$).

The day 33 mid-diaphysis cortical BMD was decreased by NC1 and NC2 relative to PC, and each phytase alleviated the adverse effect of the NC1 and NC2 diets ($P = 0.005$; Table 5). Relative to the PC, the day 33 mid-diaphysis total BMC was maintained by NC1 but tended to be decreased by NC2, which tended to be alleviated by both BSP and CBP at 1,000 FTU/kg ($P = 0.073$). Also, the day 33 mid-diaphysis cortical

Table 4. Effect of dietary Ca, available P, and phytase source and dose on broiler femur densitometry at 22 D of age.[1]

	Bone mineral density (mg/cm3)			Cross-sectional area (mm^2)			Bone mineral content (mg/mm)[2]		
Diets[3,4]	Total	Trabecular	Cortical	Total	Trabecular	Cortical	Total	Trabecular	Cortical
Mid-diaphysis[5]									
PC	539	61.4	840^{a}	31.2	10.6	18.1	16.8^{a,b}	0.63	15.2^{a,b}
NC1	525	52.9	803^{b}	28.7	9.59	17.1	15.0^{c,d}	0.49	13.6^{d,e}
NC1+500BSP[3]	551	58.7	838^{a}	32.2	10.4	19.2	17.7^{a}	0.58	16.1^{a}
NC1+500CBP[3]	513	49.7	820^{a,b}	30.4	11.2	16.9	15.4^{b-d}	0.62	13.9^{d,e}
NC2	514	46.4	767^{c}	28.5	8.57	17.1	14.6^{d}	0.39	13.2^{e}
NC2+1,000BSP[3]	551	63.9	836^{a}	30.4	9.76	18.1	16.7^{a,b}	0.61	15.1^{a-c}
NC2+1,000CBP[3]	543	61.1	837^{a}	29.5	9.85	17.4	16.0^{b}	0.59	14.5^{b-d}
Pooled SEM	14.94	9.51	10.69	0.89	0.61	0.66	0.56	0.10	0.53
Source of variation	*P*-value								
Diet	0.227	0.820	<0.001	0.055	0.086	0.107	0.001	0.668	<0.001
Covariate (BW)	0.774	0.820	0.739	0.620	0.981	0.534	0.761	0.706	0.612
Proximal metaphysis[6]									
PC	365	99.8^{a,b}	747^{a,b}	38.4	21.3	13.7	13.9^{a}	2.15^{a,b}	10.2^{a}
NC1	347	93.6^{a-c}	733^{b}	35.9	20.2	12.5	12.4^{c}	1.87^{a-c}	9.19^{b,c}
NC1+500BSP[3]	359	96.8^{a,b}	754^{a}	38.6	21.6	13.4	13.7^{a,b}	2.14^{a,b}	10.1^{a,b}
NC1+500CBP[3]	357	85.5^{b,c}	749^{a,b}	36.7	20.4	13.1	12.9^{b,c}	1.71^{b,c}	9.81^{a,b}
NC2	325	76.6^{c}	707^{c}	35.7	20.0	12.1	11.5^{d}	1.55^{c}	8.53^{c}
NC2+1,000BSP[3]	358	107.3^{a}	739^{a,b}	38.3	21.4	13.4	13.7^{a,b}	2.25^{a}	9.93^{a,b}
NC2+1,000CBP[3]	343	78.3^{c}	748^{a,b}	36.6	20.8	12.8	12.5^{c}	1.64^{c}	9.61^{a,b}
Pooled SEM	10.36	6.63	7.51	1.16	0.97	0.65	0.36	0.18	0.34
Source of variation	*P*-value								
Diet effect	0.153	0.005	0.001	0.315	0.852	0.075	<0.001	0.020	0.011
Co-variate (BW)	0.087	0.245	0.021	0.111	0.075	0.408	0.954	0.042	0.145

[a–e]Treatment means with no common superscript within a column differ ($P \leq 0.05$).

[1]Each treatment mean was calculated from right femurs of 2 birds in each of 9 cage replicates ($n = 126$) on day 22.

[2]Bone mineral content was calculated by multiplying the respective bone cross-sectional area by bone mineral density to yield the amount of mineral (mg) contained in 1 mm-thick x-ray scan for each of bone fraction.

[3]BSP = *Buttiauxella* sp. phytase and CBP = *Citrobacter braakii* phytase.

[4]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet, respectively (NC1), or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (BSP) or phytase B (CBP) at 500 FTU/kg (NC1+500BSP and NC1+500CBP); and the NC2 + BSP or CBP at 1,000 FTU/kg (NC2+1,000BSP and NC2+1,000CBP).

[5]Mid-point of femur length.

[6]30% point from the proximal end of femur.

Table 5. Effect of dietary Ca, available P, and phytase source and dose on broiler femur densitometry traits at 33 D of age.[1]

Diets[3,4]	Bone mineral density (mg/cm^3)			Cross-sectional area (mm^2)			Bone mineral content (mg/mm)[2]		
	Total	Trabecular	Cortical	Total	Trabecular	Cortical	Total	Trabecular	Cortical
Mid-diaphysis[5]									
PC	466	50.6	891^{a,b}	49.2	23.3	23.4	22.8	1.17	20.8^{b}
NC1	453	60.0	860^{c}	50.9	24.3	24.0	23.0	1.47	20.6^{b}
NC1+500BSP[3]	468	48.6	885^{a,b}	49.4	23.0	23.9	23.0	1.13	21.1^{a,b}
NC1+500CBP[3]	465	49.3	884^{a,b}	48.8	23.2	23.1	22.4	1.11	20.4^{b}
NC2	466	51.4	871^{b,c}	47.4	22.2	23.1	22.0	1.23	20.1^{b}
NC2+1,000BSP[3]	483	48.0	897^{a}	50.7	23.2	25.0	24.4	1.10	22.4^{a}
NC2+1,000CBP[3]	484	47.5	901^{a}	48.1	22.5	23.6	23.2	1.07	21.2^{a,b}
Pooled SEM	9.31	6.0	8.56	1.19	0.8	0.54	0.54	0.15	0.45
Source of variation	*P*-value								
Diet	0.238	0.811	0.005	0.347	0.644	0.174	0.073	0.523	0.014
Covariate (BW)	0.069	0.739	<0.001	<0.001	<0.001	<0.001	<0.001	0.108	<0.001
Proximal metaphysis[6]									
PC	325	74.1	784	60.9	37.4	19.2	19.7	2.77	15.0^{a-c}
NC1	319	65.9	789	58.9	38.9	18.7	18.8	2.44	14.8^{b,c}
NC1+500BSP[3]	334	74.1	797	58.5	35.6	19.1	19.5	2.63	15.1^{a,b}
NC1+500CBP[3]	330	81.6	784	59.0	36.4	18.5	19.3	2.96	14.5^{b,c}
NC2	310	73.6	777	60.1	37.8	18.0	18.5	2.8	14.0^{c}
NC2+1,000BSP[3]	338	71.4	805	59.6	36.2	19.8	20.1	2.59	15.9^{a}
NC2+1,000CBP[3]	330	65.9	793	59.7	36.3	19.6	19.5	2.38	15.5^{a,b}
Pooled SEM	8.48	4.03	6.94	1.41	1.59	0.48	0.51	0.18	0.41
Source of variation	*P*-value								
Diet	0.286	0.117	0.096	0.921	0.793	0.191	0.225	0.292	0.037
Covariate (BW)	0.605	0.671	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	<0.001

[a–c]Treatment means with no common superscript within a column differ ($P \leq 0.05$).

[1]Each treatment mean was calculated from right femurs of 2 birds in each of 9 cage replicates ($n = 126$) on day 33.

[2]Bone mineral content was calculated by multiplying the respective bone cross-sectional area by bone mineral density to yield the amount of mineral (mg) contained in 1 mm-thick x-ray scan for each of bone fraction.

[3]BSP = *Buttiauxella sp.* phytase and CBP = *Citrobacter braakii* phytase.

[4]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet, respectively (NC1), or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (BSP) or phytase B (CBP) at 500 FTU/kg (NC1+500BSP and NC1+500CBP); and the NC2 + BSP or CBP at 1,000 FTU/kg (NC2+1,000BSP and NC2+1,000CBP).

[5]Mid-point of femur length.

[6]30% point from the proximal end of femur.

BMC was maintained by NC1 and NC2 relative to PC and was increased by NC2+1,000BSP compared to PC, NC1, NC2, and NC1+500CBP ($P = 0.014$). Proximal metaphysis cortical BMD was nearly significantly decreased ($P = 0.096$) by NC2 relative to the PC but was restored by supplementation of either phytase at 1,000 FTU/kg feed. The day 33 proximal metaphysis cortical BMC was maintained by NC1 and NC2 relative to PC, but each phytase at 1,000 FTU/kg increased cortical BMC compared to NC2 ($P = 0.037$). None of the other day 33 bone densitometry parameters was affected by diet.

Relative to the PC, the day 8 BBS was maintained by the NC1 diet but decreased by the NC2 diet, and the use of BSP or CBP at 1,000 FTU/kg alleviated the adverse effect ($P < 0.001$ Table 6). The day 22 BBS was decreased by the NC1 and NC2 diets relative to PC. At 500 FTU/kg, only BSP alleviated the adverse effects of NC1, while at 1,000 FTU/kg, both BSP and CBP alleviated the adverse effects of NC2 ($P < 0.001$). The day 33 BBS was decreased in NC1 and NC2 birds relative to PC. Both BSP and CBP at 500 and 1,000 FTU/kg alleviated the adverse effect of the NC1 and NC2 diets, respectively; however, the day 33 BBS was higher in the NC2+1,000BSP birds than in the NC2+1,000CBP birds ($P < 0.001$). Relative to the PC, the day 22 bone ash was decreased by the NC1 diet and maintained by NC1+500BSP and NC1+500CBP, with a similar effect on NC2 and the respective phytase-supplemented diets ($P < 0.001$). There was no diet effect on day 8 or day 33 bone ash.

Apparent Ileal Digestibility of P and Ca

The day 8 AIDP was maintained by NC1 but decreased by NC2 relative to PC and was increased by NC1+500BSP and NC1+500CBP relative to NC1 and PC ($P < 0.001$; Table 7). Relative to NC2, each phytase increased AIDP. The day 22 and 33 AIDP were each decreased by NC1 and NC2 relative to PC and were alleviated by supplemented phytase regardless of type and dose. However, day 22 and 33 AIDP were each higher with BSP relative to CBP, regardless of dose ($P < 0.001$). The day 22 AIDCa was increased by NC1, maintained by NC2 compared to PC, and was increased by BSP and CBP at 1,000 FTU/kg. There were no treatment effects at day 8 and 33 ($P < 0.001$).

Table 6. Effect of dietary Ca, available P, and phytase source and dose on femur traits of broilers.[1]

Diets[2,3]	Bone breaking strength (kgF)			Bone ash content (%)		
	Day 8	Day 22	Day 33	Day 8	Day 22	Day 33
PC	3.18^{a}	14.7^{a}	20.2^{b,c}	33.4	36.1^{a,b}	31.5
NC1	3.06^{a}	13.0^{b,c}	18.7^{c,d}	30.4	33.5^{d}	31.8
NC1+500BSP[2]	3.22^{a}	14.3^{a}	21.3^{a,b}	30.2	35.2$^{a–c}$	33.3
NC1+500CBP[2]	3.17^{a}	13.7^{a,b}	20.9^{b}	30.1	35.1^{b,c}	31.8
NC2	2.70^{b}	12.4^{c}	17.9^{d}	29.9	34.2^{c,d}	31.3
NC2+1,000BSP[2]	3.24^{a}	14.5^{a}	22.7^{a}	30.0	36.3^{a}	33.4
NC2+1,000CBP[2]	3.22^{a}	14.4^{a}	20.5^{b}	32.2	36.3^{a}	31.5
Pooled SEM	0.09	0.39	0.58	1.63	0.46	0.77
Source of variation	*P*-value					
Diet	<0.001	<0.001	<0.001	NS	<0.001	NS
Covariate (BW)	<0.001	<0.001	<0.001	NS	NS	0.01

$^{a–d}$Treatment means with no common superscript within a column differ ($P \leq 0.05$).

[1]Treatment means were calculated for each of bone breaking strength and ash content from right femurs of 2 birds in each of 9 cage replicates per treatment ($n = 126$) on each of days 22 and 33.

[2]BSP = *Buttiauxella sp.* phytase and CBP = *Citrobacter braakii* phytase.

[3]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet respectively (NC1), or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (BSP) or phytase B (CBP) at 500 FTU/kg (NC1+500BSP and NC1+500CBP); and the NC2 + BSP or CBP at 1,000 FTU/kg (NC2+1,000BSP and NC2+1,000CBP).

Table 7. Effect of dietary Ca, available P, and phytase source and dose on apparent ileal digestibility of phosphorus (AIDP) and calcium (AIDCa) in broilers.[1]

Diets[2,3]	AIDP%			AIDCa%		
	Day 8	Day 22	Day 33	Day 8	Day 22	Day 33
PC	45.5^{d,e}	48.3^{c}	44.6^{d}	40.5	41.2^{b,c}	32.6
NC1	42.8^{e,f}	39.6^{d}	36.8^{e}	41.4	50.1^{a}	33.5
NC1+500BSP[2]	56.3^{b}	58.9^{b}	56.7^{a,b}	46.9	50.7^{a}	32.2
NC1+500CBP[2]	54.5^{b,c}	48.2^{c}	51.5^{c}	43.8	40.9^{b,c}	34.9
NC2	39.5^{f}	42.6^{d}	34.6^{e}	35.7	37.6^{c}	38.4
NC2+1,000BSP[2]	63.3^{a}	66.9^{a}	61.4^{a}	50.9	53.2^{a}	28.5
NC2+1,000CBP[2]	49.3^{d}	50.1^{c}	54.7^{b,c}	39.7	46.8^{a,b}	29.0
Pooled SEM		2.21			3.09	
Source of variation	*P*-value					
Diet × age		<0.001			<0.001	

$^{a–d}$Treatment means with no common superscript for each parameter differ ($P \leq 0.05$).

[1]Treatment means were calculated for each of AIDP, and AIDCa from 9 cage replicates ($n = 63$) each pooled from 11 and 5 birds for days 22 and 33, respectively.

[2]BSP = *Buttiauxella* sp. phytase and CBP = *Citrobacter braakii* phytase.

[3]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet, respectively (NC1), or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (BSP) or phytase B (CBP) at 500 FTU/kg (NC1+500BSP and NC1+500CBP); and the NC2 + BSP or CBP at 1,000 FTU/kg (NC2+1,000BSP and NC2+1,000CBP).

IP Degradation Across the GIT and IP_6 Disappearance in the Distal Ileum

In each section of the GIT, concentrations of each $IP_{6–3}$ ester were maintained by NC1 and NC2 relative to PC, except for proventriculus + gizzard IP_5, which was lower in NC1 than in PC and NC2 ($P < 0.001$; Table 8). Relative to PC and NC1, each of crop $IP_{6–3}$ concentrations was not different for NC1+500BSP, but both IP_6 and IP_5 concentrations were decreased, and each of IP_4 and IP_3 concentrations was increased by NC1+500CBP. Relative to the PC and NC2, crop IP_6 concentration was decreased by each of NC2+1,000BSP and NC2+1,000CBP, the IP_5 concentration was only decreased by NC2+1,000CBP, and each of the $IP_{4–3}$ concentrations was increased by each of the 2 phytases except for NC2+1,000BSP, which had similar crop IP_3 to the controls. Overall, concentrations of each IP ester in the crop were decreased, and both IP_4 and IP_3 concentrations were greater for CBP relative to BSP at each dose. Relative to PC and NC1, proventriculus + gizzard IP_6 concentration was decreased by each of NC1+500BSP and NC1+500CBP. The IP_5 concentration was decreased by NC1+500BSP, the IP_4 concentration was increased by NC1+500CBP, and the IP_3 concentration was increased by each phytase at 500 FTU/kg. Relative to PC and NC2, proventriculus + gizzard IP_6 and IP_5 concentrations were decreased by NC2+1,000BSP and NC2+1,000CBP each, and IP_4 and IP_3 concentrations were maintained by NC2+1,000BSP, but increased by NC2+1,000CBP. The proventriculus +

Table 8. Effect of dietary Ca, available P, and phytase source and dose on digesta $IP_{6–3}$ concentrations in the crop, proventriculus + gizzard, and distal ileum of broilers at day 22.[1]

	Crop, % of DM				Proventriculus + gizzard, % of DM				Distal ileum, % of DM			
Diets[2,3]	IP_6	IP_5	IP_4	IP_3	IP_6	IP_5	IP_4	IP_3	IP_6	IP_5	IP_4	IP_3
PC	0.85^{a}	0.10^{a,b}	0.08^{c}	0.06^{c}	0.26^{a}	0.04^{a}	0.03^{b,c}	0.04^{b}	1.69^{a}	0.23^{a}	0.13^{d}	0.09^{d}
NC1	0.83^{a}	0.09^{b,c}	0.05^{c}	0.05^{c}	0.30^{a}	0.02^{b}	0.02^{b,c}	0.03^{b}	1.83^{a}	0.19^{a,b}	0.09^{d}	0.11^{c,d}
NC1+500BSP[2]	0.80^{a}	0.11^{a,b}	0.11^{c}	0.05^{c}	0.03^{c}	0.00^{c}	0.04^{b}	0.06^{a}	0.88^{c}	0.27^{a}	0.43^{a}	0.17^{b}
NC1+500CBP[2]	0.58^{b}	0.06^{d}	0.23^{a,b}	0.08^{a,b}	0.10^{b}	0.02^{b}	0.07^{a}	0.06^{a}	1.23^{b}	0.28^{a}	0.28^{c}	0.15^{b}
NC2	0.84^{a}	0.10^{a,b}	0.08^{c}	0.05^{c}	0.27^{a}	0.03^{a}	0.03^{b,c}	0.04^{b}	1.81^{a}	0.21^{a}	0.11^{d}	0.12^{c,d}
NC2+1,000BSP[2]	0.61^{b}	0.13^{a}	0.18^{b}	0.06^{c}	0.02^{c}	0.00^{c}	0.02^{c}	0.04^{b}	0.48^{d}	0.13^{b}	0.38^{a,b}	0.21^{a}
NC2+1,000CBP[2]	0.52^{b}	0.05^{d}	0.28^{a}	0.09^{a}	0.07^{b}	0.01^{b}	0.06^{a}	0.06^{a}	0.89^{c}	0.30^{a}	0.31^{b,c}	0.16^{b}
Pooled SEM	0.04	0.01	0.02	0.01	0.02	0.01	0.01	0.01	0.07	0.03	0.04	0.01
Source of variation	*P*-value											
Diet	<0.001	<0.001	<0.001	0.004	<0.001	<0.001	<0.001	0.004	<0.001	0.006	<0.001	<0.001

[a–d]Treatment means with no common superscript within a column differ ($P \leq 0.05$).

[1]$IP_{6–3}$ (IP_6 = myo-inositol hexa-phosphates, IP_5 = myo-inositol penta-phosphates, IP_4 = myo-inositol tetra-phosphates, and IP_3 = myo-inositol tri-phosphates) means were calculated for each of crop, proventriculus + gizzard, and distal ileum from 9 cage replicates (n = 189) each pooled from 11 birds.

[2]BSP = *Buttiauxella* sp. phytase and CBP = *Citrobacter braakii* phytase.

[3]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet respectively (NC1), or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (BSP) or phytase B (CBP) at 500 FTU/kg (NC1+500BSP and NC1+500CBP); and the NC2 + BSP or CBP at 1,000 FTU/kg (NC2+1,000BSP and NC2+1,000CBP).

gizzard IP_3 concentration was not affected by phytase type at 500 FTU/kg, but was decreased by BSP relative to CBP at 1,000 FTU/kg; the $IP_{6–4}$ concentrations were also decreased by BSP relative to the CBP at both doses. Also, the proventriculus + gizzard IP_4 and IP_3 concentrations were lower in the NC2+1,000BSP birds than in the NC1+500BSP birds. Relative to PC and NC1, distal ileum IP_6 concentration was decreased, the IP_5 concentration was maintained, and the $IP_{4–3}$ concentrations were each increased by each of NC1+500BSP and NC1+500CBP. Relative to PC and NC2, the distal ileum IP_6 concentration was decreased by NC2+1,000BSP and NC2+1,000CBP, the IP_5 concentration was decreased only by NC2+1,000BSP, and the IP_4 and IP_3 concentrations were increased by each phytase. Overall, distal ileum $IP_{6–5}$ concentrations were decreased by the BSP relative to the CBP at both doses and by NC2+1,000BSP relative to NC1+500BSP. Also in the distal ileum, however, IP4 concentration was increased by NC1+500BSP relative to NC1+500CBP but similar at the 1,000 FTU/kg dose, and the IP_3 concentration was increased in the NC2+1,000BSP compared to NC2+1,000CBP but similar at the 500 FTU/kg dose. Also, the distal ileum IP_6 disappearance was maintained in the NC1 and NC2 diets relative to the PC and was increased by each respective phytase supplementation (Figure 1). Relative to the control diets, the distal ileum IP_6 disappearance was also further increased by BSP relative to CBP at both doses and by 1,000 FTU/kg relative to 500 FTU/kg with both phytases.

DISCUSSION

Diets were formulated using the analyzed Ca and P levels of the corn, soybean meal, and canola meal to achieve the desired dietary Ca and avP levels. Hence, the differences between the formulated and analyzed Ca and P levels may be due to sampling error, and are not anticipated to have any influence on interpretation of the treatment effects. The higher phytase recovery than expected in the starter diets might indicate an excessive dietary inclusion of the enzyme following the diets being re-mixed after low initial phytase recoveries were noted before the start of the trial. However, while IP ester degradation and bone densitometry were not evaluated during this phase, interpretation of treatment effects on bone breaking strength and ash and AIDP and AIDCa at day 8 would not be compromised by the higher analyzed phytase activity than expected in the starter diets. The phytase recovery from the grower and finisher diets was in close agreement to the expected activities. Overall, the high analyzed phytase activity applied to both phytases; however, the enzyme activities tended to be higher for the CBP diets than in the BSP diets. Also, the analyzed phytase activity in the finisher PC diet tended to be higher than expected. Because the phytase activity in the finisher PC diet was well below that in the phytase-supplemented diets and the analyzed enzyme activity in the 500 FTU/kg phytase-supplemented diets was substantially lower than in the 1,000 FTU/kg phytase-supplemented diets, the variances in analyzed phytase activity in each of the diets do not compromise the objectives of the study.

Bone Quality Traits

The avP and Ca reductions in the NC1 and NC2 diets relative to the PC decreased bone mineralization and strength of broilers (Tables 4–6). Reduced dietary Ca and avP in broilers decreased bone mineralization and strength (Powell et al., 2011; Mello et al., 2012; Amerah et al., 2014). The reductions in avP and Ca levels in the NC1 and NC2 diets also increased the Ca: avP ratio relative to that in the nutrient-adequate PC

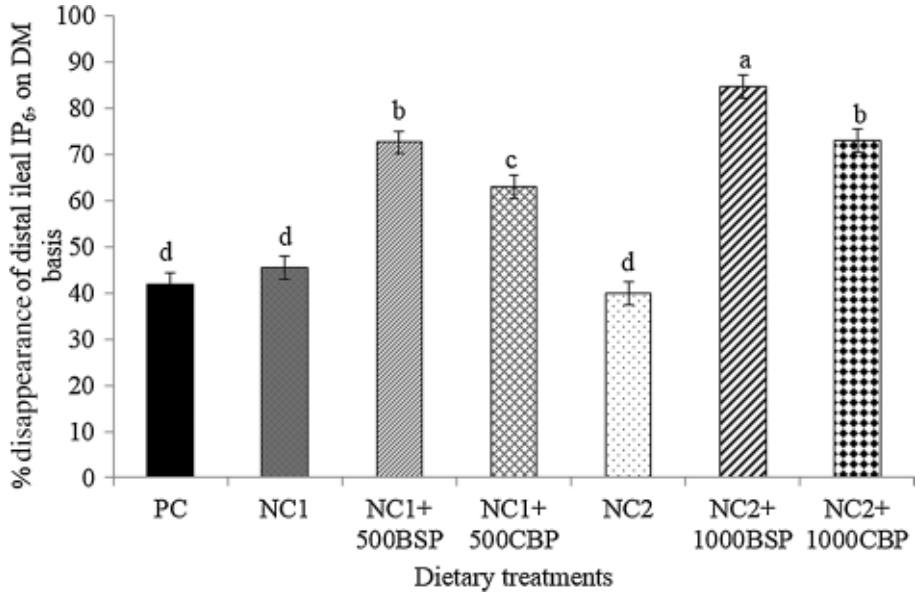

Figure 1. Effect of dietary Ca, available P, and phytase source and dose on the disappearance of inositol hexa-phosphate (IP_6) in the distal ileum of broilers at day 22. [a–d]Treatment means with no common superscript differ ($P \leq 0.05$). [1]Each treatment mean for distal ileum IP_6 concentration was calculated from 9 replicates each pooled from 11 birds at 22 D of age ($n = 63$). [2]BSP = *Buttiauxella* sp. phytase and CBP = *Citrobacter braakii* phytase. [3]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet, respectively (NC1), or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (BSP) or phytase B (CBP) at 500 FTU/kg (NC1+500BSP and NC1+500CBP); and the NC2 + BSP or CBP at 1,000 FTU/kg (NC2+1,000BSP and NC2+1,000CBP).

diet, which may have resulted in a mineral imbalance. The Ca: avP ratios in the grower PC, NC1, and NC2 diets of the current study were 1.98, 2.55, and 2.73, respectively. Hence, the decreased femur bone density, ash and strength of the NC1 and NC2 birds could be related to reduced availability, and utilization of dietary P and Ca. Cortical and trabecular bone densitometry measures, as well as BBS and bone ash, were decreased by the avP and Ca reductions of the NC1 and NC2 diets relative to the PC diets at days 22 and 33. Similarly, a decrease in dietary Ca from 9.1 to 4.5 g/kg and in total P from 7.2 to 3.7 g/kg linearly decreased dual-energy x-ray absorptiometry-measured total BMD and BMC (Onyango et al., 2003). At day 42, a reduction of dietary avP from 0.37 to 0.13% linearly increased broiler bone tartrate-resistant acid phosphatase concentration (de Souza Nakagi et al., 2013), an indicator of bone resorption activity by osteoclasts (Dacke et al., 1993). Cortical and trabecular bone tissues are actively remodeled throughout the life cycle of broiler chickens (Pratt and Cooper, 2018). The decreased femur cortical and trabecular BMD and BMC in the NC1 and NC2 chickens relative to the PC chickens at days 22 and 33 in the current study indicated that the reduction in dietary avP induced higher osteoclastic activity.

Regardless of source and dose, dietary phytase supplementation alleviated the decrease in the bone quality of NC1 and NC2 birds. Phytase use in a diet reduced in avP and Ca alleviated the decrease in BBS and bone ash in day 21 broilers (Powell et al., 2011). Although there were no effects of phytase source nor dose on BBS, bone ash, and total and cortical densitometry, the proximal metaphysis trabecular BMD and BMC were each increased to a greater extent by NC2+1,000BSP than by NC2+1,000CBP. Trabecular bone tissue is concentrated towards the proximal and distal ends of the long bones of broilers (Aguado et al., 2015), which explains the proportionally higher trabecular BMD, BCSA, and BMC at the proximal metaphysis than at the mid-diaphysis at days 22 and 33. The higher trabecular densitometry values at the proximal metaphysis than at the mid-diaphysis enabled the opportunity to better understand the diet effects on overall structural bone densitometry. There was no diet effect on mid-diaphysis trabecular densitometry, while proximal metaphysis trabecular BMD and BMC were each higher in NC1+500BSP or NC2+1,000BSP birds than those of the NC2+1,000CBP at day 22. Also, relative to the PC, the day 22 trabecular BMD and BMC were each maintained by NC1 but decreased by the NC2, which allowed the chance to assess phytase effects on the moderate avP and Ca deficiency. The diet effects showed that the cortical bone densitometry was generally not affected by phytase source or dose. Hence, the increase in day 22 trabecular BMD and BMC by NC2+1,000BSP relative to NC2+1,000CBP resulted in the higher day 33 BBS in the NC2+1,000BSP birds than in NC2+1,000CBP birds, while there was no difference between the day 22 BBS of NC1+500BSP and NC2+1,000BSP birds. The moderate avP and Ca reduction in the NC2 diet was completely alleviated by the BSP at 1,000 FTU/kg but not by CBP at the same dose. Not only does phytase supplementation increase P and Ca digestibility to subsequently increase mineral

availability and utilization (Bedford et al., 2015), effective phytase usage in avP- and Ca-deficient diets also maintains overall bone health (Powell et al., 2011). In the current study, phytase supplementation in avP- and Ca-deficient diets increased AIDP at each age and day 22 AIDCa to alleviate the adverse effects on bone mineralization and strength. Overall, usage of the highly efficient BSP at 1,000 FTU/kg in broiler diets deficient in avP and Ca fortified trabecular bone densitometry traits to increase load-bearing integrity of bone.

IP Degradation Across the GIT, IP_6 Disappearance in the Distal Ileum, and P and Ca Digestibility

Phytase liberates phosphate molecules through a stepwise dephosphorylation pathway to degrade IP_6 to IP_{3-1} (Greiner and Konietzny, 2011; Menezes-Blackburn et al., 2015). The effects of diets on P and Ca digestibility (Table 7), IP_{6-3} in the crop, proventriculus + gizzard, and distal ileum (Table 8), IP_6 distal ileum disappearance (Figure 1) indicated that phytase degraded phytate in a stepwise manner to increase P and Ca availability. This in vivo phytate-degrading activity of phytase was consistent with findings of previous studies (Walk et al., 2014; Li et al., 2016; Beeson et al., 2017; Sommerfeld et al., 2018). However, the magnitude of the increases in P and Ca digestibilities, proventriculus + gizzard and distal ileum IP_{6-3} degradation, and distal ileum IP_6 disappearance was greater for BSP than CBP and for 1,000 FTU/kg than the 500 FTU/kg dose. Specifically, Li et al. (2016) also evaluated the IP_6 degradation efficacy of the BSP at 500 and 1,000 FTU/kg in the crop, proventriculus+gizzard, and distal ileum. Overall, IP_6 degradation by the BSP at the 2 doses in the proventriculus+gizzard and distal ileum in the current study were in line with those reported by Li et al. (2016). However, crop IP_6 degradation by BSP at 500 and 1,000 FTU/kg was 3.6 and 27%, respectively, in the current study and 32.8 and 44.8%, respectively, as reported by Li et al., 2016. The differences between the 2 studies may be related to differences in diet composition. The current study used corn-soybean meal diets with 7.5% canola meal (0.29 phytate P), whereas Li et al. (2016) used a corn-soybean meal with 6% rice bran (0.34% phytate P). Hence, the reduced crop IP_6 degradation with BSP in the current study may be due to the differences in dietary phytate P sources and levels. However, the consistently high IP_6 degradation by BSP in the proventriculus+gizzard and distal ileum in both studies indicates a high efficacy of BSP in the low pH of the proventriculus and the high pH of the small intestine, regardless of phytate P source and level.

Overall, IP_6 was the most abundant IP ester in the diet (Table 3); this has the greatest anti-nutritional effects relative to other IP esters (Li et al., 2016). The IP_6 concentrations in each of the crop, proventriculus + gizzard, and distal ileum were decreased relative to the NC2 birds by 27.4, 92.6, and 73.5%, respectively, with BSP at 1,000 FTU/kg and 38.1, 74.1, and 50.8%, respectively, with CBP at the same dose. Hence, the majority of IP degradation by phytase occurred in the proventriculus + gizzard, showing the importance of low pH on phytase activity. The pH values in the crop, proventriculus + gizzard, and ileum in broilers are 4.5, 2.8, and 5.8, respectively, indicating high variability in pH across the GIT of broilers (Amerah et al., 2014). Compared to overall IP_{6-3} concentration across NC1 and NC2, the overall IP_{6-3} degradation in the crop, proventriculus + gizzard, and distal ileum were 2.6, 73.0, and 33.2%, respectively for BSP diets and 10.8, 37.9, and 18.6%, respectively, for CBP diets. Phytase is supplemented in animal feed based on activity at pH 5.5; however, the activity of different phytases in the lower pH range significantly differ. Menezes-Blackburn et al. (2015) tested 7 commercial phytase products and observed that at pH 3, the activity of BSP was 235%, and for CBP, the activity was 146% relative to activity at pH 5.5. This explains the higher IP ester degradation efficacy in the proventriculus + gizzard with BSP compared to CBP. Also, CBP may have lost considerable activity in the low pH of the proventriculus + gizzard, and in interactions with pancreatic enzymes (Onyango et al., 2005), thereby reducing phytate degradation relative to the BSP across the GIT segments. The activities of *E. coli* and *Peniophora lycii* phytases were decreased in the proventriculus + gizzard by 59.4 and 93.7%, respectively, relative to the dietary phytase activity (Onyango et al., 2005). Thus, the high acidity of the proventriculus + gizzard may negatively affect some phytases, particularly those with optimal activity at relatively higher pH. On the other hand, the lower IP_{6-3} in the proventriculus + gizzard for BSP than CBP showed that BSP exhibited the highest activity at the low pH of the proventriculus + gizzard. Although Menezes-Blackburn et al. (2015) reported from the in vitro assessment that 80% of optimal activity of CBP and BSP ranged at pH 3–4.5 and 3 (expressed as % of maximum activity), respectively. However, the findings of the in vitro assessment also showed that when activity of each of the phytases was optimized at 100% based on pH 5.5, the relative activity at pH 3 was 235% for BSP and 147% for CBP (Menezes-Blackburn et al., 2015). This supports the findings of the current study, which indicated that BSP may have had a broader pH spectrum of efficacy relative to CBP in the low pH environment of the proventriculus + gizzard and in the higher pH environment of the distal ileum. Dietary BSP degraded IP_{6-3} across the crop, proventriculus + gizzard, and distal ileum to increase disappearance of distal ileum IP_6, and the apparent ileal digestibility of P and Ca to subsequently increase some aspects of broiler bone mineralization relative to the CBP. Hence, these findings imply that the IP ester-degrading efficacy and nutrient digestibility and utilization of phytases are dependent on the enzyme source.

Relative to 500 FTU/kg of BSP, the 1,000 FTU/kg dose exhibited higher degradation of IP_6 and IP_5 across the GIT, although the effects of each dose were similar in the proventriculus + gizzard. The 1,000 FTU/kg dose of BSP also showed higher degradation of $IP_{4–3}$ in the proventriculus + gizzard but resulted in an increase of IP_4 in the crop and IP_3 in the distal ileum, compared to 500 FTU/kg. This likely resulted from the greater degradation of $IP_{6–5}$ across the GIT by the higher dose. Because of the linear decrease in the number of chelated phosphate groups from IP_6 to IP_1 (Greiner and Konietzny, 2011), $IP_{4–3}$ exhibit significantly lower affinity to complex with cations such as Ca relative to IP_6 (Persson et al., 1998). Hence, the decreased $IP_{6–5}$ and increased $IP_{4–3}$ across the GIT of birds with phytase, particularly BSP at 1,000 FTU/kg relative to 500 FTU/kg, suggest that the higher phytase dose would reduce the cumulative anti-nutritional effects of phytate in the diet (Beeson et al., 2017). Effective phytase usage requires an enzyme with high efficiency, at dose sufficient to optimally decrease IP esters, particularly IP_6 which is the most potent (Li et al., 2016), to increase phytate P availability. The 1,000 FTU/kg does of CBP degraded $IP_{6–3}$ in each GIT segment to a similar extent as 500 FTU/kg, except that the higher dose further degraded distal ileum IP_6. Particularly with BSP, 1,000 FTU/kg increased degradation of $IP_{6–5}$, with a smaller increase of $IP_{4–3}$ across the GIT segments to increase P availability compared to 500 FTU/kg. Hence, 1,000 FTU/kg of BSP had higher efficacy to reduce the anti-nutritional effects of phytate and its metabolites and increase P and Ca availability for absorption than the 500 FTU/kg in the GIT of broilers. The effects of 500, 1,000, or 1,500 FTU/kg of *E. coli* phytase on gizzard $IP_{6–4}$ concentrations were similar, but 1,000 and 1,500 FTU/kg each decreased gizzard IP_3 concentration relative to 500 FTU/kg (Walk et al., 2014). Similarly, 1,000 FTU/kg BSP phytase decreased crop and proventriculus + gizzard IP_6 concentration relative to 500 FTU/kg but observed no difference between the 2 doses on distal ileum IP_6 disappearance (Li et al., 2016). Also, 1,500 and 500 FTU/kg ECP phytases had similar effects on gizzard $IP_{6–5}$ degradation, but the lower dose increased IP_4 concentration, while no difference was observed for distal ileum IP_6 disappearance between the 2 doses (Beeson et al., 2017). The level of dietary phytate and inorganic P usage also influence the efficacy of phytase on the substrate degradation. Unlike the aforementioned studies (Walk et al., 2014; Li et al., 2016; Beeson et al., 2017), in the current study greater reductions in P and Ca were used for the higher phytase dose, which may have contributed to the higher GIT $IP_{6–3}$ degradation for the 1,000 FTU/kg dose of BSP relative to the 500 FTU/kg dose. Overall, 1,000 FTU/kg degraded IP to a greater extent across the GIT than 500 FTU/kg, possibly because the higher phytase dose may have exhibited a faster rate of reaction with the IP substrate. Phytate was completely solubilized within 6 min by a 0.3 FTU/mL phytase dose, within 8.5 min by a 0.2 FTU/mL dose, and in 14 min by a 0.1 FTU/mL dose in an in vitro assessment (Tran et al., 2011). Also, the higher IP-degrading efficacy of phytase at 1,000 FTU/kg than at 500 FTU/kg could be related to a higher residual activity at each subsequent GIT segment of broilers with higher phytase doses (Nyannor et al., 2013). Residual phytase activity decreased as digesta passed through the GIT of broilers fed phytase-supplemented diets (Nyannor et al., 2013); hence, the 1,000 FTU/kg phytase dose used in the current study may also have retained a higher residual enzyme activity in each GIT segment than the 500 FTU/kg dose. Dietary phytase diet at 1,000 FTU/kg was more effective at degrading IP than 500 FTU/kg, particularly with BSP.

To efficiently degrade and decrease the anti-nutritional effects of IP on nutrient digestibility and utilization, the source and dose of dietary phytase are essential factors to consider. Both BSP and CBP at 500 and 1,000 FTU/kg each decreased IP_6 across the crop, proventriculus + gizzard, and distal ileum to increase P availability and bone quality traits relative to the non-phytase containing diets. However, BSP at 1,000 FTU/kg had the greatest effect on $IP_{6–4}$ degradation across the digestive tract segments and increased P availability and bone quality. The poultry industry can effectively use phytase to reduce inorganic P supplementation, which could decrease feed cost and P excretion, without reducing bone quality in broilers.

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