Phytase dosing affects phytate degradation and Muc2 transporter gene expression in broiler starters

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ABSTRACT This study was conducted to determine effects of high phytase use on growth performance, amino acid (**AA**) digestibility, intestinal phytate breakdown, and nutrient transporter expression in starter broiler chickens. Male Ross 308 chicks were allocated to 24 pens, at 15 birds/pen and assigned to one of 4 dietary treatments. Treatments were: a control diet (PCa+) that contained adequate levels of calcium (**Ca**) and phosphorus (**P**) for growing broiler chicks; a reduced Ca and P diet (PCa–:–1.5 g P/kg and –1.6 g Ca/kg), and 2 additional diets in which phytase was supplemented in the PCa– diet at 1,500 (PCa-Phy1500) and 3,000 (PCa-Phy3000) FTU/kg feed. A common starter diet was fed from day 1 to 8. From day 8 to 22, birds were fed the 4 experimental diets. On day 22, birds were killed for sample collection. From day 8 to 15, average daily gain and average daily feed intake were not different across treatments ($P < 0.05$) but gain-to-feed ratio (**G:F**) was reduced ($P < 0.006$) in the PCa– treatment compared with other treatments. There were no further performance differences, but a tendency of phytase treatments improving the overall G:F ($P = 0.079$; day 8–22). Up to both the duodenum–jejunum and ileum, phytate, P, and Ca disappearance were increased ($P < 0.05$) in the PCa-Phy1500 and PCa-Phy3000 treatments compared with PCa– treatment. Phytase dose dependently increased myoinositol (**MI**) concentration in the digesta from both the duodenum–jejunum and ileum ($P < 0.001$). The highest concentration of MI was found in the PCa-Phy3000 treatment. Plasma MI concentration was increased by phytase supplementation ($P < 0.001$). Prececal disappearance of Cys was lower ($P < 0.05$) in the PCa– treatment than in PCa1and PCa-Phy3000 treatment. Expression of MUC2 in the duodenum–jejunum was higher ($P < 0.05$) in the PCa-Phy3000 treatment than in other treatments. Phytase-induced hydrolysis of phytate led to elevated digesta and plasma MI concentrations and reduced digesta concentrations of phytate breakdown intermediates

Key words: amino acid, broiler starter, inositol phosphate, myoinositol, phytase

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INTRODUCTION

Phosphorus (**P**) is an important macro mineral that must be present in poultry diets in adequate amounts to support the need for bone formation and a variety of other metabolic needs (NRC, 1994). A diet that is deficient in P, especially in the early stages of growth in the broiler chicken, can result in suboptimal health, impaired bone and skeletal development, and reduced growth performance (Edwards and Veltmann, 1983; Atia et al., 2000, de Jong et al., 2014). However, P is only partially available from plant-based ingredients because the majority of it (approximately 60–70%) is bound as the salt form of phytic acid (myoinositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) [**InsP$_6$**]), called phytate (Wonyengo and Nyachoti, 2013). Breakdown of phytate to release bound P in the gastrointestinal tract of chickens requires phytase. Exogenous phytases of bacterial and fungal origins have been used in the industry since the 1980s and are effective in increasing release of phytate-bound P and other nutrients (Lei et al., 2013). Phytase is typically added to chicken diets at about a concentration of 500 FTU/kg of diet, resulting in increased digestibility of P and calcium (**Ca**) and improvement in growth performance (Viveros et al., 2002; Cowieson et al., 2004). However, there is renewed interest in the use of higher levels of

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phytase (>1,500 FTU/kg) because these supplementation levels have been associated with further improvements in performance that cannot be solely attributed to increased release of P (Józefiak et al., 2010).

Use of high levels of phytase has been associated with improvement in antioxidant status (Karadas et al., 2010), complete breakdown of antinutritive factors (Dos Santos et al., 2013; Walk et al., 2014), and alteration of expression levels of genes involved in growth regulation (Woyengo et al., 2011). Phytate breakdown leads to release of myoinositol (**MI**), a precursor for membrane phosphatidylinositol, a major membrane phospholipid (Huber, 2016). The relative composition of the membrane phospholipids phosphatidylserine, phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine affects membrane properties such as fluidity and receptor expression (Fajardo et al., 2011). Indeed, our study in pigs (Lu et al., 2019) showed that phytase supplementation increased the expression of GLUT4 in the longissimus dorsi muscle. Others have shown that phytase supplementation increased expression of sodium-dependent MI transporter, SMIT2, in the jejunum of chickens (Walk et al., 2018). Thus, regulation of expression of nutrient transporters may represent an important mechanism behind the growth-enhancing effect of high levels of supplemental phytase.

In addition, current evidence indicates that MI regulates glucose homeostasis and insulin-regulated metabolism (Lee and Bedford., 2016). Cowieson et al. (2013) found that supplementation of a low P and Ca poultry diet with both MI and phytase over 42 days resulted in elevated insulin and glucagon concentrations with a concomitant increase in serum glucose. Therefore, we also determined the impact of phytase on metabolites such as glucose and MI and serum insulin concentration.

Phytate breakdown by phytase is associated with a change in the pattern of lower inositol phosphate esters such as $InsP_5$, $InsP_4$, $InsP_3$, $InsP_2$, and $InsP_1$, some of which might exert antinutritive effects by binding other nutrients and impairing their digestibility (Yu et al., 2012). There is need for increased understanding of the impact of phytase on phytate breakdown into the lower inositol esters and effects on prececal amino acid (**AA**) digestibility in the broiler starter, especially with regard to the potential benefit that may be obtained in diets with reduced AA, as demonstrated recently by Walk and Ramo Rao (2018). Therefore, a major objective of this study was to investigate the expression of nutrient transporters in phytase-supplemented starter chickens. Another objective of the present experiment was to evaluate the effects of phytase supplementation on the concentrations of InsP esters and the prececal AA digestibility in broiler starters.

Table 1. Ingredient composition of the starter diet and calculated concentrations.

Ingredient	g/kg
Corn	516.5
Soybean meal	410
Soybean oil	30
D,L-Methionine	1.5
Monocalcium phosphate	21
Sand	-
Limestone (fine)	12
Sodium chloride	1.5
Choline chloride	2.0
Sodium bicarbonate	3.0
Vitamin premix	2.0
Mineral premix	0.5
Calculated composition, g/kg of DM	
Crude protein	256
ME (MJ/kg)	13.8
Total P	9.1
Non-phytate Phosphorus (**NPP**)	6.8
Ca	11.6
Ca:P	1.3

Abbreviations: Ca, calcium; P, phosphorus.

MATERIALS AND METHODS

Bird Use

All procedures and protocols were approved by Regierungspräsidium Tübingen, Germany (project no. HOH 51/17 TE), in accordance with the German Animal Welfare Legislation. The study was performed at the university experimental station, Unterer Lindenhof. In total, 360 male Ross 308 hatchlings obtained from a commercial hatchery (Brüterei Süd GmbH & Co. KG, Regenstauf, Germany) were allocated to 24 floor pens (115*230 cm ground area, 2.60-m height) on deep litter bedding, each comprising 15 hatchlings. From day 15 until the end (day 22), the birds were kept on perforated floors. The lighting program was as follows: continuous light (24L:0D) from hatch to day 3, 18 h of light and 6 h of darkness (18L:6D) from day 4 until the end. The temperature in the barn was set at 34°C on the first day and then gradually decreased every 3–5 D to achieve a temperature of 27°C on the last 4 D. The well-being of the animals was checked twice daily. They were fed a starter diet (Table 1) from day 1 to 8. From day 8 until the end, the birds were fed with the experimental diets (Table 2). Six pens were randomly allocated to each of the 4 treatments in a completely randomized block design. Feed and water were provided for ad libitum consumption. Birds and feed were weighed on days 1, 8, 15, and 22 to determine average daily feed intake (**ADFI**), average daily gain (**ADG**), and gain-to-feed ratio (**G:F**). The animals were deprived of feed 2 h before slaughter, and then, 1 h before the slaughter, feeders were moved back into the pens to standardize intestinal fill. On day 22, 13 birds per pen were stunned with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2 and euthanized by CO_2 asphyxiation. Two birds per pen were also stunned with the same gas mixture but decapitated to collect blood samples for MI, inorganic P ($\mathbf{P_i}$), Ca, glucose, and insulin analysis. Tubes containing sodium fluoride and heparin were centrifuged for 10 min at 2,000 × *g* to separate the plasma. Tubes without chemical supplements were centrifuged for 10 min at 2,000 × *g* to separate the serum. Mucosa samples were

Table 2. Ingredient composition of the experimental diets, calculated and analyzed concentrations.

Ingredient, g/kg	PCa+	PCa–	PCa-Phy1500	PCa-Phy3000
Corn	581	581	581	581
Soybean meal	350	350	350	350
Soybean oil	30	30	30	30
D,L-Methionine	3.5	3.5	3.5	3.5
Monocalcium phosphate	14.5	8.2	8.2	8.2
Sand	-	7.1	7.1	7.1
Limestone (fine)	7.5	6.7	6.7	6.7
Sodium chloride	1.0	1.0	1.0	1.0
Choline chloride	2.0	2.0	2.0	2.0
Sodium bicarbonate	3.0	3.0	3.0	3.0
Vitamin premix[1]	2.0	2.0	2.0	2.0
Mineral premix[2]	0.5	0.5	0.5	0.5
Titanium dioxide	5.0	5.0	5.0	5.0
Calculated concentration, g/kg of DM				
Crude protein	232	232	232	232
ME (MJ/kg)	14.1	14.1	14.1	14.1
Total P	7.4	5.8	5.8	5.8
Non-phytate Phosphorus (**NPP**)	5.1	3.6	3.6	3.6
Ca	8.2	6.6	6.6	6.6
Ca:P	1.1	1.1	1.1	1.1
Analyzed concentration				
Total P, g/kg of DM	7.95	6.44	6.35	6.35
$InsP_6$-P, g/kg of DM	2.55	2.57	2.55	2.59
Ca, g/kg of DM	8.35	6.77	6.76	6.74
Myoinositol, µmol/g DM	1.72	1.67	1.67	1.67
Ins(1,2,3,4,5)P_5[3], µmol/g DM	0.4	0.4	0.4	0.5
Ins(1,2,4,5,6)P_5[3], µmol/g DM	0.9	0.9	1.0	0.9
$InsP_6$[3], µmol/g DM	13.7	13.8	13.7	13.9
Phytase activity, FTU/kg	<50	<50	1,530	3,190

Abbreviations: Ca, calcium; $InsP_6$, myoinositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); P, phosphorus.

[1]Vitamin premix (Miavit GmbH, Essen, Germany), provides per kg of complete diet: 10,000 IU vitamin A, 3,000 IU vitamin D3, 30 mg vitamin E, 2.4 mg vitamin K3, 100 mcg biotin, 1 mg folic acid, 3 mg vitamin B1, 6 mg vitamin B2, 6 mg vitamin B6, 30 mcg vitamin B12, 50 mg nicotinamide, 14 mg calcium-D-pantothenate.

[2]Trace element premix (Gelamin Gesellschaft für Tierernährung mbH, Memmingen, Germany), provides per kg of complete diet: 80 mg manganese from manganese(II) oxide, 60 mg zinc from zinc oxide, 25 mg iron from ferrous(II) sulfate monohydrate, 7.5 mg copper from cupric(II) sulfate pentahydrate, 0.6 mg iodine from calcium iodate, 0.2 mg selenium from sodium selenite.

[3]No other InsP isomers were detected.

collected from the duodenum–jejunum area and the terminal two-thirds of the ileum of 4 birds per pen for RNA isolation and PCR analysis. Digesta was rinsed with cold double-distilled water from the whole duodenum–jejunum and the terminal part of the ileum (last two-thirds of the section between Meckel's diverticulum and 2 cm before the ileo–ceco–colonic junction) of the remaining 11 birds per pen and pooled. All samples were immediately frozen at −20°C, freeze-dried, and pulverized using a vibrating cup mill. Pulverized samples were stored in airtight containers until further analysis at a temperature lower than 6°C. Digesta from both sections were used for analysis of Ca, P, Ti, InsPs, and MI, and digesta from the terminal part of the ileum, for analysis of AA.

Experimental Diets

The ingredient and nutrient composition of the experimental diets are shown in Table 2. PCa+ is a corn–soy diet that contained adequate levels of all nutrients according to the GfE (1999). Titanium dioxide at a concentration of 5 g/kg was included in the diets as an indigestible marker. The PCa– diet had reduced P and Ca levels (–1.5 g P/kg and –1.6 g Ca/kg) according to the recommendations of the supplier (AB Vista, Marlborough, UK) of the phytase (modified *Escherichia coli*–derived 6-phytase, Quantum Blue). The PCa-Phy1500 and PCa-Phy3000 diets were supplemented with 1,500 and 3,000 FTU/kg phytase, respectively. The experimental diets were produced by first mixing all ingredients, with the exception of variable ingredients. This mix was divided into 4 parts. Each part was then supplemented with an individual mixture of monocalcium phosphate, limestone, sand, and phytase. The diets were remixed and pelleted using a 3-mm pelleting matrix without using steam conditioning. The pelleting temperature stayed lower than 65°C, which was confirmed by the temperature measurement of the pellets immediately after release from the press. Representative samples were taken from every feed bag of each diet using a sampler and then pulverized using a vibrating cup mill (PULVERISETTE 9, Fritsch GmbH, Idar-Oberstein, Germany).

Table 3. Primers used in real-time quantitative PCR.

Gene	Forward	Reverse	Accession #	Product size (bp)
PEPT1	TACAATTGGGCAGGCAGTCA	TCCAAGCGCGATGAGAATCA	NM_204365.1	133
GLUT2	GCACAGGACCTTGCATTTGG	CTGGACAGGCTTGAGAGGTG	NM_207178.1	72
GLUT5	GTAGGTGTGGGCGTGTGTTG	TTTAACGAGCCCTGCTCTCT	XM_004947446.2	99
MUC2	AGGTAATTGTCTGGCCGTGG	GTGGTTGTACCTTCGGTGCT	NM_001318434.1	111
SLC5A11/SMIT2	ATGACCATCCCGTCCCTGT	CCTTGGCGTGTGAGAGGTT	XM_01529447	88
SLC5A3/SMIT1	GGCTGTACTTCGTGCTTGTAAT	CCTGCCAAGAAGTAGCCACT	000282	88
SLC2A13/HMIT	CATCTATGACAGTGCCTGTGTAC	CTCCAGTGATGAACAGAGTGTTAAT	XM_001232939.5	93
ALPI	AGTCACTTCTCCCTGACTCTG	GCCTTCTGTGTCCATGAAGC	XM 0.152,9148	84
GAPDH	GGGGAAAGTCATCCCTGAGC	TTGGCTGGTTTCTCCAGACG	NM_204305	111

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT2, solute carrier family 2 (facilitated glucose transporter), member 2; GLUT5, solute carrier family 2 (facilitated glucose transporter), member 5; MUC2, mucin2; PEPT1, peptide transporter; SLC5A11/SMIT2, solute carrier family 5 (sodium/glucose cotransporter/sodium/myoinositol cotransporter; SLC5A3/SMIT1, solute carrier family 5 (inositol transporters) member 3; SLC2A13/HMIT, solute carrier family 2 member 13/(H+/myoinositol transporter; ALPI, alkaline phosphatase, intestinal.

Total RNA Extraction, Reverse Transcription and Quantitative Real-Time PCR Analysis

Total RNA was extracted from the duodenal–jejunal border (hereinafter referred to as duodenum–jejunum) and the distal ileum, approximately 2 cm from the ileocecal junction using the TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Concentrations of RNA were determined using the NanoDrop OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Integrity of RNA was verified by agarose gel electrophoresis. Then, 2 μg of total RNA from each sample was reverse transcribed into cDNA using the MMLV reverse transcriptase (Thermo Scientific, Waltham, MA).

Expression of genes was determined by real-time PCR using the Bio-Rad CFX Connect Real-Time PCR system with the SYBR Select Master Mix for CFX (Applied Biosystems, Waltham, MA). PCR programs for all genes were designed as follows: initial denaturation for 10 min at 95°C; extension for 40 cycles at 95°C for 15 s; and primer-specific annealing temperature for 30 s; followed by melt curve analysis. The primer sequences used for real-time PCR are listed in Table 3. Primer specificity and efficiency were determined using pooled samples. Primer sets with DNA amplification efficiencies between 90 and 110% were used for analysis. Reactions were performed in duplicate, and coefficient of variation equal to or less than 5% was considered acceptable. Quantification of target transcripts was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using GAPDH as the housekeeper. For comparison of relative expression levels, the PCa + treatment was set at 1 or 100%, and all other treatments were expressed relative to this treatment.

Chemical Analysis

Pulverized feed and digesta samples were analyzed for P, Ca, and Ti using a modified method from Boguhn et al. (2009), as described by Zeller et al. (2015). The extraction and measurement of $InsP_{3–6}$ isomers in feed and digesta were conducted according to the method of Zeller et al. (2015) with slight modifications. In short, the samples were extracted twice using a solution of 0.2 mol EDTA and 0.1 mol sodium fluoride (pH 8.0; 4°C) for 30 min under constant agitation and centrifuged after each extraction at 12,000 × *g* for 15 min. The supernatants obtained were combined, and a 1-mL sample was centrifuged at 14,000 × *g* for 15 min and then filtered before another centrifugation at 14,000 × *g* for 30 min. Filtrates were analyzed by high-performance ion chromatography and UV detection at 290 nm after postcolumn reaction with $Fe(NO_3)_3$ in $HClO_4$ using an ICS-3000 system (Dionex, Idstein, Germany). With this methodology, separation of enantiomers is not possible, and therefore, we were unable to distinguish between the D- and L-forms.

Some $InsP_3$ isomers could not be identified because standards were unavailable. A clear separation between the isomers Ins(1,2,6)P_3, Ins(1,4,5)P_3, and Ins(2,4,5)P_3 was not possible because of coelution. Therefore, henceforth, the term $InsP_{3x}$ is used for these $InsP_3$ isomers of unknown proportions. Myoinositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) was used for quantification, and correction factors for differences in detector responses for $InsP_{3–5}$ were used according to Skoglund et al. (1997). Myoinositol in feed, digesta, and plasma samples was analyzed according to Sommerfeld et al. (2018a) using a gas chromatograph or mass spectrometer after derivatization of the samples. Analysis of AA was performed according to Rodehutscord et al. (2004). In brief, the samples were oxidized in an ice bath using a mixture of hydrogen peroxide, phenolic formic acid solution, and phenol. The samples were then hydrolyzed at 113°C for 24 h in a mixture containing HCl and phenol. Norleucine was used as an external standard. Amino acids were separated and detected using an L-8900 Amino Acid Analyzer (VWR, Hitachi Ltd., Tokyo, Japan). Methionine and cysteine were determined as methionine sulfone and cysteic acid, respectively. The concentrations of tyrosine, histidine, and phenylalanine may be affected to some extent by the oxidation procedure (Mason et al., 1980).

Calcium, P_i, and glucose levels in blood serum were analyzed at the IDEXX BioResearch Vet Med Labor GmbH (Ludwigsburg, Germany). Calcium was measured photometrically by the Arsenazo method using a Beckman Olympus AU480 (Beckman Coulter Inc., Brea, CA). Inorganic P was measured photometrically as phosphomolybdate complex using a Beckman Olympus AU480. Glucose was measured by the hexokinase method using a Beckman Olympus AU480.

Feed samples were analyzed for phytase activity by AB Vista Laboratory Services (Ystrad Mynach, UK) using the analytical method of the supplier (pH 4.5 and 60°C), followed by transfer of the result to commonly used phytase unit (**FTU**) by a validated transfer factor.

Calculations

ADG, ADFI, and G:F were calculated for the experimental periods from day 8 to 15 and day 8 to 22 on a pen basis with accounting for mortality. The disappearance of $InsP_6$, P, Ca, and AA was calculated based on the analyzed concentrations of $InsP_6$, P, Ca, AA, and Ti in feed and digesta.

The following generally accepted equation was used:

$$y(X) = 100 - 100 * \left(\frac{Ti\ in\ feed\ (g/kg\ DM)}{Ti\ in\ digesta\ (g/kg\ DM)} * \frac{X\ in\ digesta}{X\ in\ feed} \right)$$

where y(X) is the disappearance of X in % and X is $InsP_6$, P, Ca, or AA in grams per kilogram of DM.

The concentrations of disappeared P and Ca (y) were calculated as follows:

y = (P or Ca disappearance × P or Ca content in feed)/100.where y is in grams per kilogram of DM, P or Ca disappearance is in percentage, and P or Ca content in feed is in grams per kilogram of DM.

Statistical Analysis

All data were subjected to a one-factor analysis of variance using the MIXED procedure and pairwise t tests using the software package SAS (version 9.3; SAS Institute Inc., Cary, NC). The pen was considered as the experimental unit. The following model was used: $Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$, where Y_i is the response variable, μ is the overall mean, α_i is the effect of dietary treatment (fixed), β_j is the effect of block (random), and ε_i is the residual error. Statistical significance was declared at $P < 0.05$. A tendency was declared when P is between 0.05 and 0.1.

RESULTS

Analyzed P concentrations in the PCa+, PCa–, PCa-Phy1500, and PCa-Phy3000 diets were 7.95, 6.44, 6.35, and 6.35 g/kg of DM, respectively (Table 2). In addition, the total Ca in the diets was 8.35, 6.77, 6.76, and 6.74 g/kg of DM for the 4 diets, respectively. These values were close to the expected reductions of approximately 1.5 and 1.6 g/kg of P and Ca, respectively, in PCa–, PCa-Phy1500, and PCa-Phy3000 diets compared with PCa+. Analyzed phytase activities of 1,530 and 3,190 in PCa-Phy1500 and PCa-Phy3000 diets, respectively, also confirmed the calculated values.

From day 8 to 15, ADG and ADFI were not different across treatments ($P > 0.05$) (Table 4). However, G:F was significantly reduced ($P < 0.006$) in the PCa- treatment compared with that in the other treatments. Overall (day 8–22), ADG and ADFI were not affected by treatment. However, there was a tendency ($P = 0.079$) for an increased G:F in both PCa-Phy1500 and PCa-Phy3000 treatment groups. Final body weight on day 22 was not affected by treatment ($P > 0.05$). The serum concentrations of P_i, Ca, glucose, and insulin were not affected by treatment ($P > 0.05$) (Table 5). In the plasma, MI concentration was higher ($P < 0.001$) in the PCa-Phy1500 and PCa-Phy3000 diets than in PCa+ and PCa– diets. The disappearance of $InsP_6$, P, and Ca up to both the duodenum–jejunum and ileum was increased ($P < 0.05$) in the PCa-Phy1500 and PCa-Phy3000 treatments compared with PCa– treatment (Table 6). Increasing phytase from 1,500 to 3,000 FTU/kg feed further increased $InsP_6$ and P disappearance by 17.7 and 5.4 percentage points, respectively, up to the duodenum–jejunum and by 8.7 and 4.5 percentage points, respectively, up to the ileum ($P < 0.001$).

There was a dose-dependent increase in MI concentration in the digesta of phytase-fed broilers from both the duodenum–jejunum and ileum ($P < 0.001$) (Table 7). The concentration of $InsP_{3x}$ was below the limit of quantification in the duodenum–jejunum in the PCa– treatment, but was significantly increased ($P < 0.005$) in

Table 4. Effect of P, Ca, or phytase on performance parameters of broiler chickens[1].

Parameter	PCa+	PCa–	PCa-Phy1500	PCa-Phy3000	Pooled SEM	*P*-value
Day 8–15						
ADG (g/D)	40.0	40.0	40.8	40.8	0.54	0.498
ADFI (g/D)	52.0	53.1	52.6	52.2	0.54	0.481
G:F (g/g)	0.77[a]	0.75[b]	0.78[a]	0.78[a]	0.006	0.006
Day 8–22						
ADG (g/D)	57.6	58.4	59.2	58.7	1.04	0.619
ADFI (g/D)	75.6	77.2	76.9	75.9	1.09	0.533
G:F (g/g)	0.76	0.76	0.77	0.77	0.005	0.079
Day 22						
Body weight (g)	979	983	996	989	14.4	0.755

[a,b]Means within a row not showing a common superscript differ ($P < 0.05$).

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; Ca, calcium; G:F, gain-to-feed ratio; P, phosphorus.

[1]Data are given as treatment means; n = 6 pens per treatment.

the PCa-Phy1500 and PCa-Phy3000 treatments. The concentration of Ins(1,2,3,4)P_4 was only quantifiable in PCa-Phy1500 treatment in the duodenum–jejunum, and its concentration was lower ($P < 0.05$) in the ileum of birds at the highest phytase level. In contrast, the concentration of Ins(1,2,5,6)P_4 was increased ($P < 0.05$) in the phytase-supplemented treatments in both the duodenum–jejunum and ileum compared with the unsupplemented treatments. The concentration of Ins(1,2,3,4,6)P_5 was below the limit of detection in the phytase-supplemented treatments, but occurred in control birds' small intestine. The concentration of Ins(1,2,3,4,5)P_5 in the duodenum–jejunum was higher ($P < 0.05$) in the PCa-Phy1500 treatment than in the other treatments. However, its concentration was not affected by treatment in the ileum ($P > 0.05$). The lowest concentration of Ins(1,2,4,5,6)P_5 in both the duodenum–jejunum and ileum was found in the PCa-Phy3000 treatment. Myoinositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) concentration was dose dependently reduced with phytase supplementation in both sections with lower levels analyzed in ileal than in duodenal–jejunal digesta.

The digestibility of most AA was numerically, but not significantly, lower in PCa– treatment than in all other treatments except for Cys that was significantly lower ($P < 0.05$) in the PCa– treatment than in PCa+ and PCa-Phy3000 treatment (Table 8). The PCa-Phy1500 treatment showed an intermediate value of Cys disappearance.

Expression of MUC2 was increased significantly in the duodenal–jejunal mucosa ($P < 0.05$) in the PCa-Phy3000 compared with other treatments (Table 9). There was no treatment effect on the expression of other genes measured.

DISCUSSION

Use of very high levels of phytase has been associated with performance improvements, especially in G:F when broilers are fed diets that are marginally limited in P and Ca (Walk et al., 2014; Manobhavan et al., 2016; Gautier et al., 2018). In this study, the observed increase in G:F in PCa-Phy1500 and PCa-Phy3000 treatments from day 8 to 15 confirmed the expected effect of the levels of phytase used. Positive G:F response to phytase has been associated with high breakdown of phytate, which leads to improvements in body weight gain without corresponding increases in ADFI (Walk et al., 2014; Sommerfeld et al., 2018a). In support of this, phytase addition in the present study resulted in near-total disappearance of phytate in the duodenum–jejunum and ileum and increased MI concentrations in the duodenum–jejunum, ileum, and plasma. The further degradation of $InsP_6$ from the duodenum–jejunum to the terminal ileum demonstrates that although $InsP_6$ is less soluble under this circumstance, it is still degraded within the small intestine either by the phytase added or phytases of the host or its microbiome. Furthermore, phytase addition also significantly increased P and Ca

Table 5. Effect of P, Ca, or phytase on blood serum or plasma parameters of broilers fed with the experimental diets from day 8 to day 22[1].

Parameter	PCa+	PCa–	PCa-Phy1500	PCa-Phy3000	Pooled SEM	*P*-value
Serum						
Inorganic P (mmol/L)	2.61	2.56	2.71	2.68	0.089	0.463
Calcium (mmol/L)	2.38	2.44	2.82	2.31	0.195	0.282
Glucose (mmol/L)	17.94	18.15	17.83	17.60	0.359	0.653
Insulin (ng/mL)	1.52	1.75	2.01	1.39	0.22	0.76
Plasma						
Myoinositol (mmol/L)	0.26[c]	0.30[c]	0.41[a]	0.36[b]	0.017	<0.001

[a-c]Means within a row not showing a common superscript differ ($P < 0.05$).

Abbreviations: Ca, calcium; P, phosphorus.

[1]Data are given as treatment means; n = 6 pens per treatment.

Table 6. Effect of P, Ca, or phytase on $InsP_6$, P, and Ca disappearance up to the duodenum–jejunum and terminal ileum of broilers fed with the experimental diets from day 8 to 22[1].

	PCa+	PCa–	PCa-Phy1500	PCa-Phy3000	Pooled SEM	*P*-value
Duodenum–jejunum						
$InsP_6$ disappearance (%)	6.3[d]	13.9[c]	66.5[b]	84.2[a]	1.86	<0.001
P disappearance (%)	43.5[c]	37.9[d]	54.4[b]	59.8[a]	1.41	<0.001
P disappearance (g/kg of DM)	3.5[b]	2.4[c]	3.5[b]	3.8[a]	0.09	<0.001
Ca disappearance (%)	42.1[b]	44.3[b]	51.3[a]	51.2[a]	1.07	<0.001
Ca disappearance (g/kg of DM)	3.5[a]	3.0[b]	3.5[a]	3.5[a]	0.08	<0.001
Ileum						
$InsP_6$ disappearance (%)	15.3[d]	26.8[c]	84.4[b]	93.1[a]	2.73	<0.001
P disappearance (%)	59.6[c]	57.2[c]	78.3[b]	82.8[a]	1.18	<0.001
P disappearance (g/kg of DM)	4.7[c]	3.7[d]	4.9[b]	5.2[a]	0.08	<0.001
Ca disappearance (%)	54.2[c]	60.3[b]	64.5[a]	64.4[a]	1.58	<0.001
Ca disappearance (g/kg of DM)	4.5[a]	4.1[b]	4.4[a]	4.3[ab]	0.11	0.017

[a-d]Means within a row not showing a common superscript differ ($P < 0.05$).
Abbreviations: Ca, calcium; InsP6, myoinositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); P, phosphorus.
[1]Data are given as treatment means; n = 6 pens per treatment.

disappearance up to the duodenum–jejunum and ileum. The effect of the higher phytase level was demonstrated in the higher disappearance of phytate and P up to the duodenum–jejunum and ileum in the PCa-Phy3000 treatment than in PCa-Phy1500 treatment. A phytase dose–related effect was not observed with Ca disappearance in both intestinal sections. This may reflect a higher sensitivity of phytate and P to increased dietary phytase than Ca. However, despite the increase in P and Ca disappearance with phytase, the lack of effect on body weight and G:F in the total trial period could indicate that the marginal reduction of Ca and P in the PCa– diet did not negatively affect growth in birds that had ad libitum access to feed and that the further release of inositol did not further improve growth over this short period.

Breakdown of phytate releases P and lower InsP esters. Theoretically, phytase could break down phytate nearly completely through a stepwise dephosphorylation reaction ($InsP_6 \rightarrow InsP_5 \rightarrow InsP_4 \rightarrow InsP_3 \rightarrow InsP_2$, followed by phosphatase dephosphorylation$\rightarrow InsP_1 \rightarrow MI + P_i$). However, hydrolysis of phytate in the gastrointestinal tract is mostly incomplete, leading to generation of a mixture of InsP esters (Zeller et al., 2015). The increased concentration of $Ins(1,2,5,6)P_4$ and $InsP_{3x}$ in the duodenum–jejunum and ileum in PCa-Phy1500 and PCa-Phy3000 treatments reflects this effect of phytase. There was also a corresponding decrease in the concentration of $InsP_5$ isomers in the ileum, with phytase supplementation compared with PCa+ treatment. This reduction in $InsP_5$ isomers is similar to the finding of Sommerfeld et al. (2018a) who

Table 7. Effect of P, Ca, or phytase on concentrations of InsP isomers (μmol/g DM) in the digesta of the duodenum–jejunum and terminal ileum of broilers fed with the experimental diets from day 8 to 22[1].

InsP isomer	PCa+	PCa–	PCa-Phy1500	PCa-Phy3000	Pooled SEM	*P*-value
Duodenum–jejunum						
Myoinositol	8.3d	9.8c	18.1b	22.3[a]	0.40	<0.001
$InsP_{3x}$[2]	0.2b	<LOQ[3]	0.5a	0.8[a]	0.11	0.005
$Ins(1,2,3,4)P_4$	<LOQ	<LOQ	0.2	n.d.[4]	0.05	-
$Ins(1,2,5,6)P_4$	0.2c	<LOQ	1.6b	2.1a	0.21	<0.001
$Ins(1,2,3,4,6)P_5$	0.4	0.3	n.d.	n.d.	0.03	0.028
$Ins(1,2,3,4,5)P_5$	1.0b	0.9b	2.1a	1.2b	0.16	<0.001
$Ins(1,2,4,5,6)P_5$	1.2a	0.7b	0.6b	0.4c	0.07	<0.001
$InsP_6$	26.4a	23.5b	9.4c	4.5d	0.69	<0.001
Ileum						
Myoinositol	2.8[d]	5.6c	16.3b	19.6a	0.85	<0.001
$InsP_{3x}$[2]	0.3	0.3	0.7	0.8	0.21	0.189
$Ins(1,5,6)P_3$	0.2	<LOQ[3]	<LOQ	<LOQ	0.02	-
$Ins(1,2,3,4)P_4$	0.7[a]	0.6ab	0.3bc	0.2c	0.10	0.007
$Ins(1,2,5,6)P_4$	0.4[b]	<LOQ	2.0[a]	1.8a	0.57	0.041
$Ins(1,2,3,4,6)P_5$	1.0	0.9	n.d.[4]	n.d.	0.08	0.11
$Ins(1,2,3,4,5)P_5$	2.0	1.7	1.8	0.7	0.32	0.068
$Ins(1,2,4,5,6)P_5$	1.5[a]	0.7b	0.5bc	0.3c	0.14	<0.001
$InsP_6$	38.2[a]	30.1b	6.8c	3.1d	1.37	<0.001

[a-d]Means within a row not showing a common superscript differ ($P < 0.05$).
Abbreviations: Ca, calcium; InsP6, myoinositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate); P, phosphorus.
[1]Data are given as treatment means; n = 6 pens per treatment.
[2]At least one of the following isomers: Ins(1,2,6)P3, Ins(1,4,5)P3, Ins(2,4,5)P3.
[3]<LOQ, not quantifiable in the majority of samples.
[4]n.d., not detectable in the majority of samples.

Table 8. Effect of P, Ca, or phytase on prececal AA disappearance (%) in broilers fed with experimental diets from day 8 to 22[1].

AA	PCa+	PCa–	PCa-Phy1500	PCa-Phy3000	Pooled SEM	*P*-value
Arg	87.8	86.3	87.2	87.3	1.13	0.280
His[2]	80.1	76.6	76.7	76.7	2.31	0.075
Ile	78.6	78.7	80.4	79.9	1.97	0.359
Leu	81.1	78.4	79.8	80.0	2.32	0.344
Lys	82.5	81.4	82.4	82.5	1.77	0.729
Met	91.7	90.5	91.0	91.1	1.24	0.494
Phe[2]	81.9	79.6	81.3	81.4	1.96	0.288
Thr	72.4	69.6	70.5	70.6	2.42	0.320
Trp	79.0	76.7	76.5	75.5	2.16	0.162
Val	77.2	76.7	78.0	77.3	2.11	0.778
Alx[2]	79.0	76.2	77.0	77.0	2.65	0.415
Asp	78.3	76.3	77.4	77.8	1.69	0.353
Cys	67.9[a]	63.3[b]	66.6[ab]	68.2[a]	2.52	0.027
Glx[2]	85.6	83.6	84.6	84.9	1.58	0.299
Gly	74.6	72.2	72.9	73.1	1.99	0.312
Pro	79.9	76.8	77.8	78.1	2.08	0.170
Ser	78.6	75.6	76.9	77.3	2.00	0.178
Tyr[3]	79.9	77.6	78.9	79.3	2.21	0.382

[a,b]Means within a row not showing a common superscript differ ($P < 0.05$).

Abbreviations: Ca, calcium; P, phosphorus.

[1]Data are given as treatment means; n = 6 pens per treatment.

[2]Asp, Asn, and Glu, Gln, respectively, were detected together because the side groups of Asn and Gln are lost during acid hydrolysis (Fontaine, 2003).

[3]The concentrations of histidine and phenylalanine may be affected to some extent by the oxidation procedure (Mason et al., 1980).

found a reduction in this group of isomers in the crop and terminal ileum of broiler chickens fed phytase-supplemented diets, demonstrating the $InsP_5$ is well degraded in the digestive tract of broilers by the phytase tested.

The effect of phytase supplementation on lower InsP esters was also reflected in the increased duodenal–jejunal, ileal, and plasma MI concentrations, demonstrating that some $InsP_6$ gets completely dephosphorylated. This observation agrees with previous reports that phytase treatment leads to elevation in plasma MI concentration in broiler chickens (Beeson et al., 2017; Sommerfeld et al., 2018a; Walk et al., 2018; Künzel et al., 2019). Although a higher duodenal–jejunal and ileal MI concentration was found in the PCa-Phy3000 treatment than in PCa-Phy1500 treatment, plasma MI concentration reached a plateau in PCa-Phy1500 treatment and significantly declined

Table 9. Gene expression of nutrient transporters, mucin, and ALPI in the duodenal–jejunal and ileal mucosa of experimental birds.

Gene	PCa+	PCa–	PCa-Phy1500	PCa-Phy3000	Pooled SEM	*P*-value
Duodenum–jejunum						
PEPT1	1.00	0.77	0.75	0.95	0.17	0.86
GLUT2	1.00	1.18	1.24	1.41	0.32	0.42
GLUT5	1.00	0.97	0.89	0.58	0.22	0.79
MUC2	1.00[b]	0.50[b]	0.25[b]	2.94[a]	0.43	0.03
SLC5A11/SMIT2	1.00	0.75	0.50	2.04	0.38	0.40
SLC5A3/SMIT1	1.00	2.00	0.83	1.21	0.06	0.21
SLC2A13/HMIT	1.00	1.51	1.13	0.91	0.07	0.24
ALPI	1.00	1.25	1.89	1.85	0.17	0.53
Ileum						
PEPT1	1.00	0.94	1.14	0.92	0.15	0.91
GLUT2	1.00	0.70	1.09	1.70	0.06	0.67
GLUT5	1.00	1.60	1.03	2.45	0.19	0.67
MUC2	1.00	1.86	1.20	3.24	0.50	0.32
SLC5A11/SMIT2	1.00	0.87	0.95	1.18	0.07	0.79
SLC5A3/SMIT1	1.00	1.04	0.61	0.88	0.09	0.58
SLC2A13/HMIT	1.00	1.01	1.18	1.09	0.12	0.88
ALPI	1.00	0.44	1.07	0.87	0.19	0.17

[a,b]Means within a row not showing a common superscript differ ($P < 0.05$).

Means are based on 2 birds per pen and 6 replicate pens per diet.

Abbreviations: ALPI, alkaline phosphatase, intestinal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT2, solute carrier family 2 (facilitated glucose transporter), member 2; GLUT5, solute carrier family 2 (facilitated glucose transporter), member 5; MUC2, mucin2; PEPT1, peptide transporter; SLC5A11/SMIT2, solute carrier family 5 (sodium/glucose cotransporter/sodium/myoinositol cotransporter; SLC5A3/SMIT1, solute carrier family 5 (inositol transporters) member 3; SLC2A13/HMIT, solute carrier family 2 member 13/(H+/myoinositol transporter.

in the PCa-Phy3000 treatment. The reasons for this dose-dependent regulation of plasma MI concentration are currently unclear. In contrast, Sommerfeld et al. (2018a), who fed the same phytase and application rates or MI did not see reduced MI plasma levels. This suggests that the increased MI clearance noted with the PCa-Phy3000 treatment is probably a consequence of differing conditions in this study compared with the trial by Sommerfeld et al (2018a). Further investigations beyond point-in-time measurements on the regulation of MI in plasma are needed to better understand the contribution of MI when released by phytase to growth performance and its metabolism.

As MI is known as a precursor for membrane phosphatidylinositol, a major membrane phospholipid, it would have been expected that phytase supplementation with the subsequent increased MI level in digesta and blood led to different transporter gene expressions. Gene expression analysis in phytase-supplemented birds revealed that phytase increased, in muscle tissue, the expression of genes involved in muscle development, via calmodulin and calcineurin, and insulin-like growth factor pathway (Schmeisser et al., 2017). Phytase supplementation to chickens also tended to increase the expression of H + -dependent MI transporter, HMIT, in the ileum and jejunum, and increase the expression of the sodium-dependent MI transporter, SMIT2, in the jejunum and intestinal alkaline phosphatase in the ileum (Walk et al., 2018). However, there was no effect of phytase on gene expression of inositol transporters or alkaline phosphatase in the present study. Gene expression responses to phytase may be related to phytase effect on growth performance. In both the studies by Schmeisser et al. (2017) and Walk et al. (2018), phytase effect was associated with performance improvements such as increased weight gain (Walk et al., 2018), ADG, and feed conversion ratio (Schmeisser et al. 2017). In the present study, only G:F was improved, perhaps due to the short trial period. Therefore, the limited performance effects observed in this study may preclude a similar effect of phytase on transporter gene expression in this study. Nevertheless, phytase supplementation at a concentration of 3000 FTU/kg increased the expression of MUC2 in the duodenum–jejunum. MUC2 is important for the generation of mucin (Jiang et al., 2013) and maintenance of intestinal mucosal health (Smith and Podolsky, 1986). However, Onyango et al. (2009) found higher mucin production in broilers fed with phytate or phytic acid, components which were successfully degraded in the present study. Therefore, further investigations are needed to better understand the effect of phytate degradation on mucin secretion and the role of phytase in this process.

Although higher MI release was observed, there was no effect of phytase supplementation on serum glucose and insulin concentrations. This result is in contrast to the findings of Cowieson et al. (2013) who found increased circulating blood glucose concentration in phytase-supplemented broilers. Differences related to the feeding period, age when birds were killed, and diet composition may explain the disparity obtained with regulation of serum glucose concentration by phytase. However, phytase effect on insulin action and circulating insulin concentrations requires further investigation as insulin concentration did not mirror glucose concentration in this study nor in the study by Cowieson et al. (2013).

In some studies, phytase has been shown to increase AA digestibility (Amerah et al., 2014; Gallardo et al., 2017; Sommerfeld et al., 2018b; Walk and Rama Rao, 2018; Borda-Molina et al., 2019). Although digestibility of most AA studied was not significantly improved by phytase supplementation in this study, PCa-Phy3000 treatment increased Cys digestibility compared with PCa– treatment. A possibility that this might be due to a selectivity of phytase on AA digestibility, as seen in studies on pigs (Adedokun et al., 2015; Zeng et al., 2016; She at al., 2018) and poultry (Gallardo et al., 2017), or simply due to the more distinct effects due to the low digestibility of Cys compared with other AA remains unclear. The finding that supplementation of microbial phytase does not always have an effect on the prececal digestibility of AA in broilers has also been shown by Sebastian et al. (1997), Peter and Baker (2001), and Rodehutscord et al. (2004). However, higher application rates as used in this trial, when fed over a longer period, can contribute to improved AA digestibility (Walk and Ramo Rao, 2018).

We conclude that the phytase used in this study increases G:F as shown in the initial period (day 8–15). Duration of the application might play a significant role in the response to phytase to growth performance in birds that are fed marginally deficient diets ad libitum. However, the reduction of P and Ca in the diets might have been insufficient to see significant phytase effects in the total trial period. Phytase caused an almost complete hydrolysis of phytate as shown by the reduced InPs levels in the digesta and additionally indicated by the elevated plasma and digesta MI concentration. This did not translate into consistent effects on AA digestibility or expression of nutrient transporters but improved apparent ileal Cys digestibility. The increased MUC2 expression accompanied by further phytate degradation in the upper intestinal tract suggests that high phytase can contribute to regulation of gut integrity.

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