Effect of phytase on intestinal phytate breakdown, plasma inositol concentrations and glucose transporter type 4 abundance in muscle membranes of weanling pigs

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ABSTRACT: The objective of this current study was to determine the effects of phytase dosing on growth performance, mineral digestibility, phytate breakdown and the level of glucose transporter type 4 (**GLUT4**) in muscle plasma membranes of weanling pigs. A total of 160 barrows were used in a randomized completely block design and assigned to four treatments for a 7-week study. Depending on the feeding phase, diets differed in dietary calcium (**Ca**) and phosphorus (**P**) levels (PC:8 to 6.8g/kg Ca; 7.3 to 6.3 g/kg P; negative control (**NC**):5.5 to 5.2 g/kg Ca; 5.4 to 4.7 g/kg P). NC diets were supplemented with phytase at 0 (NC); 500 (NC+500 FTU) or 2000 FTU/kg (NC+2000 FTU) phytase units/kg. Blood was collected after fasting (d 48) or feeding (d 49) for measurement of plasma inositol concentrations. On d 49, two pigs per pen were euthanized, duodenal and ileal digesta samples were collected to determine inositol phosphates ($InsP_{6-2}$) concentrations. High phytase supplementation increased body weight (**BW**) on d 21, 35 and 49 (P <0.05). Over the entire feeding period, average daily gain (**ADG**), average daily feed intake (**ADFI**) and feed efficiency were increased by NC+2000 FTU compared to the other treatments (P <0.05). Postprandial plasma inositol concentration was increased in NC+2000 (P < 0.01), but there was only a tendency (P = 0.06) of a higher fasting plasma inositol concentration in this group. Inositol concentrations in the portal vein plasma (d 49) were not different among treatments. Duodenal digesta $InsP_5$ and $InsP_6$ concentrations were similar in PC and NC, but higher in these two treatments (P < 0.05) than those supplemented with phytase. Phytase supplementation decreased $InsP_{6-4}$, resulting in increased $InsP_{3-2}$ and *myo*-inositol concentrations. Similar effects were found in ileal contents. Compared to NC, phytase supplementation resulted in greater cumulative $InsP_{6-2}$ disappearance (93.6% vs. 72.8% vs. 25.0%, for NC+2000 FTU, NC +500 FTU and NC, respectively, P < 0.01) till the the distal ileum. Longissimus dorsi muscle plasma membrane GLUT4 concentration was increased by

NC+2000 FTU ($P < 0.01$) compared to NC. In summary, high phytase supplementation increased growth performance of nursery pigs. The higher *myo*-inositol release from phytate could contribute to the increased expression of GLUT4 in muscle plasma membranes. Further investigation is needed to determine if this is associated with enhanced cellular glucose uptake and utilization.

Key words: Phytase, Nursery pigs, Inositol, Growth performance; GLUT4

INTRODUCTION

Plant seeds store phosphorus mainly as phytate (Kumar et al., 2012). The phytate molecule is essentially a salt of *myo*-inositol (a.k.a. inositol) phosphate because it is able to bind minerals within the molecule. Phytate is also a major antinutrient in monogastric animals as it cannot be efficiently degraded due to low phytase activity in the gastro-intestinal tract. Inositol phosphates (**InsPs**) can bind minerals and this reduces digestibility of bound cations, including calcium (**Ca**), phosphorus (**P**),and Zinc (Woyengo et al., 2009; Woyengo and Nyachoti, 2013) as well as iron (Laird et al , 2018). Phytate also binds proteins and enzymes such as trypsin and α-amylase and inhibits their activities, consequently reducing protein and carbohydrate digestibility (Singh and Krikorian, 1982; Deshpande and Cheryan, 1984). This effect ultimately reduces growth performance of animals. Recently, feeding high levels of phytase in the diet has become more common and effects attributable to the released *myo*-inositol from phytate degradation have been recently discussed (Sommerfeld et al, 2018; Laird et al, 2018). Apart from these positive effects of phytate degradation and the enhanced P utilization by the animal, phytase use can have beneficial effects on the environment by limiting the release of undigested phosphorus into soil and ground water.

Use of microbial (fungal or bacterial derived) phytase has led to a reduction in the level of inorganic phosphorus used in animal diets. Currently, phytase is mostly added at 500 FTU/kg to pig diets as the standard inclusion rate (Wilcock et al., 2016). It has been reported that 500 FTU/kg phytase is equal to 0.3-1.7 g/kg inorganic P depending on the phytase source (Augspurger et al., 2003). It has been shown that 2000-2500 FTU phytase/kg feed could lead to an additional increase in growth performance of pigs beyond the expected growth increase from just releasing adequate P to support growth (Santos et al., 2014; Laird et al., 2018). Zeng et al. (2016) reported that phytase up to 20,000 FTU/kg feed could increase digestibility of crude protein and amino acid (**AA**). However, the mechanism of the extra phosphoric effect of higher phytase inclusion level than recommended (super dosed phytase) is still poorly defined. One possible reason is that the more complete phytate degradation by super dosed phytase releases higher levels of *myo*-inositol, which is known to have insulin-like effects (Lee and Bedford, 2016; Huber, 2016). *Myo*-inositol may increase insulin sensitivity by enhancing the concentration of PIP3 in the cell (Jiang et al., 1998). In addition, *myo*-inositol may also promote insulin secretion from pancreatic β cells. In mammalian cells, pyrophosphates (**$InsP_7$**) are synthesized from *myo*-inositol through a series of chemical reactions involving many enzymes such as inositol hexakisphosphate (**$InsP_6$**) kinases, diphosphoinositol pentakisphosphate (**$PP\text{-}IP_5$**) kinases (Wilson et al., 2013). It has been reported that overexpression of $InsP_6$ kinases in pancreatic β cells increased production of $InsP_7$, and this stimulated exocytosis of insulin in a dose-dependent manner (Illies et al., 2007). Insulin is known to stimulate glucose uptake by increasing the translocation of intracellular glucose transporter type 4 (**GLUT4**) vesicles to the plasma membrane of myocytes (Kahh, 1996), and it is suggested that glucose transport is the rate limiting step in muscle glycogen synthesis (Richter and Hargreaves, 2013). Thus, by promoting

glucose utilization in muscle, and perhaps other tissues, phytase could be contributing to increased animal growth, through a mechanism that is different from its effect in increasing P availability. Therefore, the objective of the current study was to determine the effect of high phytase level on growth performance, mineral digestibility and inositol phosphate disappearance, and to determine effects on plasma metabolites and GLUT4 muscle plasma membrane concentration.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Purdue Animal Care and Use Committee. A total of 160 weanling barrows (initial body weight 5.6 ± 0.5 kg) were used in a randomized complete block design. The experiment was performed in two replicate runs using 80 pigs per run. In each run, treatments were replicated in 5 pens with pigs of similar body weight (block) in the same pen. There were 4 pigs per replicate pen. Therefore, for the two runs of the experiment, there was a total of 10 replicate pens per treatment. Pigs were fed according to a three-phase feeding program post weaning; phase 1 (d 0-21), phase 2 (d 21-35) and phase 3 (d 35-49).

Dietary treatments

All diets were fed as mash diets. During phase 1, pigs in the positive control (PC) and negative control (NC) treatments were fed a common control diet which met their nutrient requirements to ensure sufficient phosphorus build up during this period. The two enzyme groups were supplemented with 500 and 2000 FTU/kg phytase on top of the PC diet. At the end of phase 1(d 0-21), half of the pigs fed with control diet during phase 1 were assigned to the PC treatment which had sufficient dietary phosphorus concentration that met the nutrient

requirement of weanling pigs (NRC, 2012), and the other half were given the NC diet which contained reduced standardized total tract digestible (**STTD**) Ca (-1.6 g/kg) and P (-1.4 g/kg) compared to PC diets. Two levels of phytase (500 FTU/kg and 2000 FTU/kg feed, Quantum™ Blue, AB Vista, Marlborough, UK) were added to the NC diet for treatments NC+500 FTU and NC+2000 FTU, respectively. Titanium dioxide (**TiO_2**) was added at 0.5% to phase 3 diets to determine apparent ileal digestibility (**AID**) of calcium, phosphorus and disappearance of inositol phosphate ($InsP_6$) and of cumulated $InsP_{6\text{-}2}$. The diet formulations are presented in Table 1.

Experiment procedure and sample collection

Pigs were weighed every week. Feed intakes were recorded and feed efficiency was calculated based on the ratio of average daily gain (**ADG**) and average daily feed intake (**ADFI**) (**G:F**). On d 47, feeders were closed for about 12 h in the evening, and blood samples were collected from the jugular vein from two pigs per pen on d 48 (fasting blood). On d 49, the same two pigs from each pen were injected with 0.5 mL of Telazol (Zoetis Inc. MI USA, reconstituted with 5 mL of xylazine (RXV Inc. CA USA)) to provide 0.2 mg tiletamine base, 0.2 mg zolazepam base and 0.2 mg mannitol per kg BW of pig to induce anesthesia and euthanized with CO_2 asphyxiation. Blood samples from the portal vein (portal blood) and jugular vein (fed blood) were collected in a vacutainer tube containing lithium heparin (BD Inc. NJ USA). Tubes were shaken slightly to mix thoroughly with the anticoagulant and immediately put on ice. Digesta from proximal duodenum (about 50 cm length from duodenal bulb) and distal ileum (about 50 cm length from ileal-cecal junction) were collected and pooled from two pigs per pen, and immediately frozen at -20°C. Longissimus dorsi muscle tissue was collected from each pig and immediately frozen in liquid nitrogen.

Phosphorus, calcium and inositol phosphates determination

Duodenal and ileal digesta were freeze dried, and ground to pass through a 0.5-mm screen before analysis. Subsamples of ileal digesta and diets were sent to the University of Missouri (MO, US) for determination of titanium concentration. Briefly, samples were digested in H_2SO_4. Then 30% H_2O_2 was added and the absorbance of samples was measured at 406 nm wavelength (Myers et al., 2004). Phosphorus concentration was determined by digesting the samples in concentrated nitric acid and 70% perchloric acid. Absorbance was measured at 620 nm using a spectrophotometer (SpectraCount, model AS1000; Packard, Meriden, CT; AOAC, 2006) as described by Zhai and Adeola (2013). Calcium concentration was determined using an atomic absorption spectrometer (AAnalyst 300; PerkinElmer, Norwalk, CT). Inositol phosphates (InsPs: $InsP_6$, $InsP_5$, $InsP_4$, $InsP_3$ and $InsP_2$) were determined by the post column UV detection method of Phillippy and Bland (1988) after Blaabjerg et al. (2010). Milled, dry feed or digesta (100mg) were extracted with 5 mL 100 mm NaF, 20 mM disodium EDTA, pH 10, for 30 min with shaking, followed by 30 min in a bath sonicator at approximately 10 °C and a further 2 h standing at 4 °C. The extract was centrifuged at 9,000 x *g* for 15 minutes at 4 °C and an aliquot of the supernatant filtered through a 13 mm 0.45 µm pore size PTFE syringe filter (Kinesis, UK). Samples (20 µL) were injected onto a 3mm x 200 mm Dionex CarboPac PA-200 column with 3 x 50 mm guard column of the same material. The column was eluted at a flow rate of 0.4 $ml.min^{-1}$ with water (A) and 0.6 M methanesulfonic acid (B), mixed according to the following schedule: time (min), % B; 0, 0; 25, 60; 28, 100; 38, 100. The post column reagent was added at a flow rate of 0.2 $mL.min^{-1}$ and the whole routed through a 192 uL volume knitted reaction coil to a UV detector set at 290 nm. For inositol measurement of feed and digesta, the acid extract was diluted 50-fold with 18.2 Mohm.cm water and aliquots (20 µL) analysed by HPLC with pulsed amperometric detection according to Lee et al. (2018). A 7-point calibration curve (0-8 µM)

gave a linear correlation coefficient $r^2 > 0.996$. AID of phosphorus, calcium, $InsP_6$ and cumulated $InsP_{6-2}$ were calculated using following equation:

$$AID, \% = [1-(Ti_i / Ti_o) \times (Y_o / Y_i)] \times 100;$$

Where Ti_i and Ti_o are the titanium concentrations of the diet and ileal digesta, respectively (mg/kg of DM); and Y_o and Y_i are the concentrations of phosphorus, calcium, $InsP_6$ or cumulated $InsP_{6-2}$ in the ileal digesta and diet, respectively (nmol/g DM).

Plasma sample preparation metabolites and inositol concentration

Heparinised blood samples were centrifuged at 2000 × g for 15 min at 4 °C and the supernatant was transferred to a new tube and stored at -80 °C before analysis. Plasma glucose concentrations were determined using the Autokit glucose kit (Wako Pure Chemical Industries Ltd., Chuo-Ku Osaka, Japan) following the manufacturer's protocol. Non-esterified fatty acid (NEFA) concentrations were determined using a NEFA kit from the same manufacturer. Plasma insulin concentration was determined using the porcine insulin ELISA kit following the manufacturer's protocol (Inter and intra-assay CV: 2.7 and 3.5% ; Mercodia, Uppsala, Sweden). Plasma triglyceride was determined with the triglyceride determination kit (Sigma Aldrich, St Louis, MO). For analysis of plasma inositol, frozen plasma samples were first thawed, then 1N perchloric acid was added (2 volume of perchloric acid to 1 volume of plasma). Samples were incubated at 4 °C for 30 min before centrifugation at 17,500 × g for 10 min. The supernatant was transferred to a new tube and sent to University of East Anglia (Norwich, UK) for *myo*-inositol analysis by HPLC-pulsed amperometry by the method of Lee et al. (2018). The acidified plasma samples were diluted 20-fold with 18.2 Mohm.cm water and 20 µl samples injected. A 5-point calibration curve of inositol standards 0-1 µM gave a linear correlation coefficient $r^2 > 0.996$.

Determination of muscle plasma membrane GLUT4 concentration and western blot analysis

Frozen muscle samples were thawed on ice, and approximately 1 g of sample was mixed with 2 mL mannitol-HEPES buffer (100 mM mannitol, 10 mM Tris-HEPES, pH 7.4) and homogenized. Homogenized samples were centrifuged at 1,800 x g for 10 min at 10 °C, and the supernatant filtered through a 70 nm nylon mesh. The infiltrate was then centrifuged at 45,000g for 45 min at 4 °C to obtain a crude membrane pellet. The membrane pellet was resuspended in 1 ml of mannitol-HEPES buffer to obtain the crude membrane protein fraction. Protein concentration in the crude membrane fraction was determined with the bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich). Membrane GLUT4 concentrations were measured using the porcine glucose transporter 4 ELISA kit (Inter and intra-assay CV: < 15%; MyBioSource, Inc. San Diego, CA) following the manufacturer's protocol. Data obtained were standardized with the total protein concentration in each sample.

Protein expression of Akt and phosphorylated-Akt was determined by western blot using SDS-PAGE (Yan and Ajuwon, 2017). Muscle samples were homogenized in 1× RIPA buffer [50 mmol/l Trizma-HCl (pH 7.4), 15 mmol/l NaCl, 0.25% deoxycholic acid, 0.1% Triton X, 10 mmol/l EDTA, 1 mmol/l Na2VO3 and protease inhibitor cocktail] (Sigma-Aldrich). Tissue homogenates were centrifuged at 10,000g for 10 min at 4°C. Protein concentration in clear homogenates was determined with the bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich). Protein samples were resolved by 10% acrylamide gel. Proteins were transferred to a 0.2- μm pore-size nitrocellulose membrane by the semidry method (Bio-Rad, Hercules, CA). Membranes were blocked in 5% BSA in TBS (50 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl). Immunoblotting was performed using a polyclonal antibody against mouse protein kinase B (**Akt**) and phosphorylated-Akt (Cell signaling, Beverly, MA) at a dilution of 1:1000. Membranes were

stripped and reblotted with rabbit anti-β-actin antibody (Cell Signaling Technology, Beverly, MA) at a dilution of 1:1000. Blots were then incubated with IRDye® 800CW secondary antibodies (LI-COR, Lincoln, NE) at a dilution of 1:2000. Signal was detected and quantified with the imaging software on the Odyssey CLx machine (Samkoe et al., 2019; LI-COR, Lincoln, NE).

Statistical Analysis

Data were analyzed using the Proc GLM procedure of SAS (SAS Inst. Inc., Cary NC) for a randomized complete block design with diet as the main effect. Pen was the experimental unit. The model included period (two runs), replicate (block) within period and diet. There was no difference between the first and second runs of the experiment. Therefore, data from both runs were pooled. Results are reported as least square means and standard errors of the means. Means were different at $P \leq 0.05$. When diet effect was significant at $P < 0.05$, differences between means were compared using the Tukey test. Superscript designations were used to indicate significant mean differences.

RESULTS

Growth performance and mineral digestibility

Phosphorus concentration in the PC and NC diets were 7.1, 6.4 and 5.2, 5.7 and 4.1 g/kg feed for phases 1, 2 and 3 respectively (Table 1). Body weights were not different between PC and NC treatments on day 35 and 49. The application of phytase at 500 FTU/kg feed had no significant effect on BW compared with NC or PC treatments. However, phytase supplementation at 2000 FTU/kg feed significantly increased BW ($P < 0.01$; Table 2 and 3).

Phytase at 500 FTU/kg feed had no significant effect on ADG, ADFI and G:F in phases 2, 3 and overall, whereas phytase at 2000 FTU/kg feed significantly increased these parameters ($P < 0.05$; Table 3). Both phytase application rates increased AID of P compared to the NC. Pigs that received the PC diet had higher daily Ca and P absorption than NC pigs ($P < 0.01$; Table 4), and these were not different from the NC+2000 FTU pigs.

Plasma metabolites and inositol concentration

Fasting blood plasma glucose, triglyceride and insulin concentrations were not different among treatments (Table 5). However, the NEFA concentration was higher in phytase-fed piglets with differences being significant only for the NC + 500 FTU treatment compared to NC and PC ($P < 0.01$; Table 5). In general, fasting blood plasma inositol concentration was 4 to 6 fold higher than levels analyzed in fed or portal blood plasma (Table 6). Fed blood plasma inositol concentration was greatest in the NC +2000 FTU treatment ($P < 0.01$; Table 6), and for this treatment, a tendency of a higher fasting blood plasma inositol concentration was observed also ($P = 0.06$; Table 6). However, inositol concentration in the portal vein blood was not different among treatments (Table 6).

Digesta inositol phosphates

Duodenal digesta $InsP_5$ and $InsP_6$ remained highest in PC and NC with no differences between these treatments. In contrast, phytase supplementation decreased duodenal $InsP_6$ (Table 7) and the 2000 FTU/kg treatment significantly decreased $InsP_5$ level as well. Lower InsPs ($InsP_4$, $_3$ and $_2$) and *myo*-inositol concentrations were increased by NC+500FTU while 2000 FTU/kg treatmentincreased ($P < 0.01$) duodenal *myo*-inositol level only (Table 7). Similarly, phytase supplementation decreased $InsP_6$ and $InsP_5$ concentrations in the ileal digesta. $InsP_6$

disappearance was greater with increasing phytase supplementation (90% and 97.9%) and cumulated $InsP_{6-2}$ disappearance was 93.6% in NC+2000 FTU and 72.8% in NC+ 500 FTU compared to 25 – 31.5% in the control treatments ($P < 0.01$; Table 7). The concentration of $InsP_5$ in the ileal digesta was higher in the NC and PC treatments compared to treatments containing phytase ($P < 0.001$, Table 7). Feeding NC+500 resulted in increased $InsP_4$, $InsP_3$, $InsP_2$ and *myo*-inositol abundance in the ileal digesta ($P < 0.01$, Table 7). However in NC+2000 piglets, the increase of lower InsPs in the ileum was reversed compared with NC+500 with the exception of $InsP_2$ and *myo*-inositol increased further ($P < 0.001$, Table 7). Although total $InsP_5$ and $InsP_4$ differed between treatments, these effects were not significant for single $InsP_4$ and $InsP_5$ isomers. Separation of inositol phosphate (InsP) isomers1 of InsP4 and InsP5 based on existing standards and probability of peak separation in duodenal and ileal digesta of piglets fed diets different in dietary P/Ca and phytase levels is presented in Table 8. No significant differences in the concentrations of these isomers were observed.

Analysis of muscle plasma membrane GLUT4 concentration by western blot

Lowest longissimus dorsi muscle plasma membrane GLUT4 concentrations were found in NC piglets but this was not different compared to NC+500 and PC ($P >0.05$). However, the highest GLUT4 concentration was found in the NC+2000 treatment ($P < 0.01$ compared to NC; Figure 1). There was no treatment effect on protein kinase B (Akt) and phosphorylated-Akt concentration in muscle (Figure 2).

DISCUSSION

Growth performance and mineral digestibility

Phytase is typically added to swine diets at 500 FTU/kg feed to release 0.3-1.7 g/kg available phosphorus (Augspurger et al., 2003; Wilcock and Walk, 2016). However, there is growing interest in supplementing higher phytase levels (>1500 FTU/kg feed) to diets. This is considered as super dosing if no further reduction in dietary P and Ca is implemented with high phytase supplementation. A deficiency of non-phytate phosphorus typically results in reduction in feed intake and exogenous phytase is known to increase feed intake in weaner pigs (Kornegay and Qian, 1996). The increased BW, ADG, ADFI and feed efficiency in pigs fed phytase at 2000 FTU/kg diet compared with the NC (and partly the PC) also agrees with reported effects of super dosed phytase. The work by Kies et al. (2006) and Zeng et al. (2015) showed that phytase at 15,000 FTU/kg or 20,000 FTU/kg diet increased ADG and feed efficiency of weanling pigs. In a study by Nyannor et al. (2007), a linear effect of phytase was found on ADG and feed efficiency when phytase was added at 16,500; 33,000 and 49,500 FTU/kg feed. In a trial with pigs fed the same phytase as used in this trial, 2500 FTU/kg feed, but not 500 FTU/kg feed, improved ADG and G:F (Laird et al., 2018). Therefore, it became necessary to investigate possible extraphosphoric effects of phytase. A probable explanation for the performance improvements in super dosed fed pigs could be related to an increased nutrient digestibility, especially minerals, proteins and amino acids. Adedokun et al. (2015) reported that phytase increased AID of DM, nitrogen, Ca, P and several amino acids in cannulated pigs using 4 levels of phytase (0, 500, 1000, and 2000 FYT/kg feed). Similar results were reported by Zeng et al. (2016) demonstrating that a high dose of phytase (20,000 FTU/kg feed) increased, in addition to Ca and P, the

digestibility of other minerals and trace minerals (Na, K, Mg and Zn). Nyannor et al. (2007) found that phytase added at 16,500, 33,000 and 49,500 FTU/kg feed linearly increased digestibility of DM, GE, Ca and P. In the current study, adding 2000 FTU/kg feed to a P-deficient corn-soybean meal based diet increased AID of Ca and P by 16.1 and 35.2%, respectively, compared to NC. Absorption of Ca and P was also greater in pigs that received 2000 FTU/kg feed (158% and 148% compared to NC, respectively) with no difference compared to the PC. Ths suggested that 2000 FTU/kg could recover the difference in Ca and P between the NC abd PC respectively at the very least.

There was no significant difference between the PC and NC treatments in growth performance. This could mean P was not sufficiently reduced in the NC diet compared to PC to have any effect on performance although if could be the case that even the PC diets were still not adequate in P. This is, to some extent, supported by the higher feed intake in pigs that received phytase at a level of 2000 FTU/kg diet. However, the calculated dietary standardized total tract digestibility (STTD) of P in the NC diet was 0.3% and 0.25% for phases 2 and 3, respectively. This was about 0.14% lower compared with the respective digestible P level in PC diets. According to NRC (2012), the STTD P requirements for pigs weighing between 7-11 kg and 11-25 kg are 0.4% and 0.33%, respectively. Alexander et al. (2008) reported that there was no difference in growth performance of growing pigs with a 20% reduction in total P compared with a P adequate diet. Santos et al. (2014) also reported the lack of a difference between PC and NC with a 0.15% reduction in available P in pigs with initial BW of about 23kg. Therefore, these results suggest that P application met the requirement and was not deficient as to reduce growth performance in PC and NC treatment groups in the current study.

The daily Ca and P absorption in the PC piglets were 60% and 35% higher compared with the absorption calculated for NC+500 FTU animals, whereas for the NC+2000FTU treatment, similar daily Ca and P absorption was noted compared with the PC (6.4 vs 6.3 g/d and 3.5 vs 3.1 g/d for Ca and P, respectively). However, there was no growth performance difference between the PC and NC+500 FTU treatments, suggesting that P was limiting and thus affecting growth. Despite the similar daily Ca and P absorption between the PC and 2000 FTU/kg diet, the latter resulted in increased ADG and G:F. Thus, it can be speculated that the positive phytase effect of the NC+2000 FTUtreatment on growth performance was not due to increased utilization of Ca and P .

Inositol phosphates

Phytate degradation by phytase releases P and finally, $InsP_1$. The final conversion of $InsP_1$ to inositol is thought to be effected by intestinal phosphatases. In theory, phytase could degrade phytate completely through a step-wise dephosphorylation ($InsP_6 \rightarrow InsP_5 \rightarrow InsP_4 \rightarrow InsP_3 \rightarrow InsP_2 \rightarrow InsP_1$). However, *in vivo*, hydrolysis of phytate is often incomplete, leading to a mixture of inositol phosphate esters (Humer et al., 2015; Zeller et al., 2015; Kühn et al., 2016; Laird et al. 2018). The increased formation of $InsP_4$ to $InsP_2$ by 500 FTU phytase/kg feed, and a numerically higher level of *myo*-inositol at the 2000 FTU/kg phytase supplementation indicated that the super dosed level of phytase resulted in a more complete breakdown of phytase compared to supplementation at 500 FTU/kg feed. These results demonstrate that intermediate inositol phosphates like $InsP_3$ and $InsP_2$ can be further hydrolyzed by application of high phytase concentration. These results are in line with the work done by Kemme et al. (2006) who found that $InsP_6$ was mainly dephosphorylated to $InsP_3$ and $InsP_2$ with 900 FTU phytase /kg feed.

Similarly, Laird et al. (2018) found a dose-dependent increase in gastro-intestinal InsP degradation and myo-inositol concentration in the digesta when feeding either 500 or 2500 FTU /kg feed of the same phytase as used in this trial.

Compared to NC the concentration of $InsP_6$ in the duodenum was about 72 and 91% and in the ileum s 87 and 96% lower in piglets fed the 500 and 2000 FTU phytase /kg feed, respectively. The optimal pH for *E. coli* phytase is around 4.5 (Igbasan et al., 2000) and the activity of mucosa phytase in pigs is very low (Jongbloed et al., 1992; Schlemmer et al. 2001). This, and the fact that phytate solubility decreases with pH above 4 to 4.5 (Angel et al 2002), indicates that the stomach is the main site for InsP6 hydrolysis by the this phytase . The $InsP_6$ disappearance in the ileal digesta was 97.9% with 2000 FTU/kg which is similar to previously reported analyses in broilers in which 12,500 FTU/kg of the same phytase resulted in 92% $InsP_6$ hydrolysis (Zeller et al., 2015). Laird et al. (2018) also found a dose-dependent effect of phytase with 67.6 and 78.1% $InsP_6$ disappearance at the terminal ileum when 500 and 2500 FTU/ kg phytase was used, respectively.

The ileal *myo*-inositol concentration was greatest in the NC+2000 FTU treatment and 7.4 fold higher compared to NC. In addition, the blood *myo*-inositol concentrations were highest in the NC+2000 FTU fed piglets. This was especially noticeable and significant in fed plasma *myo*-inositol concentration in the NC+2000 FTU treatment that was 1.6 fold higher compared to that in NC piglets. Others (Guggenbuhl et al 2016; Laird et al 2018) have also found increased plasma inositol concentrations with increased dietary phytase application. A study done by Cowieson et al. (2017) reported an immediate increase of plasma *myo*-inositol concentration after oral ingestion of *myo*-inositol, and 3000 FTU phytase /kg feed significantly increased plasma *myo*-inositol 6 h after feed intake. Therefore, the increased *myo*-inositol concentration in

the fed status at 2000 FTU/kg was likely due to the released *myo*-inositol by phytase. However, only a numerical increase of portal vein myo-inositol concentration was observed in the current study with a large variability in the data set. This is in contrast to Laird et al 2016, who found higher *myo*-inositol concentration in portal, compared to, peripheral pig plasma. However, such a difference was not seen in another trial carried out by Laird et a (2018). Taken together, the inconsistency in the effect of phytase on plasma *myo*-inositol concentrations could be due to differences in experimental conditions in different studies, and perhaps due to variability in data sets. Factors such as timing of sampling relative to feeding, differences in dietary phytate, P and Ca level could contribute to this variability. Additional experiments are needed to investigate effects of these factors, and the potential role that saturation of the transporter for *myo*-inositol (Na+ dependent transporter SMIT2, Sasseville et al., 2014) may play in regulating plasma *myo*-inositol concentrations.

Muscle plasma membrane GLUT4 concentration

Longissimus dorsi muscle is a good tissue target for measuring energy sensing and insulin signaling (Manjarín et al., 2016). Insulin is a peptide hormone that activates PI 3-kinase signaling, which, through conversion of PIP_2 to PIP_3, recruits PDK1 to the plasma membrane with resultant phosphorylation of AKT and subsequent translocation of GLUT4 to the plasma membrane of skeletal muscle. As a metabolic precursor of PIP3 it can be speculated that *myo*-inositol may have distal insulin-like effects. Indeed, while *myo*-inositol supplementation reduces fasting blood glucose level in humans with type 2 diabetes (Pintaudi et al., 2016), there was no treatment effect of phytase on fasting blood glucose and insulin concentrations in this piglet study.The lack of a phytase effect on blood glucose concentration in the current study suggests that physiological blood glucose concentrations may not be under significant regulation by

inositol levels generated in this study. In addition, treatment had no effect on the level of phosphorylated-Akt. However, GLUT4 concentration in muscle plasma membranes was increased by high phytase level (NC+2000 FTU, $P < 0.05$) supplementation. Chukwuma et al. (2016) reported that *myo*-inositol could promote rat muscle glucose uptake. Therefore, although phytase did not affect serum glucose and insulin levels in this study, possibly due to a complex metabolic regulation of these levels, it still might affect certain aspects of insulin signaling, such as an increase in membrane GLUT4 concentration. This effect has a potential to increase muscle tissue glucose uptake to support tissue growth. Dang et al. (2010) reported that oral injection of *myo*-inositol could induce the translocation of GLUT4 to the membrane of skeletal muscle in mice. In a subsequent study, the same group (Yamashita et al., 2013) showed that 1g/kg oral *myo*-inositol raised plasma *myo*-inositol from < 0.1mM to 2.67 ± 0.72mM also induced GLUT4 translocation. It is plausible, therefore, that the increased GLUT4 level in muscle plasma membranes of NC+2000 compared to NC piglet might be due to species variation in sensitivity to inositol and the increased *myo*-inositol release from phytate. However, there may also be an interaction between P and *myo*-inositol in regulating GLUT4 abundance as higher GLUT4 level was also found in the PC treatment, despite the lower blood plasma *myo*-inositol concentration in this treatment. Thus, the regulation of GLUT4 by *myo*-inositol and P requires further investigation.

We conclude that super dosed phytase (2000 FTU/kg feed) improved growth performance of weanling pigs and caused an almost complete hydrolysis of phytate ($InsP_6$, 97.8%; 93.6% $InsP_{6-2}$). The increased released of *myo*-inositol with high phytase supplementation may explain some of the extra phosphoric effect seen at this level, and this may suggest increased muscle glucose uptake. Future studies will be needed to better understand the

effects of *myo*-inositol, its absorption, utilization and regulatory factors on growth performance of weanling pigs.

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FIGURE LEGENDS

Figure 1: Longissimus dorsi muscle plasma membrane GLUT4 concentration in piglets fed diets different in P, Ca and phytase levels sampled at the end of the overall feeding period of 49 days.

Figure 2: Longissimus dorsi muscle Akt and phosphorylated-Akt (p-Akt) protein expression in piglets fed diets different in P, Ca and phytase levels at the end of the overall feeding period of 49 days.

Table 1. Ingredient composition and analyzed nutrient composition of experimental diets on an as-fed basis of PC and NC diets

	Day 0-21	Day 21-35		Day 35-49	
	PC	PC	NC	PC	NC
Ingredient, g/kg					
Corn	380.25	458.9	472.8	441.9	456.3
Soybean Meal	250	300	300	300	300
Soy protein concentrate	88	58.5	58.5	58.5	58.5
Whey, dried	180	100	100	100	100
Lactose	30	51	51	51	51
Fish meal	40	0	0	0	0
Soybean oil	12	9	3.5	9	3.4
Limestone	7.5	8.75	7.6	9	8.2
Monocalcium Phosphate	6.5	11.25	4	9	1
L-lysine-HCl	0	1	1	1	1
DL Met	0.6	0.6	0.6	0.6	0.6
TiO_2 premix, [1]	0	0	0	15	15
Salt	1	1	1	1	1
ZnO	0.15	0	0	0	0
Vitamin premix, [2]	2.5	2.5	2.5	2.5	2.5
Mineral premix, [3]	1.5	1.5	1.5	1.5	1.5
Total	1000	1000	1000	1000	1000
Calculated composition					
ME (kcal/kg)	3415	3354	3354	3310	3311
CP, g/kg	254	231	232	230	231
Ca, g/kg	8.0	7.1	5.5	6.8	5.2
Total P, g/kg	7.3	6.8	5.4	6.3	4.7
STTD P, g/kg	4.8	4.4	3.0	3.9	2.5
Analyzed composition					
CP, g/kg	248	225	228	231	229
Ca, g/kg	8.2	7.3	5.7	7.5	5.1
Total P, g/kg	7.1	6.4	5.2	5.7	4.1
InsP6-2, µmol/kg	-	-	-	17224	14859
myo-inositol, µmol/kg	-	-	-	422	340

[1]TiO_2 premix resulting in 3g Ti/kg feed .
[2]Vitamin premix supplied per kilogram of diet: 3,635 IU vitamin A, 363 IU vitamin D3, 26.4 IU vitamin E, 3.6 mg vitamin K, 1,206 µg menadione, 21.2 µg vitamin B12, 4.2 mg riboflavin, 13.5 mg d-pantothenic acid, and 19.5 mg niacin.
[3]Mineral premix supplied per kilogram diet: 9 mg Cu (as copper sulfate),0.34 mg I (as Ca iodate), 97 mg Fe (as ferrous sulfate), Fe (as ferrous sulfate), 12 mg Fe (as ferrous sulfate), and 97 mg Zn (as zinc oxide).

Table 2. Growth performance of pigs fed experimental diets during day 0-21

	PC	PC + 500 FTU	PC + 2000 FTU	SEM	*P* -value
BW, kg					
Day 0	5.6	5.6	5.6	0.08	0.99
Day 21	8.2[b]	8.8[ab]	9.5[a]	0.81	< 0.01
Day 0-21					
ADG, g/d	120[b]	148[ab]	186[a]	39.8	< 0.01
ADFI, g/d	234[b]	244[ab]	281[a]	40.3	0.02
G:F, g/kg	504[b]	600[ab]	650[a]	100.9	< 0.01

[1]Values are means of each treatment (n=20 for PC, a combination of each of the 10 replicates in the PC and NC pigs treatments because both groups were on the same diet during this period; n=10 for each of the PC+500 FTU and PC +2000 FTU treatments), means with different superscript differ significantly ($P < 0.05$). PC: positive control, PC+500 FTU and PC+2000 FTU: PC added with phytase by 500 or 2000 FTU/kg feed, respectively. BW: body weight, ADG: average daily gain, ADFI: average daily feed intake, G:F: gain to feed ratio.

Table 3. Growth performance of pigs fed experimental diets different in dietary P, Ca and phytase levels

	PC	NC	NC + 500 FTU	NC + 2000 FTU	SEM	*P* -value
BW, kg						
Day 21	8.2[b]	8.2[b]	8.8[ab]	9.5[a]	0.26	< 0.01
Day 35	14.1[b]	13.7[b]	14.4[ab]	16.1[a]	0.45	< 0.01
Day 49	23.1[ab]	21.4[b]	23.4[ab]	26.0[a]	0.69	< 0.01
Phase 2						
ADG, g/d	417[ab]	388[b]	404[ab]	468[a]	18.89	0.03
ADFI, g/d	696[b]	711[b]	716[b]	811[a]	22.52	< 0.01
G:F, g/kg	594	542	561	578	16.87	0.18
Phase 3						
ADG, g/d	636[a]	556[b]	637[a]	708[a]	21.01	< 0.01
ADFI, g/d	1163[ab]	1070[b]	1143[ab]	1235[a]	29.96	< 0.01
G:F, g/kg	551[ab]	521[b]	558[ab]	574[a]	11.83	0.02
Overall						
ADG, g/d	352[b]	321[b]	361[b]	416[a]	14.09	< 0.01
ADFI, g/d	628[b]	612[b]	636[b]	705[a]	17.43	< 0.01
G:F, g/kg	559[ab]	522[b]	565[ab]	588[a]	12.36	< 0.01

[1]Values are means of 10 replicate pens per treatment (5 replciate pens per run), means with different superscript differ significantly ($P < 0.05$). PC: positive control, NC: negative control, NC+ 500FTU and NC+2000 FTU: NC added with phytase added by 500 or 2000 FTU/kg feed, respectively. BW: body weight, ADG: average daily gain, ADFI: average daily feed intake, G:F: gain to feed ratio.

Table 4. Apparent ileal Ca and P digestibility and calculated absorption thereof in piglets fed diets different in P, Ca and phytase levels at the end of the overall feeding period of 49 days.

	PC	NC	NC + 500 FTU	NC + 2000 FTU	SEM	*P* -value
Ca digestibility, %	76.6^{ab}	70.3^{b}	69.6^{b}	81.6^{a}	2.70	< 0.01
P digestibility, %	55.3^{ab}	45.4^{c}	54.9^{ab}	61.4^{a}	2.54	< 0.01
Ca absorption, g/d	6.4^{a}	4.0^{b}	4.0^{b}	6.3^{a}	0.23	< 0.01
P absorption, g/d	3.5^{a}	2.1^{c}	2.6^{bc}	3.1^{ab}	0.16	< 0.01

Values are means of 10 replicate pens per treatment (5 replicate pens per run), means with different superscripts are different ($P < 0.05$). PC: positive control, NC: negative control, NC+500 FTU and NC+2000 FTU: NC added with phytase added by 500 or 2000 FTU/kg feed, respectively The absorption of Ca and P was calculated by multiplying the analysed digestibility of Ca and P with average daily mineral feed intake. Ca: Calcium, P: Phosphorus.

Table 5. Blood plasma metabolite concentrations of piglets fed diets different in P, Ca and phytase levels at the end of the overall feeding period of 49 days.

	PC	NC	NC + 500 FTU	NC + 2000 FTU	SEM	*P* -value
Glucose,mg/dL	100.1	97.3	89.5	92.7	3.81	0.21
TG,mg/ml	0.24	0.29	0.26	0.28	0.02	0.32
NEFA,mmol/L	0.51^{b}	0.59^{b}	0.78^{a}	0.68^{ab}	0.06	0.02
Insulin,ug/L	0.023	0.021	0.025	0.023	0.002	0.55

Data are means of 10 replicate pens pooled from 2 pigs per pen in each treatment, means with different superscripts are different ($P < 0.05$). PC: positive control, NC: negative control, NC+500 FTU and NC+2000 FTU: NC added with phytase added by 500 or 2000 FTU/kg feed, respectively TG: Triglycerol, NEFA: Non-esterified fatty acid.

Table 6. Plasma inositol concentration in fasting, fed and portal vein bloodof piglets fed diets different in P, Ca and phytase levels sampled at the end of the overall feeding period

	PC	NC	NC + 500FTU	NC + 2000 FTU	SD	*P* - value
Portal plasma inositol, nmol/ml	4.17	6.38	4.78	8.53	5.24	0.14
Fasting plasma inositol, nmol/ml	25.05	23.00	21.88	37.49	16.32	0.06
Fed plasma inositol, nmol/ml	5.87^{b}	5.97^{b}	6.08^{ab}	9.71^{a}	6.67	0.01

Data are means of 10 replicate pens pooled from 2 pigs per pen in each treatment, means with different superscripts differ significantly ($P < 0.05$). PC: positive control, NC: negative control, NC+500 FTU and NC+2000 FTU: NC added with phytase added by 500 or 2000 FTU/kg feed, respectively Values are means of each treatment with pigs were fasted overnight at d47, and fasting blood were collected on the following day (d 48, n=20). Fed blood and portal vein were collected on day 49 after slaughter, and (n=20,20,20,19 for PC, NC, 500, and 2000 phytase, respectively for fed blood), for portal vein (n=18, 16, 20, 17 for PC, NC, 500, and 2000 phytase, respectively.

Table 7. Inositol phosphate concentrations (nmol/g DM) in duodenal and ileal digesta and disappearance (%) of different inositol phosphates down to the ileum of piglets fed diets different in P, Ca and phytase levels sampled at the end of the overall feeding period of 49 days.

		PC	NC	NC+500 FTU	NC+2000 FTU	SEM	*P* -value
Duodenum, nmol/g DM							
	$InsP_6$	7615.7^{a}	7232.7^{a}	2014.3^{b}	661.8^{c}	657.8	<0.0001
	$InsP_5$	1283.4^{a}	1160.3^{a}	579.6^{a}	200.6^{b}	101.1	<0.0001
	$InsP_4$	234.7^{b}	285.2^{b}	970.8^{a}	377.4^{b}	78.9	0.0002
	$InsP_3$	313.6^{ab}	278.9^{b}	527.4^{a}	317.4^{ab}	32.1	0.02
	$InsP_2$	45.8^{b}	97.1^{ab}	200.9^{a}	247.7^{a}	21	<0.0001
	myo-inositol	1186.4^{b}	685.9^{b}	1472.8^{ab}	2153.8^{a}	178.2	0.0021
Ileum, nmol/g DM							
	$InsP_6$	28895.4^{a}	29265.0^{a}	3685.7^{b}	1012.8^{b}	2287.5	<0.0001
	$InsP_5$	5451.2^{a}	4295.5^{a}	1013.7^{b}	269.6^{b}	380.5	<0.0001
	$InsP_4$	1239.1^{b}	925.1^{b}	3856.9^{a}	1708.8^{b}	346.6	0.01
	$InsP_3$	369.9^{b}	380.3^{b}	1535.6^{a}	816.3^{ab}	109.1	<0.0001
	$InsP_2$	210.9^{c}	204.2^{c}	2118.6^{a}	1005.3^{b}	162.7	<0.0001
	myo-inositol	1531.7^{ab}	1168.5^{b}	6092.3^{a}	8689.4^{a}	547.9	0.001
Disappearance, %							
	$InsP_6$	35.7^{b}	25.4^{b}	90.0^{a}	97.9^{a}	3.2	<0.0001
	$InsP_{6-2}$	31.5^{c}	25.0^{c}	72.8^{b}	93.6^{a}	4.1	<0.0001

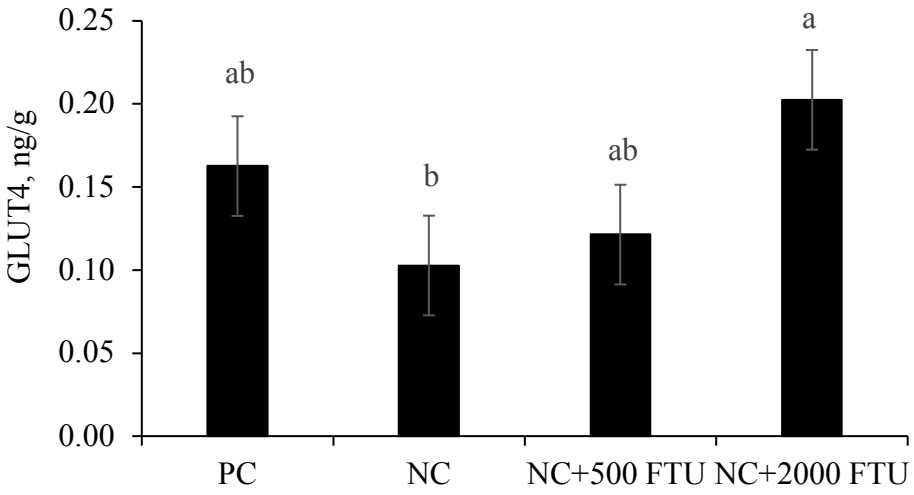
Values are means of 10 replicate pens with digesta pooled from two pigs per pen, means with different superscript differ significantly ($P < 0.05$). Disapperance rate was calculated using same formula for AID calculation. PC: positive control, NC: negative control, NC+500 FTU and NC+2000 FTU: NC added with phytase added by 500 or 2000 FTU/kg feed, respectively InsP: inositol phosphates.

Table 8. Separation of inositol phosphate (InsP) isomers[1] of InsP 4 and InsP 5 based on existing standards and probability of peak separation in duodenal and ileal digesta of piglets fed diets different in dietary P/Ca and phytase levels (nmol/g DM; n=10).

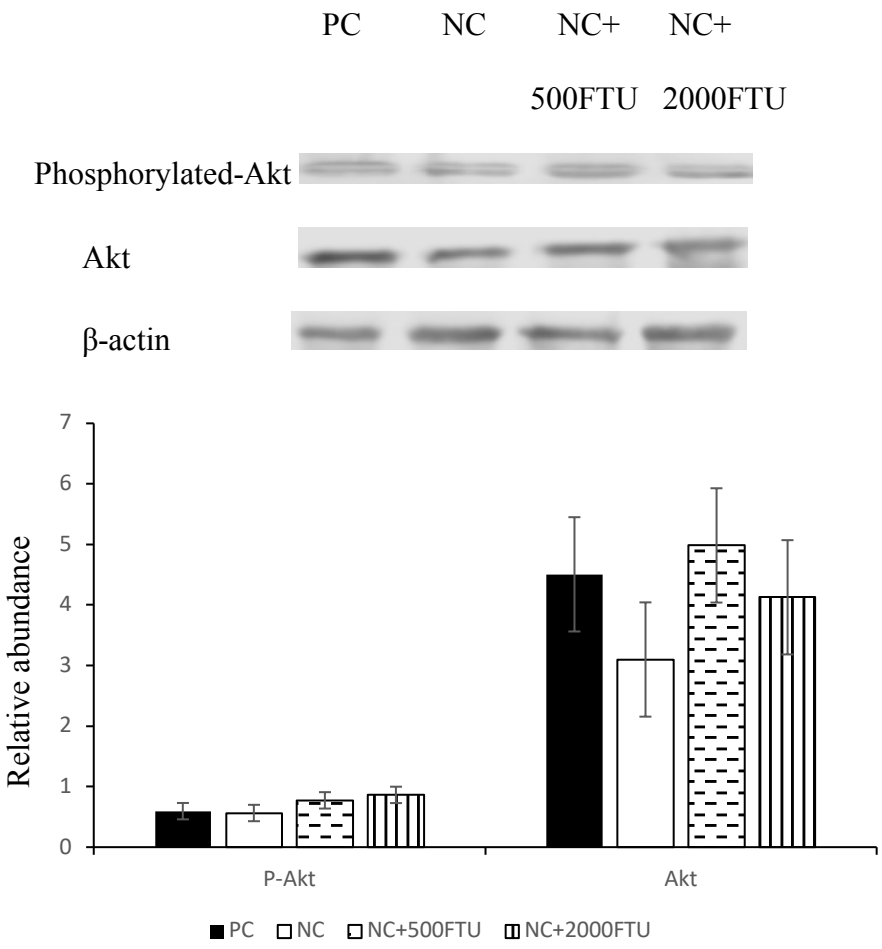
		PC	NC	NC+500 FTU	NC+2000 FTU	SE	*P* -value
Duodenum							
	Ins(1246/2346) P_4	41	0	45	0	-	-
	Ins(1234/1236) P_4	84	44	77	187	17	0.18
	Ins(1256/2345) P_4	172	241	905	378	158	0.89
	Ins(12346) P_5	134	119	66	31	21	0.92
	Ins(12356/12345) P_5	424	394	310	153	69	0.24
	Ins(23456/12456) P_5	639	578	192	107	105	0.25
	Ins(13456) P_5	86	91	63	48	13.6	0.98
Ileum							
	Ins(1246/2346) P_4	42	36	0	0	-	-
	Ins(1234/1236) P_4	314	270	426	398	65	0.12
	Ins(1256/2345) P_4	899	651	3664	1090	663	0.96
	Ins(12346) P_5	531	497	86	67	55	0.66
	Ins(12356/12345)P_5	1761	1318	647	174	263	0.47
	Ins(23456/12456)P_5	2943	2553	355	171	377	0.58
	Ins(13456) P_5	215	207	94	35	19	0.12

[1] Numbers in brackets give the positioning of remaining P group in the 2 possible enantiomers that cannot be separated analytically

PC: positive control, NC: negative control, NC+500FTU and NC+2000 FTU: NC added with phytase added by 500 or 2000 FTU/kg feed, respectively.



Data are expressed as nanogram of GLUT4 protein per gram of total protein and are means of 20 samples per treatment, means with different superscript are different ($P < 0.05$). PC: positive control, NC: negative control, NC+500FTU and NC+2000 FTU: NC added with phytase added by 500 or 2000 FTU/kg feed, respectively GLUT4: glucose transporter type 4.



PC: positive control, NC: negative control, NC+500 FTU and NC+2000 FTU. NC added with phytase added by 500 or 2000 FTU/kg feed, respectively. Y axis is the ratio of band density of P-akt and Akt to β-actin. Data are means of twenty samples per treatment.