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Effect of phytase on growth performance, phytate degradation and gene expression of *myo*-inositol transporters in the small intestine, liver and kidney of 21 day old broilers

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Running head: Superdoses of phytase and *myo*-inositol transporters

ABSTRACT An experiment was conducted to evaluate phytase on growth performance, phytate degradation and the gene expression of *myo*-inositol transporters in 21-day old broilers. Ross 308, male broilers (n = 240) were obtained and assigned to one of four diets, with 10 pens/diet and six birds/pen from day one to 21. The diets consisted of a negative control (NC) formulated to meet or exceed Ross 308 nutrient requirements, with the exception of calcium (Ca) and available P (avP), which was reduced by 0.16 and 0.15%, respectively. The NC diet was supplemented with 0, 500, 1,500 or 4,500 FTU/kg of phytase to create four experimental diets. On day 21, all birds per pen were euthanized to obtain digesta and tissue samples for phytate degradation and gene expression. Data were analysed as an analysis of variance using the fit model platform in JMP v 13.0. The model included phytase and significant means were separated using orthogonal linear and quadratic contrasts. Phytase supplementation increased gain (linear, $P < 0.05$) but had no effect on feed intake or feed conversion ratio. Phytate (IP6; quadratic, $P < 0.05$), phytate ester (IP5, IP4, IP3; quadratic, $P < 0.05$) and inositol (linear, $P < 0.05$) concentration in the gizzard was influenced by phytase supplementation. Phytase supplementation decreased IP6 (linear, $P < 0.05$) and IP5, IP4, IP3 (linear or quadratic, $P < 0.05$) and increased inositol (quadratic, $P < 0.05$) concentration in the ileal digesta. The expression of the H^+-dependent *myo*-inositol transporter, HMIT, was decreased (linear, $P < 0.05$) in the kidney and increased (linear, $P < 0.05$) in the ileum as phytase dose increased. Expression of the sodium-dependent *myo*-inositol transporter, SMIT2, increased in the liver (quadratic, $P < 0.10$) and the jejunum (quadratic, $P < 0.05$) as phytase dose increased. Intestinal alkaline phosphatase expression increased in the ileum (linear, $P < 0.05$) as phytase dose increased. The influence of phytase on phytate, phytate esters and inositol may influence intestinal alkaline phosphatase activity and the expression of *myo*-inositol transporters in the small intestine and kidney.

Key words: broiler, gene expression, *myo*-inositol, phytase, phytate

Introduction

Data evaluating the efficacy of phytase in poultry nutrition, to liberate phytate-bound phosphorus is readily available and spans a period of more than 50 years (Nelson, 1967; Dersjant-Li et al., 2015). Recent interest in supplementing poultry diets with higher doses of phytase, sometimes referred to as "superdoses" of phytase, has led to further understanding of phytate hydrolysis and reported benefits in feed conversion (Walk et al., 2013, 2014). These benefits are thought to be predominantly associated with the near complete destruction of phytate (iP6) and lower phytate esters (iP5, iP4, iP3) in the proximal gastrointestinal tract, alleviation of their antinutritional properties (Bedford and Walk, 2016), and the provision of *myo*-inositol (Walk et al., 2014; Cowieson et al., 2015; Lee and Bedford, 2016).

Myo-inositol is considered an essential constituent of cellular phosphoinositides and is involved in many cellular functions, such as insulin sensitivity, lipid metabolism, and cell survival, structure and growth (Huber, 2016). *Myo*-inositol can be synthesised in the body from glucose, released from cellular phospholipids, and absorbed in the intestinal tract from the diet (Huber, 2016). Free *myo*-inositol can be actively transported with high efficiency via three co-transport systems, two are sodium dependent (SMIT1 or SLC5A3 and SMIT2 or SLC5A11) and one is proton dependent (HMIT or SLC2A13; Aouameur et al., 2007). Using rabbits and rats, previous studies have demonstrated that the expression of each cotransport system is variable between the tissues; SMIT1 is primarily expressed in the brain and renal medulla, SMIT2 is expressed in the brain, intestine, and renal cortex and HMIT is predominantly expressed in the brain (Aouameur et al., 2007; Huber, 2016) with lower levels found in white and brown adipose tissues and the kidney (Mueckler and Thorens, 2014).

The location and expression of these cotransport systems in the various tissues may indicate the importance of *myo*-inositol on cellular metabolism and function. Evaluation of the expression of *myo*-inositol cotransport systems in tissues may help to further elucidate the

beneficial effects of *myo*-inositol provision through phytate destruction from superdoses of phytase. Therefore, the objective of this trial was to determine the influence of superdoses of phytase on broiler performance, mineral digestibility, specifically Ca, P, Na and K, the concentration of iP6, iP5, iP4, iP3, iP2, and *myo*-inositol in the gizzard and ileum, and the expression of the *myo*-inositol cotransporters in the kidney, liver and small intestine in 21-day old broilers.

M**aterials and Methods**

All animal care procedures used in this experiment were approved by the Scotland's Rural College Animal Experiment Committee (SRUC) before initiation of the experiment.

Animals and Management Practices

Two-hundred and forty male Ross 308 commercial broiler chicks were obtained and allocated to four dietary treatments in a randomized complete block design with six chicks per cage and 10 replicate cages per treatment. Birds were housed at the SRUC poultry farm in thermostatically-controlled brooder battery cages with raised-wire floors with a lighting program of 23L:1D from hatch to day 7 and 14L:10D for the remainder of the 21-day trial. Temperature in the battery cages was maintained at 32°C for the first day of the study and decreased to 21°C by day 21.

Experimental Diets

Chicks were fed one of four dietary treatments that consisted of a low Ca and avP basal diet supplemented with 0, 500, 1,500 or 4,500 FTU/kg of phytase (Table 1). The phytase was a third generation microbial phytase (Quantum Blue, AB Vista, Marlborough, Wiltshire, UK) with an expected activity of 5,000 FTU/g. All diets were formulated to meet Ross 308 nutrient recommendations, with the exception of Ca and avP, which were reduced by 0.16 and 0.15%, respectively (Table 1). Titanium dioxide was included in all diets at 0.5% as an indigestible marker to permit calculation of nutrient digestibility by the index

method. Access to feed and water was provided *ad libitum* throughout the 21-d feeding period. Feed was fed in mash form via a feed trough and water was provided via a nipple and cup drinker.

Measurements

Chicks were weighed and randomly allotted such that average initial group weights were distributed similarly across dietary treatments. Birds were monitored daily for morbidity and mortality throughout the study. Dead or culled birds were recorded and these values were used to adjust FI and FCR according to the number of bird days. At the end of the 21-day feeding period, all birds and feeders were weighed to determine BWG, FI, and calculate FCR.

Collection and Analyses

On day 21, four birds per cage were euthanized by injection of pentobarbital and gizzard and ileal digesta were collected by gently flushing the entire gizzard contents and the terminal ileum (30 cm proximal to the ileo-cecal junction) with deionized water. The digesta samples were pooled per section per cage and immediately frozen (-20°C) for later analysis. Frozen gizzard and ileal digesta samples were lyophilized and ground using a 1 mm screen prior to mineral and phytate ester analyses.

Quantification of inositol phosphates in gizzard and ileal digesta samples were determined with a modified method from Kwanyuen and Burton (2005) using high-performance liquid chromatography. Freeze dried samples were extracted with 10 mL of 0.5 M HCl for 1 h at 20°C by ultrasonication. The extracts were then centrifuged for 10 minutes at 2,200 × g, and 5 mL of the supernatant was evaporated to dryness in a vacuum centrifuge. The samples were then re-dissolved in 1 mL of distilled, deionized water by ultrasonication for 1 h at 20°C and centrifuged for 15 minutes at 18,000 × g. The resulting supernatant was filtered through a 13-mm syringe filter with a 0.45 µm membrane (GH Polypro Acrodisc®,

Pall Corporation, Ann Arbor, MI) and placed in a 30 kDa centrifugal filter (Microcon® Ultracel YM-30, Millipore Corporation, Bedford, MA) and finally centrifuged for 30 minutes at 9,000 × g. The samples were then analysed for inositol phosphate moieties (iP2–iP6) using a standard HPLC analytical column (4 × 250 mm CarboPac PA1 column, Thermo Scientific, Sunnyvale, CA). Phytic acid dodecasodium salt hydrate (Sigma-Aldrich, St. Louis, MO) was used as the standard for both iP6 and the lower iP esters to calculate the ratio between peak area and concentration of iP6 and lower esters in nmol/g in the isolated digesta fractions.

Titanium dioxide concentrations of diet and ileal digesta were determined following the procedures of Short et al. (1996). Duplicate samples were weighed into crucibles, dried at 105°C for 24 h, and subsequently ashed at 550°C for 24 h. The ashed samples were then dissolved in 7.4 M sulfuric acid. Hydrogen peroxide (30% vol./vol.) was subsequently added to produce a yellow color with an intensity proportional to the titanium dioxide concentration in each sample. Duplicate aliquots of these sample solutions were analyzed using a UV spectrophotometer by measuring the absorbance at 410 nm. Calcium, total P, Na and K were analysed in the diet and ileal digesta samples using Inductively Coupled Plasma – Optical Emission Spectroscopy (AOAC Method 990.08; AOAC, 2006) following digestion, in turn, in concentrated HNO_3 and HCl.Apparent nutrient digestibility (AND, %) was calculated according to the following equation: $AND = [1- [(M_i / M_o) \times (X_o / X_i)] *100$,

where M_i = concentration of TiO_2 (marker) of the diet sample,

M_o = concentration of TiO_2 (marker) of the ileal digesta,

X_o = nutrient concentration of the ileal digesta sample,

X_i = nutrient concentration of the diet sample.

The remaining 2 birds per cage were euthanized by a lethal injection of pentobarbital to permit collection of tissue samples. An incision was made below the sternum to expose the abdominal cavity as previously described (Olukosi and Dono, 2014). The entire liver and

kidney and sections of the jejunum and ileum were collected from each bird, stored in RNAlater and frozen until PCR analyses.

The genes analysed in the liver and kidney were sodium/glucose cotransporter 11 (SLC5A11 or SMIT2); sodium *myo*-inositol cotransporter (SLC5A3 or SMIT1) and H+/*myo*-inositol transporter (SLC2A13 or HMIT). The genes analysed in the intestine were the three listed previously as well as intestinal alkaline phosphatase (ALPI).

RNA were extracted from the tissues and total RNA (5 µl) was reverse-transcribed onto cDNA using 20µl 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 Techhonologies, UK). 1µl of each primer/probe mix was combined with 10µl Precision 2× Mastermix and 4µl PCR water (all from PrimerDesign, Southampton, UK). 5µl diluted cDNA was used in each reaction. All PCR were performed in duplicate in Stratagene PCR plates (Agilent Techhonologies, 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 (**C_T**) method (Livak and Schmittgen, 2001). Glyceraldehyde-3-phosphate dehydrogenase gene (**GAPDH**) was used to normalize variations in the amount of mRNA for the target genes. The ΔC_T value was calculated as the difference between the C_T value of each GAPDH and the average C_T value for GAPDH, this value was used to calculate GAPDH fold (i.e. $\Delta C_T^{1.97}$). The same mathematical treatment was done for the C_T value of the target genes and these values were normalized against the value for GAPDH.

Statistical Analyses

Cage served as the experimental unit for all parameters. Performance, apparent ileal digestibility and phytate and phytate ester data are presented as least square means per treatment group. Gene expression data are presented as the relative fold change when compared to the housekeeping gene, GADPH. All data were analysed as analysis of variance using the fit model platform in JMP Pro v 13.0 (SAS Institute, Cary, NC). The model included phytase and means were separated using linear and quadratic orthogonal polynomial contrasts. Statistical significance was considered when $P \leq 0.05$ and trends discussed at $P \leq 0.10$.

Results

Phytase activity recovered in the experimental diets was higher than expected at < 50, 907, 2,050, and 6,120 FTU/kg for 0, 500, 1,500 and 4,500 FTU/kg, respectively. Overall mortality was 5%. Analysed total P, Ca, Na and CP are presented in Table 1 and were within the expected levels for all the diets. Overall feed intake or feed conversion ratio were not influenced by phytase dose (Table 3). Body weight gain from hatch to 21-days post-hatch increased (linear, $P < 0.05$) as phytase dose increased from 0 to 4,500 FTU/kg (Table 3).

Apparent ileal digestibility of dry matter (linear, $P < 0.05$) and Ca (quadratic, $P < 0.05$) decreased and P (quadratic, $P < 0.05$) and Na (quadratic, $P < 0.05$) increased as phytase dose increased from 0 to 4,500 FTU/kg (Table 4). Increasing phytase dose from 0 to 4,500 FTU/kg decreased (quadratic, $P < 0.05$) the concentration of iP6 and iP5 in the gizzard digesta (Table 5). The concentration of iP4, iP3 and iP2 in the gizzard digesta increased and then decreased (all quadratic, $P < 0.05$) as phytase supplementation in the diet increased from 0 to 4,500 FTU/kg (Table 5). Inositol concentration increased (linear, $P < 0.05$) in the gizzard digesta as phytase supplementation increased in the diet (Table 5). In the ileal digesta, the concentration of iP6 (linear, $P < 0.05$) decreased and iP5 (quadratic, $P < 0.05$) and iP4 (quadratic, $P < 0.05$) increased and then decreased as phytase dose increased in the

diets (Table 6). In contrast to the other phytate esters, the concentration of iP3 (linear, $P < 0.05$) and inositol (quadratic, $P < 0.05$) increased as phytase dose in the diet increased to 4,500 FTU/kg (Table 6). There was no effect of phytase dose on the concentration of iP2 in the ileal digesta.

The relative changes in the gene expression of inositol transporters and intestinal alkaline phosphatase in the jejunum and ileum are presented in Table 7. In the jejunum, increasing phytase dose from 0 to 4,500 FTU/kg up-regulated the relative expression of SLC5A11 (quadratic, $P < 0.05$), tended to up-regulate the relative expression of SLC5A3 (quadratic, $P = 0.10$), and there was a tendency ($P < 0.10$) for phytase to up-regulate the relative expression of SLC2A13. There was no effect of phytase dose on the relative expression of iALP in the jejunum. In the ileum, the effect of phytase dose approached significance ($P < 0.06$) towards an up-regulation of the relative expression of SLCA13 (linear, $P < 0.05$) and significantly increased the expression of iALP (linear, $P < 0.05$). There was no effect of phytase dose on the relative expression of SLC5A11 or SLC5A3 in the ileum. The effect of phytase dose on gene expression of *myo*-inositol transporters in the liver and kidney were not significant (Table 8).

Discussion

Growth performance of broilers at the conclusion of the trial was 30-39% below Ross 308 standards (Ross 308 Broiler Performance Objectives, 2012) and this may be associated with the use of mash diets (Kilburn and Edwards, 2001). Body weight gain and P digestibility increased as phytase supplementation increased and this has been previously reported in low avP diets supplemented with 0 to 12,500 FTU/kg (Karadas et al., 2010) or 0 to 24,000 FTU/kg (Cowieson et al., 2006) of phytase indicating further benefits in nutrient digestibility and growth are attainable with higher doses of phytase.

Contradictory to previously published research (Walk et al., 2013, 2014), there was no significant effect of phytase dose on FCR in the current trial. Numeric improvements in FCR were noted however, with the highest dose of phytase improving feed efficiency by approximately 14%. Mechanisms by which high doses of phytase elicit beneficial effects on performance are proposed to be related to 1) destruction of the anti-nutritive effects of phytate with generation of more soluble lower phytate esters and 2) generation of *myo*-inositol (Cowieson et al., 2011). Phytate, phytate ester, and inositol concentrations in the gizzard and ileal digesta in the current experiment would partially support the above proposed mechanisms of superdosing. For example, in the gizzard and ileal digesta the concentration of iP6 decreased and the concentration of iP5 and iP4 increased and then decreased, while iP3 and *myo*-inositol concentration increased as phytase supplementation increased in the diet and this has been previously reported (Walk et al., 2014; Beeson et al., 2017). Using *in vitro* models, other authors have reported that phytate, as well as the lower phytate esters, have the capacity to bind minerals and to interfere with pepsin activity, particularly as pH increases, as summarised by Bedford and Walk (2016). While phytate is considered a more potent anti-nutrient than the lower esters, the anti-nutritive effects of these lower esters on minerals (Xu et al., 1992) and pepsin (Yu et al., 2012) requires further consideration, and continued reduction of these phytate esters with high doses of phytase may be a factor contributing to the increase in BWG and numeric improvements in FCR.

In addition, the continued destruction of phytate and the lower phytate esters as phytase dose increased also resulted in significant increases in *myo*-inositol in the gizzard and ileal digesta. *Myo*-inositol is an important component of cellular phospholipids and is involved in many cellular functions including survival, structure and signalling (Huber, 2016). Previous authors have loosely correlated an increase in *myo*-inositol concentrations in the gizzard with significant improvements in FCR (Walk et al., 2014). Cowieson et al.

(2013) reported supplementation of broiler diets with 0.15% *myo*-inositol resulted in significant improvements in FCR of 42-day old broilers. Others have also reported significant increases in plasma *myo*-inositol as phytase supplementation increased in the diet (Cowieson et al., 2015; Laird, 2016). Therefore, it is likely one of the beneficial effects of feeding high doses of phytase would be the provision of *myo*-inositol through phytate and phytate ester destruction.

Free *myo*-inositol in the gastrointestinal tract is absorbed with great efficiency, 99.8% (Holub, 1986; Croze and Soulage, 2013) by an active, Na-dependent process. Sodium is transported across the brush border together with *myo*-inositol via SLC5A11 (SMIT2) at a ratio of 2 Na to 1 *myo*-inositol (Huber, 2016). In the current trial, the expression of SLC5A11 was up-regulated in the jejunum and the apparent ileal Na digestibility was significantly increased as phytase supplementation increased in the diet. In addition, at least in the jejunum, there was also a tendency toward an up-regulation of both SLC5A3 (SMIT1) and SLC2A13 (HMIT) as dietary phytase increased from 0 to 4500 FTU/kg, but there was no effect on the gene expression of iALP. In contrast, in the ileum there was no effect of phytase on SLC5A11 (SMIT2) or SLC5A3 (SMIT1) and a tendency for a linear increase in SLC2A13 (HMIT) with a significant increase in iALP expression. These results are interesting, especially when considering previous authors reported the expression of SLC5A3 (SMIT1) and SLC2A13 (HMIT) are most noted in the brain and/or kidney with SMIT2 predominantly found in the small intestine (Aouameur et al., 2007; Mueckler and Thorens, 2013; Huber, 2016). However, species differences exist in *myo*-inositol transporter expression in the tissues, with SMIT2 being expressed in high concentration in the kidney of both rats and rabbits, but barely detectable in the small intestine of rabbits (Aouameur et al., 2007).

In the current experiment, the expression of *myo*-inositol transporters was not measured in the brain and more work is needed to confirm the effects reported in herein, particularly in poultry. Regardless, a few interesting points can be discussed based on the gene expression data, specifically:

1) M*yo*-inositol appears to be actively transported in the small intestine and transporter expression is influenced by *myo*-inositol concentration, with an up-regulation of the gene expression of SMIT2 or HMIT in the jejunum and ileum as phytase dose and production of *myo*-inositol increased. *Myo*-inositol uptake in the brush border vesicles of rats has been previously reported through SMIT2 with no evidence of uptake from HMIT or SMIT1 (Aouameur et al., 2007). However, as previously mentioned, these same authors reported barely any SMIT2 detection in the rabbit intestine, indicating species differences exist and these results need to be confirmed in subsequent trials. Regardless, it would appear there is an effect of *myo*-inositol concentration in the intestinal lumen on the up-regulation of transporters in the jejunum of poultry.

2) In the ileum however, only HMIT expression was up-regulated as phytase dose increased. This could also be related to the concentration of *myo*-inositol present in the ileum but also due to the reduction of Na concentration (as depicted by an increase in Na digestibility) and the concentration and type of soluble lower phytate esters present in the ileal lumen, which subsequently resulted in an up-regulation of intestinal alkaline phosphatase (Schlemmer et al., 2009); all of which resulted in an increase in HMIT, the proton dependent *myo*-inositol transporter. Previous authors have reported phytase specific activity along the intestinal brush border of broilers and layers, with intestinal phytase activity decreasing from the duodenum to the ileum (Maenz and Classen, 1998). These results may be contradictory to the current trial; however specific phytase activity was not evaluated and effects cannot be compared directly. Furthermore, the iALP expression in the

jejunum and the ileum of birds fed 0 FTU phytase/ kg diet was 0.949 vs 0.932, respectively and phytase had a significant effect in the ileum, suggesting the response to HMIT and iALP in the ileum was associated with lower phytate esters and the production of inositol by iALP.

Interestingly, even by the terminal ileum the concentration of inositol was remarkably high (53% of the total), indicating there is a rate-limiting step in inositol absorption within the gastrointestinal tract. This is contradictory to previous estimates of free *myo*-inositol absorption in the human small intestine at around 99.8% (Holub, 1986; Croze and Soulage, 2013). However, the differences may be dependent on the availability of Na and H^+ and the location in the GIT. For example, previous authors reported phytase supplementation significantly increased pH in the distal ileum from 6.56 in birds fed 0 FTU/kg phytase to 6.99 in birds fed 5,000 FTU/kg phytase (Walk et al., 2012). Taking the anti-log of these pH values indicates an almost 40% reduction in H^+ ion concentration (2.754E-07 vs 1.023E-07) in the ileum of broilers fed 5,000 FTU/kg of phytase compared with that of broilers fed 0 FTU/kg phytase. In effect, this means that while phytase supplementation increases *myo*-inositol concentration, it also creates a rate-limiting step in myo-inositol uptake by the ileum by increasing Na digestibility and reducing H^+ ions which are needed for co-transport of *myo*-inositol. The lack of an effect of phytase dose on SMIT1 or SMIT2 support this as they are both Na dependent co-transporters.

3) Finally, notable is the non-significant effect of phytase dose on *myo*-inositol transporter gene expression in the kidney and liver. Both the liver and kidney play important roles in *myo*-inositol metabolism and *de novo* synthesis and the kidney is the main site of *myo*-inositol excretion (Holub, 1986; Lahjouji et al., 2007; Croze and Soulage, 2013). The lack of an effect of phytase dose may be indicative of a reduced need for endogenous synthesis or excretion of *myo*-inositol due to the provision of dietary *myo*-inositol. These

304 results require further evaluation but may be indicative of the pathways and the regulation of
305 *myo*-inositol provided from phytate destruction in the diet.

306 In conclusion, supplementation of broiler diets with phytase up to 4,500 FTU/kg
307 significantly increased weight gain and resulted in nearly complete phytate and phytate ester
308 destruction and the significant increases in *myo*-inositol. This influenced and up-regulated
309 the gene expression Na^+ of H^+-dependent *myo*-inositol transporters within the jejunum and
310 the ileum, respectively. These results may indicate *myo*-inositol is predominantly taken up in
311 the broiler proximal small intestine via a Na^+-dependent transporter, whereas in the distal
312 intestine phytate esters created from phytate destruction may up-regulate the expression of
313 alkaline phosphatase, which in turn yields *myo*-inositol and increases the expression of the
314 proton dependent *myo*-inositol transporter, HMIT. Data from the liver and kidney need
315 further evaluation but may indicate complex pathways in regards to regulation of *myo*-
316 inositol in tissues beyond the intestine.

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403 **Table 1.** Formulated and analysed nutrient composition of the
404 experimental diets (%, as fed)

Ingredient	Basal diet
Wheat	61.15
Soybean meal	30.04
Soya oil	4.83
Salt	0.32
Limestone	0.92
Dicalcium phosphate	1.05
Sodium bicarbonate	0.15
Lysine HCl	0.14
DL-Methionine	0.24
Threonine	0.07
Vitamin and trace minerals premix[1]	0.50
Inert or phytase	0.09
TiO marker	0.50
Total	100.00
Formulated nutrient composition	
Crude protein	21.50
ME, kcal/kg	3100.00
Dry matter	87.30
Ca	0.80
P	0.55
Available P	0.30
Phytate P	0.23
Digestible Met + Cys	0.84
Digestible Lys	1.10
Digestible Thr	0.73
Digestible Val	0.84
Sodium	0.18
Chloride	0.28
Analyzed nutrient composition	
Crude protein	22.2
Calcium	0.83
Total phosphorus	0.50
Sodium	0.18

405 [1]Supplied the following per kilogram of diet: vitamin A, 5,484 IU;
406 vitamin D_3, 2,643 ICU; vitamin E, 11 IU; menadione sodium
407 bisulfite, 4.38 mg; riboflavin, 5.49 mg; d-pantothenic acid, 11 mg;
408 niacin, 44.1 mg; choline chloride, 771 mg; vitamin B_{12}, 13.2 μg;
409 biotin, 55.2 μg; thiamine mononitrate,2.2 mg; folic acid, 990 μg;
410 pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu,
411 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 250 μg.

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

Target	Accession number	Primer sequence	Size (bp)
SLC5A11	XM_01529447	F: 5'-ATGACCATCCCGTCCCTGT-3' R: 5'-CCTTGGCGTGTGAGAGGTT-3'	88
SLC5A3	000282	F: 5'-GGCTGTACTTCGTGCTTGTAAT-3' R: 5'-CCTGCCAAGAAGTAGCCACT-3'	88
SLC2A13	XM_00123293	F: 5'-CATCTATGACAGTGCCTGTGTAC-3' R: 5'-CTCCAGTGATGAACAGAGTGTTAAT-3'	93
ALPI	XM_01529148	F: 5'-AGTCACTTCTCCCTGACTCTG-3' R: 5'-GCCTTCTGTGTCCATGAAGC-3'	84
GAPDH	NM_204305	F: 5′-CCCCA CTCCAATTTCTTC-3′ R: 5′-CAGATGGTGAACACTTTTATTGATG-3′.	105

414 SLC5A11 = SMIT2 (sodium/glucose cotransporter 11).
415 SLC5A3 = SMIT1 (sodium/*myo*-inositol cotransporter).
416 SLC2A13 = HMIT (H^+/*myo*-inositol transporter).
417 ALPI = intestinal alkaline phosphatase.
418 GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

419 **Table 3.** Growth performance of broilers fed phytase from hatch to 21-days
420 post-hatch

Phytase, FTU/kg	Feed intake, g	Weight gain, g	FCR, g:g
0	938.0	581.6	1.635
500	887.3	603.0	1.500
1500	978.3	641.3	1.552
4500	949.0	675.1	1.410
SEM	30.6	22.6	0.09
P-values			
Phytase	0.483	0.012	0.351
Linear	0.251	0.003	0.151
Quadratic	0.729	0.783	0.969

421 Means are based on 6 birds per pen and 10 replicate pens per diet.

Table 4. Apparent ileal nutrient digestibility of broilers fed phytase from hatch to 21-days post-hatch

Phytase, FTU/kg	Dry matter, %	Ca, %	P, %	K, %	Na, %	Na, g/kg DMI[1]
0	73.60	69.87	69.41	88.48	-15.98	0.25
500	71.24	56.82	66.95	86.15	-27.66	0.25
1500	68.92	56.26	73.95	86.08	-19.30	0.24
4500	70.24	59.43	81.42	85.89	-0.20	0.21
SEM	0.97	1.79	1.83	1.08	7.31	0.02
P-values						
Phytase	0.005	< 0.001	< 0.001	0.196	0.038	0.314
Linear	0.006	< 0.001	< 0.001	0.136	0.091	0.191
Quadratic	0.066	< 0.001	0.010	0.329	0.043	0.423

Means are based on 4 birds per pen and 10 replicate pens per diet.

Table 5. Phytate, phytate esters and inositol concentration (umol/g DM) in the gizzard digesta of broilers fed phytase from hatch to 21-days post hatch

Phytase, FTU/kg	Inositol	iP2[1]	iP3[2]	iP4[3]	iP5[4]	iP6[5]	∑iP6-iP2[6]
0	0.936	1.448	0.319	0.920	1.918	4.296	8.902
500	1.353	1.807	0.753	1.845	0.683	0.463	5.290
1500	1.542	1.761	0.765	0.610	0.030	0.063	3.228
4500	2.305	1.635	0.635	0.386	0.014	0.050	2.719
SEM	0.090	0.072	0.066	0.143	0.159	0.227	0.298
P-values							
Phytase	< 0.001	0.003	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Linear	< 0.001	0.161	0.004	< 0.001	< 0.001	< 0.001	< 0.001
Quadratic	0.062	0.002	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Means are based on 4 birds per pen and 10 replicate pens per diet.

[1] Inositol bisphosphate.

[2] Inositol triphosphate.

[3] Inositol tetraphosphate.

[4] Inositol pentaphosphate.

[5] Inositol hexakisphosphate (phytate, phytic acid).

[6] Sum of iP2 to iP6 concentration.

Table 6. Phytate, phytate esters and inositol concentration (umol/g DM) in the ileal digesta of broilers fed phytase from hatch to 21-days post hatch

Phytase, FTU/kg	Inositol	iP2[1]	iP3[2]	iP4[3]	iP5[4]	iP6[5]	∑iP6-iP2[6]
0	6.820	7.269	0.231	0.820	2.704	30.325	41.349
500	8.307	7.529	0.499	2.055	4.076	23.519	37.677
1500	11.489	7.040	0.726	2.504	2.933	12.475	25.678
4500	15.594	7.341	0.796	1.553	0.560	2.757	12.927
SEM	0.644	0.345	0.082	0.271	0.322	1.973	2.503
P-values							
Phytase	< 0.001	0.950	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Linear	< 0.001	0.754	< 0.001	0.032	< 0.001	< 0.001	< 0.001
Quadratic	0.050	0.953	0.238	< 0.001	< 0.001	0.466	0.078

Means are based on 4 birds per pen and 10 replicate pens per diet.

[1] Inositol bisphosphate.

[2] Inositol triphosphate.

[3] Inositol tetraphosphate.

[4] Inositol pentaphosphate.

[5] Inositol hexakisphosphate (phytate, phytic acid).

[6] Sum of iP2 to iP6 concentration.

Table 7. Expression of genes in the small intestine mucosa of broilers fed phytase from hatch to 21-days post hatch

Phytase,	Jejunum				Ileum			
FTU/kg	SLC5A11[1]	SLC5A3[2]	SLC2A13[3]	iALP[4]	SLC5A11[1]	SLC5A3[2]	SLC2A13[3]	iALP[4]
0	0.661	0.923	0.962	0.949	1.218	0.994	0.975	0.932
500	1.147	0.804	0.834	1.041	1.344	0.846	0.837	1.463
1500	3.094	1.455	1.476	1.140	1.096	0.636	0.975	1.036
4500	2.890	1.232	1.331	1.159	1.128	1.139	1.452	2.295
SEM	0.38	0.20	0.17	0.16	0.17	0.11	0.12	0.23
P-values								
Phytase	0.002	0.149	0.090	0.703	0.760	0.263	0.055	0.027
Linear	0.003	0.236	0.314	0.521	0.897	0.206	0.007	0.009
Quadratic	0.017	0.101	0.112	0.703	0.731	0.146	0.965	0.265

Means are based on 2 birds per pen and 10 replicate pens per diet.

[1] SLC5A11 = SMIT2 (sodium/glucose cotransporter 11).

[2] SLC5A3 = SMIT1 (sodium/*myo*-inositol cotransporter).

[3] SLC2A13 = HMIT (H^+/*myo*-inositol transporter).

[4] iALP = intestinal alkaline phosphatase.

Table 8. Expression of genes in the kidney and liver of broilers fed phytase from hatch to 21-days post hatch

Phytase,	Kidney			Liver		
FTU/kg	SLC5A11[1]	SLC5A3[2]	SLC2A13[3]	SLC5A11[1]	SLC5A3[2]	SLC2A13[3]
0	0.971	0.936	1.026	0.948	1.116	1.148
500	1.195	0.708	0.887	0.943	1.157	0.826
1500	0.912	0.819	0.870	1.396	0.936	0.967
4500	0.641	0.734	0.744	1.160	0.820	0.924
SEM	0.20	0.09	0.09	0.16	0.13	0.12
P-values						
Phytase	0.410	0.234	0.174	0.247	0.334	0.379
Linear	0.170	0.390	0.044	0.846	0.103	0.481
Quadratic	0.921	0.516	0.565	0.069	0.528	0.443

Means are based on 2 birds per pen and 10 replicate pens per diet.

[1] SLC5A11 = SMIT2 (sodium/glucose cotransporter 11).

[2] SLC5A3 = SMIT1 (sodium/*myo*-inositol cotransporter).

[3] SLC2A13 = HMIT (H^+/*myo*-inositol transporter).