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Influence of graded concentrations of phytase in high-phytate diets on growth performance, apparent ileal amino acid digestibility, and phytate concentration in broilers from hatch to 28 D post-hatch

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ABSTRACT An experiment was conducted to evaluate graded doses of phytase in high-phytate diets. Ross 308, male broilers (n = 600) were assigned to one of 4 diets, with 10 replicate pens/diet and 15 birds/replicate pen. Diets were a nutrient adequate positive control (PC), a negative control (NC) diet with a reduction of Ca by 0.22%, available P by 0.20%, energy by 120 kcal/kg, and amino acids by 1 to 5% compared with the PC. The NC diet was supplemented with 0, 2,000, or 4,000 phytase units (FTU)/kg. Phytase increased (linear, $P < 0.05$) weight gain from hatch to day 18. Birds fed the NC + 4000 FTU/kg ate and gained more ($P < 0.05$) than birds fed the PC. The apparent ileal digestibility (AID) of all nutrients and amino acids were reduced ($P < 0.05$) in birds fed the NC compared with birds fed the PC. Phytase increased (linear, $P < 0.10$) AID of most nutrients. Digestibility was lower ($P < 0.10$) in birds fed the NC + 4000 FTU/kg compared with birds fed the PC. Using daily intake and AID to determine digestible nutrient intake resulted in no differences between birds fed the PC or NC + 4000 FTU/kg diets. Digestible intake of methionine or glutamate was better correlated with BW gain ($P < 0.0001$) than AID ($P > 0.10$). Phytase reduced (linear, $P < 0.01$) phytate concentration and increased inositol (linear, $P < 0.01$), phytate hydrolysis (linear, $P < 0.05$), and jejunal expression (linear, $P < 0.05$) of SNAT-1 and LAT-4 transporters. Supplementation of increasing doses of phytase in high-phytate, low-nutrient dense diets improved gain and digestibility through nearly complete phytate destruction. Digestible nutrient intake may be a better indication of broiler gain than AID alone.

Key words: apparent ileal digestibility, broiler, digestible nutrient intake, phytate, phytase

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INTRODUCTION

Fully phosphorylated phytate contains 12 dissociable and highly reactive protons capable of binding amino acids, minerals, and endogenous enzymes at pH ranges throughout the gastrointestinal tract. This negative effect of phytate appears to be magnified as the concentration of phytate increases in the diet (Cowieson et al., 2006a, 2017). However, even the lower, less phosphorylated phytate esters (IP5, IP4, and IP3) can bind endogenous enzymes (Yu et al., 2012) and minerals (Xu et al., 1992), and thereby reduce amino acid and mineral uptake by the animal and increase endogenous secretions of both amino acids and minerals (Cowieson et al., 2006a). The overall effect of dietary phytate is a reduction in growth performance, feed efficiency, and nutrient utilization with a corresponding increase in energy requirements and mineral and protein excretion. All at a cost to the animal in growth and energy expenditure and to the producers as lost yield or production efficiency.

Phytase supplementation, through the mitigation of the anti-nutrient effects of phytate, significantly improves growth performance and digestibility of minerals and amino acids. The improvements in nutrient utilization by phytase may be responsible for the extra-phosphoric effects noted with higher doses of phytase (Walk et al., 2014; Lee et al., 2017) or allow for further reductions of Ca, P and specific amino acids from the diet (Walk and Rama Rao, 2018). The reduction in diet nutrient density and improvements in their availability and uptake in the small intestine made possible by phytase use substantially reduces nutrient excretion in the feces while reducing feed costs with no loss in animal growth performance. The beneficial effects of phytase are particularly relevant as higher doses of phytase are supplied into the diet, supporting nearly complete phytate destruction (Walk et al., 2014; Sommerfeld et al., 2018) or further mitigation of the anti-nutritional effects of phytate in diets containing high-phytate P ingredients, such as

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canola meal, de-oiled rice bran, or sunflower meal. The objective of this work was to determine the influence of increasing phytase dose, up to 4,000 FTU/kg, in diets containing high-phytate P ingredients and severely restricted in Ca, P, amino acids, and energy. Response parameters included growth performance, nutrient and amino acids digestibility, gene expression profile of some amino acid transporters, digestible nutrient intake, and phytate degradation from hatch to day 28.

MATERIALS AND METHODS

All experimental procedures used in the study were approved by Scotland's Rural College Animal Experiment Committee.

Animals and Husbandry

Ross 308 male broilers (n = 600) were obtained at day of hatch and placed in floor pens on clean pine shavings at a stocking density of 6 chicks/m^2. There were 15 birds/pen from hatch to day 18 and 8 birds/pen from days 19 to 28. Each experimental diet was fed to 10 replicate pens. The birds were maintained on a lighting program of 23L:1D for the first 2 D, stepped up to 18L:6D on day 5 which was maintained for the remainder of the study. The birds were allowed ad libitum access to feed and water.

Dietary Treatments

Experimental diets were based on wheat, soybean meal, rapeseed meal, sunflower meal, and wheat bran and fed in crumble from hatch to day 18 or pelleted form from days 19 to 28 (Table 1). Dietary treatments consisted of a nutrient-adequate positive control (**PC**), formulated to meet or exceed the Ross 308 breed guidelines (Aviagen, 2014), a negative control (**NC**) diet formulated with a reduction of Ca by 0.22%, available P by 0.20%, energy by 120 kcal/kg, and amino acids by 1 to 5% when relative to the PC. The NC diet was then supplemented with 2,000 or 4,000 phytase units (FTU)/kg to create a total of 4 experimental diets. Wheat was exchanged with phytase in the phytase supplemented diets to equal 100%. The phytase was an enhanced *Escherichia coli* 6-phytase expressed in *Trichoderma reesei* with an expected activity of 5,000 FTU/g (Quantum Blue, AB Vista, Marlborough UK). One phytase unit is defined as the amount of enzyme required to release 1 μmol of inorganic P/min from sodium phytate at 37°C and pH 5.5. All diets contained a xylanase at 9,600 BXU/kg (Econase XT, AB Vista, Marlborough, UK).

Response Variables

Birds were weighed by pen prior to placement (day 0), days 18 and 28 to determine mean BW and calculate mean BW gain (**BWG**). Feed addition and feed leftover were weighed at day 0, feed changes (day 18), and the conclusion of the trial (day 28) to calculate feed intake (**FI**). Body weight gain and FI were used to calculate feed conversion ratio (**FCR**). Mortality was recorded daily. Any culled or dead birds were weighed. Treatment FI and thus FCR were adjusted according to the number of bird d/pen, where bird d is defined as the number of days each bird survived. Dry matter was determined by drying the samples in a drying oven at 100°C for 24 h (Method 934.01, AOAC, 2006). Nitrogen was determined by the combustion method (Method 968.06, AOAC, 2006). Ether extraction was done in Soxhlet apparatus (Method 920.39, AOAC, 2006). Mineral content was determined using inductively coupled plasma–optical emission spectroscopy (AOAC, 2006) following digestion, in turn, in concentrated HNO_3 and HCl.

The analysis of gross energy was done using a bomb calorimeter (Parr adiabatic bomb calorimeter, model 6200, Parr instruments, Moline, IL) using benzoic acid as a calibration standard. Titanium dioxide was analyzed using Short et al. (1996) method. For amino acid analysis, the samples were hydrolyzed in 6 *N* HCl for 24 h at 110°C under N atmosphere. For methionine and cysteine, performic acid oxidation was carried out before acid hydrolysis. The amino acid in the hydrozylate were determined by HPLC after post-column derivatization [Method 982.30 E (a,b,c); AOAC, 2006]. Phytase activity recovered in the diets was analyzed according to modified methods of Engelen et al. (2001). Phytate content of the diets was analyzed using K-PHYT kits from Megazyme (Bray, Ireland) according to manufacturer's recommendations and phytate P was determined as 28.2% of the total phytate.

On day 18, seven birds of average BW/pen were anaesthetized, euthanized by cervical dislocation prior to gizzard and ileal digesta collection. Digesta was obtained from the entire gizzard and the distal half of the ileum (defined as Meckel's diverticulum to the ileocecal junction) and digesta from each section was pooled/section/pen and immediately frozen at –20°C. Digesta was freeze-dried and ground to pass a 1-mm screen. Dried, ground diets, and gizzard and ileal digesta were analyzed for phytate (IP6), phytate esters (IP5, IP4, IP3), and inositol according to methods described in Laird et al. (2016). Ileal digesta was also analyzed for titanium, Ca, total P, Zn, gross energy, and total amino acids, as described previously, for determination of apparent ileal digestibility (**AID**) and digestible nutrient intake.

On day 18, jejunum tissues were obtained from 2 of the birds from which ileal digesta had been collected. The tissues were immediately fixed in RNA later and frozen prior to PCR analysis. The genes analyzed in the jejunum were mucin (MUC2) as well as cationic and neutral amino acid transporters. For cationic amino acids transporters, the following genes were analyzed: CAT-1 [solute carrier (**SLC**) A1]

Table 1. Calculated nutrient content of the basal diets.

	Starter phase		Grower phase	
	Positive control	Negative control	Positive control	Negative control
Ingredient, % of diet (as-fed basis)				
Wheat	46.81	53.43	48.95	55.57
Wheat bran	4.00	4.00	5.00	5.00
Soybean meal	27.49	24.78	23.89	21.18
Rapeseed meal	5.00	5.00	5.00	5.00
Sunflower meal	5.00	5.00	5.00	5.00
Soybean oil	6.57	3.77	7.81	5.01
Salt	0.41	0.31	0.41	0.31
Limestone	0.70	0.98	0.66	0.94
Dicalcium phosphate	2.16	0.85	1.88	0.57
Sodium bicarbonate	0.20	0.20	0.20	0.20
Lysine-HCl	0.33	0.34	0.28	0.29
DL-methionine	0.30	0.26	0.26	0.22
Threonine	0.16	0.14	0.12	0.10
Valine	0.06	0.05	0.03	0.02
Premix[1]	0.50	0.50	0.50	0.50
Titanium dioxide	0.30	0.30		
Inert (wheat/phytase)[2]		0.08		0.08
Xylanase[3]	0.01	0.01	0.01	0.01
Nutrient composition, %				
Crude protein	23.00	22.35	21.50	20.85
ME, kcal/kg	3000.00	2880.00	3100.00	2980.00
Dry matter	88.20	87.62	88.52	87.94
Calcium	0.96	0.74	0.87	0.65
Total phosphorus	0.86	0.61	0.80	0.55
Available phosphorus	0.48	0.28	0.44	0.24
Phytate phosphorus	0.32	0.32	0.32	0.32
Total methionine + cysteine	1.05	1.00	0.96	0.91
Total lysine	1.42	1.36	1.28	1.22
Digestible methionine + cysteine	0.95	0.90	0.87	0.82
Digestible lysine	1.28	1.23	1.15	1.10
Sodium	0.23	0.19	0.23	0.19
Analyzed nutrient composition, %				
Calcium	0.75	0.65	0.77	0.59
Total phosphorus	0.70	0.55	0.71	0.53
Phytate phosphorus	0.33	0.36	0.33	0.34
Crude protein	22.50	23.30	22.40	21.50
Total lysine	1.31	1.30	1.25	1.18
GE, kcal/kg	4171.00	4100.00	4293.00	4167.00

[1]Supplied per kilogram of diet: iron (ferrous sulfate), 80 mg; manganese (manganese oxide), 85 mg; zinc (zinc sulfate), 60 mg; copper (copper sulfate), 12 mg; iodine (iodate), 0.8 mg; selenium (sodium selenite), 0.15 mg; vitamin A (retinyl acetate), 10,000 IU; vitamin D_3 (cholecalciferol), 2500 IU; vitamin E (dl-alpha-tocopherol), 50 mg; vitamin B_{12} (cyanocobalamin), 20 μg; riboflavin, 7.5 mg; niacin, 35 mg; d-pantothenic acid, 12 mg; vitamin K (menadione), 1.5 mg; folic acid, 1.0 mg; vitamin B_6 (pyridoxin-HCl), 3.5 mg; thiamine, 2.0 mg; biotin, 0.2 mg, choline chloride, 460 mg.

[2]Wheat was added in place of phytase in the diets without enzyme supplementation. The phytase used was Quantum Blue (AB Vista, Marlborough, UK) with an expected activity of 5,000 FTU/g.

[3]The xylanase used was Econase XT (AB Vista, Marlborough, UK) with an expected activity of 160,000 BXU/g.

and CAT-2 (SLC7A2). For the neutral amino acids transporters, the following genes were analyzed B^0AT-1 (SLC6A19), LAT-1 (SLC7A5), LAT-4 (SLC43A2), SNAT-1 (SLC38A1), and SNAT-2 (SLC38A2). RNA was extracted from the tissues and total RNA (5 μL) was reverse transcribed onto cDNA using 20 μL RT premix (PrimerDesign, Southampton, UK). The reaction was performed at 55°C for 20 min and 72°C for 10 min. The Gallus gallus gene-specific primers for all the genes of interest (Table 2) were designed by PrimerDesign (Southampton, UK). Quantitative real-time PCR was performed using Stratagene Mx3005p (Agilent Technologies, UK). A total 1 μL of each primer/probe mix was combined with 10 μL Precision 2× Mastermix and 4 μL PCR water (all from PrimerDesign, Southampton, UK) and 5 μL diluted cDNA was used in each reaction. All PCR were performed in duplicate in Stratagene PCR plates (Agilent Technologies, UK) under the following conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Relative target gene expression level was determined by the comparative cycle threshold (**CT**) method (Livak and Schmittgen, 2001). Glyceraldehyde3-phosphate dehydrogenase gene (GAPDH) was used to normalize variations in the amount of mRNA for the target genes. The ΔCT value was calculated as the difference between the CT value of each GAPDH and the average CT value for GAPDH; this value was used to calculate GAPDH fold (i.e., $\Delta CT^{1.97}$). The same mathematical treatment was done for the CT value of the target genes and these values were normalized against the value for GAPDH.

Table 2. GenBank accession number, sequences of forward and reverse primers and fragments sizes used for real-time PCR.

Target	Accession number	Primer sequence	Size (bp)
MUC2	XM_00123458	F: 5′-TCTTTGGGGAAGTGTGTTTGAG-3′; R: 5′-CAGGTCTAAGTCGGGAAGTGTA-3′	103
SLC7A2	EU360448	F: 5′-GTGGACATAATGCTATTGGCACA-3′; R: 5′-GGTTGCTCATAGGTTAAACTGGG-3′	98
SLC7A5	NM_00103057	F: 5′-CTTGGCTTCGTTCAGATTGCA-3′; R: 5′-CACGATGTTCCCCACACCC-3′	93
SLC43A2	XM_415803	F: 5′-CAGCCTCTGTTTATGGCATTAACA-3′; R: 5′-GTCTGTAGCAGACCAGGTAGATTG-3′	121
SLC6A19	XM_419056	F: 5′-GCCTCCCTAATGTGGTATTTCTTC-3′; R: 5′-GCTCTTGGCACATTCTTCTATGTAA-3′	108
SLC38A1	NM_00119960	F: 5′-TGAGCATATGTGTAAGCCAAAGTATG-3′; R: 5′-GCTGTAAATTGGAAGGACCGATG-3′	112
SLC38A2	NM_00103074	F: 5′-TGCCTTCTATATCAAACTAGTCAAGA-3′; R: 5′-CCAGTCATCACAAGTATACCACTTAG-3′	99

Calculations and Statistical Analyses

Percent phytate hydrolysis in the gizzard was calculated using the following equation:

$$\text{Gizzard phytate hydrolysis (\%)} = \frac{[(\sum IP6 - IP3)\,\text{diet} - (\sum IP6 - IP3)\,\text{digesta}]}{(\sum IP6 - IP3)\,\text{diet}} \times 100$$

Percent phytate hydrolysis in the ileum was calculated using titanium ratios in the diets and digesta (Zeller et al., 2015).

$$\text{Ileal phytate hydrolysis (\%)} = \frac{\left[\left(\frac{\sum(IP6-IP3)}{Ti}\right)\text{diet} - \left(\frac{\sum(IP6-IP3)}{Ti}\right)\text{ileal}\right]}{\left(\frac{\sum(IP6-IP3)}{Ti}\right)\text{diet}} \times 100,$$

where $(\frac{\sum(IP6-IP3)}{Ti})$diet = the ratio of the sum of IP6, IP5, IP4, and IP3 in the diet to the concentration of titanium in the diet and $(\frac{\sum(IP6-IP3)}{Ti})$ileal = the ratio of the sum of IP6, IP5, IP4, and IP3 in the ileal digesta to the concentration of titanium in the ileal digesta.

Apparent ileal amino acid digestibility was calculated using titanium ratios in the diets and digesta (Ravindran et al., 1999).

$$\text{AID (\%)} = \frac{\left[\left(\frac{AA}{Ti}\right)\text{diet} - \left(\frac{AA}{Ti}\right)\text{ileal}\right]}{\left(\frac{AA}{Ti}\right)\text{diet}} \times 100,$$

where $(\frac{AA}{Ti})$diet = the ratio of amino acid to chromium in the diet and $(\frac{AA}{Ti})$ileal = the ratio of amino acid to chromium in the ileal digesta.

To calculate digestible nutrient intake in g/day, the following equation was used (Walk et al., 2018b).

$$\text{Digestible intake (g/day)} = \frac{\left[(\text{nutrient,\%})\text{diet} \times \left(\frac{\text{AID nutrient,\%}}{100}\right)\right]}{100} \times \text{daily intake, g}$$

where (nutrient, %) diet = the analyzed nutrient concentration of the diet; and AID nutrient = the previously calculated apparent ileal nutrient digestibility.

Data were analyzed as a one-way analysis of variance using the fit model platform of JMP Pro 14.0 (SAS Institute, Cary, NC). Outliers were determined as 3 times the root mean square error plus or minus the mean of response. Plotting the growth performance, gene expression, AID, or digestible intake data using a normal quantile plot indicated the means were normally distributed. Mortality, phytate, phytate ester, and inositol data were analyzed as non-parametric data using the fit Y by X platform of JMP Pro 14.0 (SAS Institute, Cary, NC). For all parameters, the statistical model included diet and block. When model differences were significant, means were separated using non-orthogonal contrast statements and post-hoc Scheffe's test for significance, to reduce the probability of a type I error (Kaps and Lamberson, 2004). Multivariate pairwise correlations were conducted using the multivariate platform of JMP Pro 14.0 (SAS Institute, Cary, NC). Pen served as the experimental unit for all parameters. Significance was accepted at $P \leq 0.05$ and trends at $P < 0.10$ are discussed.

RESULTS AND DISCUSSION

Diets were formulated to contain high-phytate P at 0.32% and analyzed phytate P content of the diets was between 0.33 and 0.36% (Table 1). In the starter diets, Ca, total P, crude protein, and total lysine content of the PC diet was lower than formulated, which was unexpected and may be due to differences between the

Table 3. Recovered phytase[1] and xylanase[2] activity in the experimental diets.

Experimental diet	Starter phase		Grower phase	
	Phytase, FTU/kg	Xylanase, BXU/kg	Phytase, FTU/kg	Xylanase, BXU/kg
Positive control	< 50	11,400	< 50	11,400
Negative control	< 50	10,700	< 50	10,300
Negative control + 2,000 FTU/kg	2,270	11,600	2,270	11,200
Negative control + 4,000 FTU/kg	5,130	11,200	4,780	10,600

[1]The phytase used was Quantum Blue (AB Vista, Marlborough, UK) with an expected activity of 5,000 FTU/g.
[2]The xylanase used was Econase XT (AB Vista, Marlborough, UK) with an expected activity of 160,000 BXU/g.

Table 4. Influence of phytase supplementation in high-phytate diets on broiler growth performance from hatch to day 18 or day 28 post-hatch.

Response variable[1]	Positive control (PC)	Negative control (NC)	NC + 2000 FTU	NC + 4000 FTU	SEM	ANOVA *P*-value	Non-orthogonal contrast *P*-value[2] PC vs NC	Linear phytase	PC vs NC + 4000 FTU
Hatch to day 18									
Feed intake, g	874	894	903	917	10	< 0.05	NS	NS	< 0.05
BW gain, g	717	716	735	755	9	< 0.05	NS	< 0.05	< 0.05
FCR[3], g:g	1.220	1.248	1.231	1.215	0.01	NS	NS	NS	NS
Mortality, %	0.00	2.67	5.34	2.68	1.4	< 0.10	NS	NS	NS
Hatch to day 28									
Feed intake, g	2,570	2,625	2,605	2,673	28	NS	NS	NS	NS
BW gain, g	1,830	1,813	1,853	1,918	32	< 0.10	NS	NS	NS
FCR[3], g:g	1.404	1.449	1.411	1.396	0.02	NS	NS	NS	NS
Mortality, %	0.00	2.67	6.68	4.02	1.6	< 0.05	NS	NS	NS

[1]Means represent the average response of 10 replicate pens/treatment and 15 birds/pen from hatch to days 18 and 8 birds/pen from days 19 to 28.
[2]Non-orthogonal contrast statements were adjusted using post-hoc Scheffe's test for significance (Kaps and Lamberson, 2004).
[3]Mortality corrected feed conversion ratio.

nutrient content of the ingredients and those employed in the feed formulation. However, the analyzed nutrient contents of the grower diets were as expected. Phytase and xylanase activities recovered in the diets were greater than formulated values (Table 3) and within acceptable ranges when product overages and assay variability are considered.

Overall mortality (hatch to day 28) was 3.34% and not significantly influenced by diet (Table 4). There was no difference in FI, BWG, or FCR of birds fed the PC compared with birds fed the NC (Table 4). These results were not expected as the NC was formulated to be deficient in Ca, P, amino acids, and ME when compared with birds fed the PC. However, the lack of difference between the analyzed nutrients in the starter diets may have contributed to the lack of significant performance differences between birds fed the 2 diets overall. On day 28, birds fed the NC were less efficient by 4.5 points compared with birds fed the PC ($P > 0.10$), and this has been previously reported in birds fed diets deficient in amino acids and/or energy (Ravindran et al., 2001; Olukosi et al., 2008; Walk and Rama Rao, 2018).

Phytase supplementation in the NC diet linearly ($P < 0.05$) increased BWG from hatch to day 18. Birds fed the NC + 4000 FTU/kg of phytase ate ($P < 0.05$) and gained ($P < 0.05$) more than birds fed the PC from hatch to day 18 (Table 4). Previous authors have reported that increasing doses of phytase, up to 10,000 FTU/kg (Augspurger and Baker, 2004), 12,500 FTU/kg (Karadas et al., 2010), or even 24,000 FTU/kg (Cowieson et al., 2006b), continued to improve BWG or FCR of birds fed diets severely reduced in P and when compared with birds fed a nutrient adequate control.

In the current experiment, reducing nutrients in the NC resulted in significant reductions ($P < 0.05$) in the AID of P, crude protein, gross energy, and all amino acids when compared with birds fed the PC (Table 5). While there was no difference in growth performance between the PC or NC, the anti-nutritional effects of high dietary phytate on AID may be exacerbated in diets of marginal nutrient sufficiency due to the negative effects of phytate on endogenous enzyme activity (Yu et al., 2012), endogenous amino acid losses (Cowieson and Ravindran, 2007), and mineral utilization (Cowieson et al., 2006a). Phytase supplementation in the NC diet linearly ($P < 0.10$) increased the AID of Ca, P, crude protein, and all amino acids evaluated, except cysteine or tyrosine (Table 5). In addition, the AID of P was greater ($P < 0.05$) in birds fed the NC + 4000 FTU when compared with birds fed the PC, indicating substantial phytate hydrolysis and uptake of P as phytase dose increased in the diet. The AID of Ca, crude protein, phenylalanine, lysine, arginine, aspartic acid, glutamate, and proline was equivalent to the PC when birds were fed the NC + 4000 FTU/kg phytase (Table 5). Previous authors have also reported an increase in AID of amino acids or minerals as phytase

Table 5. Influence of phytase supplementation in high-phytate diets on apparent ileal nutrient digestibility of 18-D old broilers.

						ANOVA	Non-orthogonal contrast *P*-value[2]		
Apparent ileal nutrient digestibility, %	Positive control (PC)	Negative control (NC)	NC + 2000 FTU	NC + 4000 FTU	SEM	*P*-value	PC vs NC	Linear phytase	PC vs NC + 4000 FTU
Calcium	49.5	41.9	40.6	53.0	3.1	< 0.050	NS	< 0.10	NS
Phosphorus	61.3	42.5	61.9	76.1	3.1	< 0.001	< 0.05	< 0.05	< 0.05
Crude protein	83.0	77.2	78.4	80.5	0.8	< 0.001	< 0.05	< 0.05	NS
Energy	75.7	66.4	67.4	67.5	1.3	< 0.001	< 0.05	NS	< 0.05
Methionine	93.0	89.6	90.7	91.8	0.4	< 0.001	< 0.01	< 0.01	< 0.10
Threonine	81.2	73.5	77.3	78.8	0.9	< 0.001	< 0.01	< 0.05	< 0.05
Valine	84.4	77.3	79.3	81.7	0.7	< 0.001	< 0.01	< 0.01	< 0.10
Isoleucine	85.4	79.2	80.3	82.5	0.7	< 0.001	< 0.01	< 0.05	< 0.10
Leucine	86.2	80.2	81.6	83.8	0.7	< 0.001	< 0.01	< 0.01	< 0.10
Phenylalanine	85.4	80.0	81.2	83.8	0.8	< 0.001	< 0.01	< 0.01	NS
Histidine	86.1	79.5	80.8	83.1	0.7	< 0.001	< 0.01	< 0.01	< 0.05
Lysine	87.3	82.3	83.8	85.6	0.6	< 0.001	< 0.01	< 0.01	NS
Arginine	87.8	83.0	84.2	85.9	0.7	< 0.001	< 0.01	< 0.05	NS
Cysteine	75.1	65.3	67.6	70.2	1.5	< 0.001	< 0.01	NS	NS
Aspartic acid	81.5	73.5	75.6	78.5	1.0	< 0.001	< 0.01	< 0.01	NS
Serine	82.7	74.4	77.3	78.8	0.8	< 0.001	< 0.01	< 0.01	< 0.05
Glutamate	90.5	86.2	87.5	89.1	0.5	< 0.001	< 0.01	< 0.01	NS
Glycine	79.5	70.4	73.1	75.6	1.0	< 0.001	< 0.01	< 0.01	< 0.05
Alanine	83.2	76.7	78.3	80.5	1.1	< 0.001	< 0.01	< 0.01	< 0.10
Tyrosine	86.4	79.5	79.2	71.7	1.0	< 0.001	< 0.01	NS	< 0.05
Proline	86.8	78.3	81.3	84.6	0.7	< 0.001	< 0.01	< 0.05	NS

[1]Means represent the average response of 10 replicate pens/treatment and 7 birds/pen from hatch to day 18.
[2]Non-orthogonal contrast statements were adjusted using post-hoc Scheffe's test for significance (Kaps and Lamberson, 2004).

concentration in the diet increased (Cowieson et al., 2006b). However, the influence of phytase on amino acid digestibility is reported to vary depending on the amino acid, with greater responses reported for amino acids prevalent in endogenous losses, such as cysteine (+4.6%) or threonine (+4.9%) and smaller, less significant responses reported for methionine (+1.0%; Cowieson and Bedford, 2009).

Interestingly, in the current experiment, even with the linear increase in the AID of amino acids with phytase supplementation, the digestibility of methionine, threonine, valine, isoleucine, leucine, histidine, serine, glycine, alanine, and tyrosine of birds fed the NC + 4000 FTU/kg was less ($P < 0.10$) than that of birds fed the PC. Digestibility coefficients are reported to vary greatly between experiments. As a point in time measurement, digestibility coefficients can vary depending on the age of the bird, presence of anti-nutritional factors in the diet and endogenous losses, assay method employed, inert marker, ingredient variability, and nutrient intake (Fan et al., 1994; Ravindran et al., 2005; Bryden and Li, 2010; Olukosi et al., 2012). In addition, Fan et al. (1994) reported dietary amino acid content and subsequently amino acid intake can influence AID. In the current experiment, FI was not different between the PC or NC diet. However, AID was significantly reduced in birds fed the NC compared with birds fed the PC and this resulted in a reduction ($P < 0.10$) of the digestible intake of P, gross energy, methionine, threonine, histidine, cysteine, aspartic acid, serine, glycine, alanine, and proline of birds fed the NC when compared with birds fed the PC (Table 6). Fan et al. (1994) and Ravindran et al. (2005) highlighted that at low amino acid concentrations and subsequent low amino acid intakes, a greater proportion of the amino acids from endogenous sources will be present in the digesta (relative to protein from dietary origin). This increase in the proportion of endogenous losses of birds fed the NC diet, particularly in diets containing high phytate, may have resulted in the lower AID of birds fed the NC diet when compared with birds fed the PC diet.

Phytase supplementation in the NC diet linearly ($P < 0.05$) increased digestible nutrient intake for all nutrients evaluated, except crude protein, gross energy, or isoleucine (Table 6). Furthermore, the digestible intake of most nutrients was not different ($P > 0.10$) between birds fed the PC and birds fed the NC + 4000 FTU/kg, with the exception of a few. For example, digestible methionine, phenylalanine, glutamate, or proline intake was greater ($P < 0.10$), and digestible energy or tyrosine intake was reduced ($P < 0.05$) in birds fed the NC + 4000 FTU/kg compared with birds fed the PC (Table 6). The different responses to phytase supplementation for growth performance (which was greater than the PC) and AID (which was less than the PC) can be attributed to a higher intake and consequently increase in the digestible intake of nutrients and the near complete elimination of phytate with phytase supplementation. Evaluating only nutrient digestibility and not considering nutrient intake may lead to conclusions that, as reported in this trial, do not correlate with animal performance.

For example, there were no differences between the concentration of phytate (IP6), phytate esters (IP5, IP4 or IP3), the sum of the IP6 or phytate esters, or percent IP6-IP3 hydrolysis, or inositol concentration in the gizzard or ileal digesta of birds fed the NC when compared with birds fed the PC (Table 7). However, phytase

Table 6. Influence of phytase supplementation in high-phytate diets on apparent digestible nutrient intake of 18-D old broilers.

						ANOVA	Non-orthogonal contrast *P*-value[2]		
Digestible nutrient intake[1], g/d	Positive control (PC)	Negative control (NC)	NC + 2000 FTU	NC + 4000 FTU	SEM	*P*-value	PC vs NC	Linear phytase	PC vs NC + 4000 FTU
Calcium	0.18	0.14	0.12	0.18	0.01	< 0.01	NS	< 0.05	NS
Phosphorus	0.21	0.12	0.16	0.22	0.01	< 0.001	< 0.01	< 0.01	NS
Crude protein	8.99	8.98	9.15	9.44	0.13	< 0.10	NS	NS	NS
Energy, kcal/d	298	256	267	271	5.94	< 0.001	< 0.01	NS	< 0.05
Methionine	0.26	0.25	0.26	0.27	0.00	< 0.001	< 0.05	< 0.01	< 0.10
Threonine	0.36	0.32	0.34	0.36	0.01	< 0.001	< 0.01	< 0.01	NS
Valine	0.43	0.41	0.42	0.45	0.01	< 0.01	NS	< 0.01	NS
Isoleucine	0.38	0.37	0.36	0.38	0.01	< 0.10	NS	NS	NS
Leucine	0.65	0.62	0.63	0.66	0.01	< 0.01	NS	< 0.05	NS
Phenylalanine	0.40	0.41	0.41	0.44	0.01	< 0.001	NS	< 0.05	< 0.01
Histidine	0.22	0.21	0.21	0.23	0.00	< 0.001	< 0.05	< 0.01	NS
Lysine	0.55	0.53	0.54	0.57	0.01	< 0.01	NS	< 0.01	NS
Arginine	0.59	0.58	0.59	0.61	0.01	< 0.10	NS	< 0.05	NS
Cysteine	0.13	0.12	0.12	0.13	0.00	< 0.05	< 0.05	< 0.05	NS
Aspartic acid	0.79	0.72	0.74	0.80	0.00	< 0.001	< 0.01	< 0.01	NS
Serine	0.42	0.37	0.40	0.41	0.01	< 0.001	< 0.01	< 0.01	NS
Glutamate	1.95	1.89	1.93	2.05	0.02	< 0.001	NS	< 0.01	< 0.05
Glycine	0.36	0.33	0.34	0.36	0.01	< 0.001	< 0.01	< 0.01	NS
Alanine	0.36	0.33	0.35	0.36	0.01	< 0.01	< 0.10	< 0.05	NS
Tyrosine	0.24	0.24	0.22	0.22	0.00	< 0.001	NS	< 0.05	< 0.01
Proline	0.54	0.43	0.48	0.56	0.01	< 0.001	< 0.01	< 0.01	< 0.10

[1]Means represent the average response of 10 replicate pens/treatment and 7 birds/pen from hatch to day 18.
[2]Non-orthogonal contrast statements were adjusted using post-hoc Scheffe's test for significance (Kaps and Lamberson, 2004).

Table 7. Influence of phytase supplementation in high-phytate diets on phytate, phytate esters and inositol concentration in the gizzard and ileal digesta of 18-day old broilers.

						ANOVA	Non-orthogonal contrast *P*-value[2]		
Digesta location[1]	Positive control (PC)	Negative control (NC)	NC + 2000 FTU	NC + 4000 FTU	SEM	*P*-value	PC vs NC	Linear phytase	PC vs NC + 4000 FTU
Gizzard concentration, μmol/g									
IP6	6.79	6.90	0.06	0.01	0.43	< 0.001	NS	< 0.01	< 0.01
IP5	1.08	1.10	0.05	0.03	0.07	< 0.001	NS	< 0.01	< 0.01
IP4	0.41	0.41	0.62	0.28	0.06	< 0.01	NS	NS	NS
IP3	0.53	0.42	0.62	0.51	0.05	< 0.05	NS	NS	NS
Sum of IP6-IP3	8.82	8.83	1.36	0.83	0.48	< 0.001	NS	< 0.01	< 0.01
Total hydrolysis,[3] %	57.95	55.85	93.81	96.13	1.18	< 0.001	NS	< 0.01	< 0.01
Total IP6 hydrolysis, %	59.60	55.58	99.64	99.91	1.01	< 0.001	< 0.10	< 0.01	< 0.01
Inositol	1.09	1.38	2.36	2.84	0.23	< 0.001	NS	< 0.01	< 0.01
Ileal digesta concentration, μmol/g									
IP6	59.79	58.27	12.60	5.24	1.64	< 0.001	NS	< 0.01	< 0.01
IP5	6.03	4.88	4.60	1.73	0.33	< 0.001	NS	< 0.01	< 0.01
IP4	4.69	4.11	9.75	8.35	0.62	< 0.001	NS	< 0.01	< 0.01
IP3	0.61	0.57	2.40	2.45	0.18	< 0.001	NS	< 0.01	< 0.01
Sum of IP6-IP3	71.11	67.83	29.35	17.77	2.31	< 0.001	NS	< 0.01	< 0.01
Total hydrolysis[3], %	14.06	16.77	51.41	70.66	4.65	< 0.001	NS	< 0.01	< 0.01
Total IP6 hydrolysis, %	12.23	14.68	72.75	88.31	3.34	< 0.001	NS	< 0.01	< 0.01
Inositol	10.19	11.76	32.48	33.47	1.02	< 0.001	NS	< 0.01	< 0.01

[1]Means represent the average response of 10 replicate pens/treatment and 7 birds/pen from hatch to d18.
[2]Non-orthogonal contrast statements were adjusted using post-hoc Scheffe's test for significance (Kaps and Lamberson, 2004).
[3]Total hydrolysis of IP6, IP5, IP4, and IP3.

supplementation in the NC diet linearly ($P < 0.01$) reduced IP6, IP5, and the sum of the IP ester concentrations in the gizzard and the ileum and increased (linear, $P < 0.01$) the percent IP6-IP3 hydrolysis and inositol. The concentration of IP6, IP5, and the sum of the IP esters in both the gizzard and ileal digesta was lower ($P < 0.01$) in birds fed NC + 4000 FTU/kg compared with birds fed the PC. Which increased ($P < 0.01$) the percent IP6 to IP3 hydrolysis and inositol concentration ($P < 0.01$) in birds fed the NC + 4000 FTU/kg of phytase when compared with birds fed the PC. There was no effect of diet on the concentration of IP4 or IP3 in the gizzard. In the ileal digesta, the concentration of IP4 and IP3 linearly ($P < 0.05$) increased as phytase dose increased in the NC diet, this was greater in birds fed the NC + 4000 FTU/kg when compared with birds fed the PC. Previous authors have reported significant reductions in IP6 and phytate ester concentration throughout the gastrointestinal tract as phytase dose increased in the diet (Walk et al., 2014, 2018; Sommerfeld et al., 2018). Sommerfeld et al. (2018) reported IP6 disappearance in the ileum as great as 94% with 3,000

Table 8. Influence of phytase supplementation in high-phytate diets on expression of genes in the jejunum of 18-day-old broilers.

Jejunum gene expression[1]	Positive control (PC)	Negative control (NC)	NC + 2000 FTU	NC + 4000 FTU	SEM	ANOVA *P*-value	Non-orthogonal contrast *P*-value[2] PC vs NC	Linear phytase	PC vs NC + 4000 FTU
Mucin									
MUC2	1.39	1.27	1.38	1.56	0.18	NS	NS	NS	NS
Neutral amino acid transporters (SNAT1, 2, LAT4, BOAT1, LAT1)									
SLC38A1 (SNAT-1)	1.60	1.14	1.76	2.16	0.20	< 0.10	NS	< 0.01	NS
SLC38A2 (SNAT-2)	1.24	0.97	1.43	1.51	0.22	NS	NS	NS	NS
SLC7A5 (LAT-1)	1.18	0.74	1.07	0.91	0.15	NS	NS	NS	NS
SLC43A2 (LAT-4)	1.21	1.13	1.51	1.63	0.12	< 0.05	NS	< 0.05	< 0.10
SLC6A19 (B(O)AT1)	1.56	1.23	1.82	1.85	0.20	= 0.10	NS	NS	NS
Cationic amino acid transporters (CAT1, 2)									
SLC7A1 (CAT-1)	1.10	1.24	1.32	1.13	0.14	NS	NS	NS	NS
SLC7A2 (CAT-2)	1.22	0.91	1.05	1.10	0.14	NS	NS	NS	NS

[1]Means represent the average response of 10 replicate pens/treatment and 2 birds/pen from hatch to day 18.
[2]Non-orthogonal contrast statements were adjusted using post-hoc Scheffe's test for significance (Kaps and Lamberson, 2004).

FTU/kg of phytase in diets containing 0.26% phytate P, and this is similar to the reported 88% IP6 hydrolysis in the current experiment. These results confirm that substantial phytate hydrolysis is achieved as phytase dose in the diet increases. These reductions in phytate and phytate esters concentration, particularly in the gizzard where phytate is soluble and can bind with nutrients, will have contributed to the improvements in digestible nutrient intake and improvement in BWG of broilers fed phytase.

In the current experiment, there were no differences in the expression of mucin or amino acid transporter genes in birds fed the PC or NC diet (Table 8). Phytase supplementation of the NC diet linearly ($P < 0.05$) increased the expression of the neutral amino acid transporters SNAT-1 and LAT-4. In addition, LAT-4 expression in birds fed the NC + 4000 FTU/kg was greater ($P < 0.10$) than that of birds fed the PC (Table 8). LAT-4 is a ubiquitous uniporter found along the small intestinal villi in mice and is responsible for absorption of essential amino acids such as leucine, isoleucine, valine, phenylalanine, and methionine (Guetg et al., 2015). The increased expression of LAT-4 as phytase dose increased may indicate that methionine was the most limiting nutrient in the diet. Particularly, since the effect of phytase on methionine digestibility or utilization is much lower than that of other amino acids (Cowieson and Bedford, 2009; Walk and Rama Rao, 2018). While phytase supplementation might have supplied some methionine to the bird, it was most likely not enough to balance the phytase-related effect on other amino acids, such as threonine or cysteine. It is possible that this imbalance in methionine supply stimulated an increase in LAT-4 expression and uptake of methionine, resulting in an improvement in gain. This would have been further supported by the increase in glutamate uptake from SNAT-1. Glutamate/glutamine is the most abundant amino acid in the body and is involved in many processes such as synthesis of amino acids and proteins, gluconeogenesis, cell signaling, and as an energy source for enterocytes (Pochini et al., 2014; Watford, 2015). Glutamate is also an amino acid that appears to be negatively influenced by phytate in the small intestine. For example, Onyango et al. (2008) reported that glutamate and leucine uptake in the jejunum were reduced by 6 or 18%, respectively, as phytate concentration increased from 0 to 500 mM. Supplementation of glutamine into broiler diets significantly improved BWG and increased intestinal villi height of 21-day-old broilers (Bartell and Batal, 2007). However, other authors reported significant reductions in BWG of broilers under heat stress and fed supplemental glutamine (Shakeri et al., 2014). In the current trial, digestible intake of glutamine and methionine were significantly greater in birds fed the NC + 4000 FTU/kg of phytase when compared with birds fed the PC. The increased availability of methionine and glutamine from phytase supplementation and up-regulated transporter expression, thereby aided the bird toward a more efficient use of amino acids digested from the diet and facilitated the improvement in day 18 BWG of birds fed diets deficient in essential amino acids and protein. Multivariate correlations support this hypothesis with a strong and significant relationship between BWG from hatch to day 18 and digestible methionine intake (r = 0.72, $P < 0.0001$; Figure 1) with a non-significant relationship between BWG from hatch to day 18 and AID of methionine (r = –0.26, $P = 0.12$). Similarly, for glutamate, there is a significant relationship between BWG from hatch to day 18 and digestible glutamate intake (r = 0.76, $P < 0.0001$; Figure 2) with a non-significant relationship between BWG from hatch to day 18 and AID of glutamate (r = –0.23, $P = 0.18$). Similar relationships between BWG and digestible amino acid intake (r = 0.33 to 0.72, $P < 0.10$), except digestible tyrosine intake (r = 0.20, $P = 0.23$) or BWG and AID of amino acids (r = –0.29 to –0.14, $P > 0.10$) were found for all other measured amino acids (data not reported). The non-significant relationships between AID and BWG suggest AID may not be the most representative factor when describing the influence of phytase or phytate on amino acid utilization. Walk et al. (2018) reported significant improvements in the AID of amino acids of poultry fed novel proteases in the absence of improvements in growth performance.

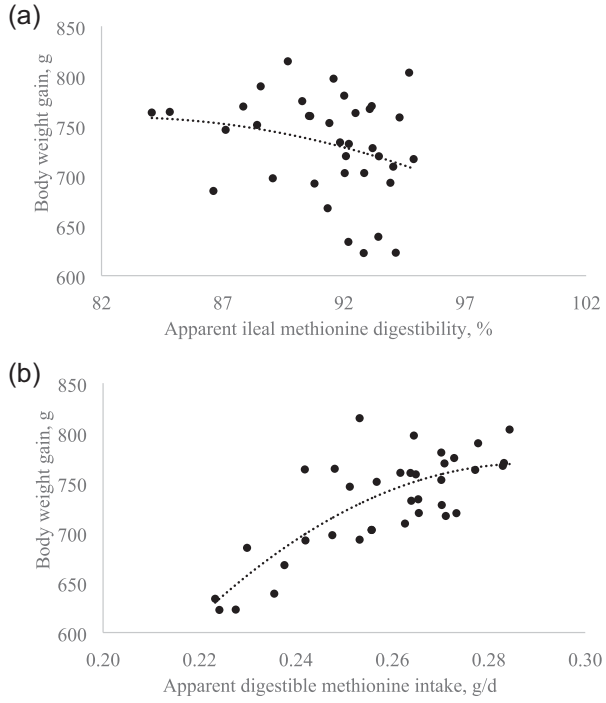

Figure 1. Scatterplot of body weight gain, g, of broilers from hatch to day 18 and apparent ileal (AID) methionine digestibility, %, $y = -1579.9 + 56.61x - 0.3426x^2$, r = –0.26, $P = 0.12$ (a) or digestible methionine intake, g/d, $y = -1982.7 + 19108x - 33,166x^2$, r = 0.72, $P < 0.0001$ (b).

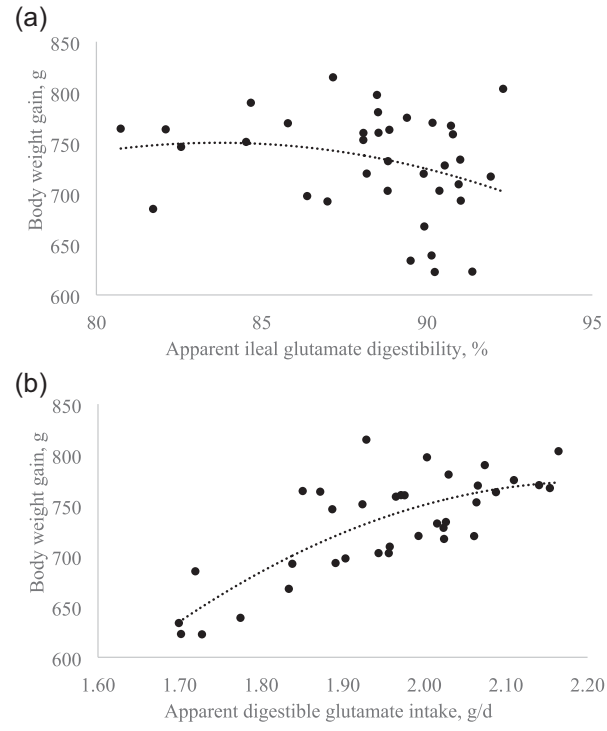

Figure 2. Scatterplot of body weight gain, g, of broilers from hatch to day 18 and apparent ileal (AID) glutamate digestibility, %, $y = -3806.9 + 108.9x - 0.6506x^2$, r = -0.23, $P = 0.18$ (a) or apparent digestible glutamate intake, g/d, $y = -1844.2 + 2371.7x - 537.2x^2$, r = 0.76, $P < 0.0001$ (b).

When the digestible amino acid intake was determined, it was apparent that even with the improvements in AID, the digestible amino acid intake of birds fed novel proteases was still significantly less than that of the PC. Based on this information, it could be concluded that digestible nutrient intake may be a better indicator of animal performance compared with AID. Determination of digestibility is a useful tool to extrapolate nutrient utilization by the animal, but it is only useful if it corresponds in similar fashion to the growth performance as digestibility is a point in time measurement and can be highly variable. To allow for the best interpretation of the results, the use of nutrient digestibility should be presented with animal performance data and this appears particularly relevant for the effects of enzymes on amino acid digestibility or endogenous losses and particularly relevant when digestibility results are used to support nutrient equivalence by additives use such as phytase.

In conclusion, supplementing high-phytate, low-nutrient dense broiler diets with increasing doses of phytase improved BWG. Phytase supplementation up to 4,000 FTU/kg resulted in approximately 96% phytate and phytate ester hydrolysis within the gizzard. This resulted in an improvement in the AID and digestible nutrient intake of minerals and amino acids. Apparent ileal nutrient digestibility may not be the most appropriate parameter when evaluating phytase or phytate due to their effects on endogenous losses, amino acid uptake, intake, and post-absorptive utilization in the tissues. Digestible nutrient intake may be a better indicator of nutrient utilization and predictor of animal performance, particularly when describing nutrients, such as amino acids, which are heavily influenced by phytate and phytase.

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