ABSTRACT

SHIELDS, MICHAEL CHRISTOPHER. Effect of Phytase on Growth Performance, Proteolytic Enzyme Activity, and Gut Morphology in Weaned Pigs. (Under the direction of Eric van Heugten and Chad Stahl).

The first two studies were designed to evaluate the effects of a bacterial phytase (Exp 1 and 2) and a fungal phytase (Exp. 2) on nutritional and physiological responses in weanling pigs. Pigs were weaned at 21d of age and housed individually. Treatments consisted of a negative control diet (0.45% total P and 0.14% available P) supplemented with either 0, 500, 1,000 and 2,000FTU/kg of phytase and a positive control (PC) diet without phytase (0.6% total P and 0.29% available P). In Exp 2, treatments were the same as Exp. 1, with the addition of a fungal phytase at 500 and 1,000FTU/kg.

In Exp 1, supplemental phytase increased bone ash % (P=0.01). Pigs fed the PC diet also had a higher bone ash (P=0.01) than pigs fed the NC. Pigs fed phytase had a linear increase in G:F (P=0.03). Pigs supplemented with phytase also tended to have an increase in the gene expression of chymotrypsinogen.

In Exp 2, pigs fed the PC diet had a higher bone ash than pigs fed the NC diet (P=0.01). There was also a linear increase for both phytases in bone ash. The *A. niger* phytase had a quadratic effect on ADG (P=0.04) and G:F (P=0.02) as phytase was added in the diet. When comparing the PC and NC, the PC tended to increase ADG (P=0.09) and G:F (P=0.09) than NC.

The third study was designed to determine the effect of high levels of phytase on performance of weanling pigs that were challenged with *Escherichia coli* F18. Pigs were weaned at 21d and were assigned to 1 of 6 dietary treatments arranged in a 2 x 3 factorial randomized complete block design. The 36 pens were held in 2 separate barns, 1 for the

control group and 1 for the challenged group. Factors consisted of: 1) disease challenge status and 2) dietary treatments (a control diet with adequate phosphorus at 0.60% total P, 0.29% available P, a diet with 0.45% total P and 0.14% available P and 500 phytase units, and a diet with 2,500 phytase units). After 2wk, pigs were either challenged with *E-coli* or not and placed in experimental pens. Because of contamination, 22 pens were used as "challenged" pens and 14 were used as control pens.

During the third weigh period (d10 to 17) there was an interaction between disease and phytase (P=0.01) indicating lower growth rates in healthy pigs fed 500FTU/kg of phytase. Phytase or disease had no other impact throughout the study.

The fourth study was designed to measure the ability of an *E. coli* phytase to improve the bioavailability of P using a slope ratio assay in nursery pigs. Pigs were weaned at 21d and fed a common diet for 14d. Dietary treatments consisted of 1) a NC diet which was deficient in P (0.55% and 0.17% Ca and available P respectively), 2) NC diet plus 0.075% additional P, 3) NC diet plus 0.15% additional P, 4) NC diet with 250 FTU of phytase, 5) NC diet with 500 FTU of phytase, 6) NC diet plus 1000 FTU of phytase.

Bone ash also had an estimated available P equivalence of 0.051, 0.071, and 0.117% for 250, 500, and 1,000FTU/g respectively. Bone P had an estimated available P equivalence of 0.041, 0.088, and 0.146% for 250, 500, and 1,000 ftu supplementation levels, respectively. Serum P had an estimated available P equivalence of 0.066, 0.106, and 0.128% for 250, 500, and 1,000ftu supplementation levels, respectively. There was an estimated available P equivalent foce apparent fecal digestibility of 0.030, 0.046, and 0.090 for 250, 500, and 1,000 ftu supplementation levels, respectively.

Effect of Phytase on Growth Performance, Proteolytic Enzyme Activity, and Gut Morphology in Weaned Pigs

by
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BIOGRAPHY

Michael Christopher Shields, son of David and Julie Shields was born on October 17, 1984 in Norfolk, VA and was raised in Ahoskie, NC. He graduated from Hertford County High School in 2003. He received his Bachelor of Science degree in Animal Science from North Carolina State University in 2007. In the fall of 2007 he re-enrolled in North Carolina State University to obtain a Master of Science degree in Animal Science/Nutrition and completed that degree in 2009. In the fall of 2009, Michael decided to further his education at North Carolina State University and pursue a PhD degree.

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Chapter 1: Literature Review

Introduction

Phytase has been fed for many years to livestock as a method to release phosphorus from phytate, which is the major storage form of phosphorus in plants. Recently, it has been shown in poultry and pigs that phytase may have other benefits in addition to liberating phosphorus. This literature review will give a back ground on phytic acid, phytase, and delve further into the extra phosphoric effects of phytase.

Phytic Acid

Phytate, which is the salt of phytic acid, was discovered in 1855 in potato starch grains (Schlemmer et al. 2009). The names, phytate, phytin, and phytic acid are used in the literature. Phytate refers to the mixed salt of phytic acid. (*myo*-inositol hexophosphate; IP_6). Phytin is the deposited complex of IP_6 with minerals, potassium, magnesium, and calcium, as it would normally appears in plants. Phytic acid refers to the free form of IP_6 (Selle and Ravindran, 2007) (Figure 1). Phytate is the major storage form of phosphorus in grain. It represents 50 to 85% of the total phosphorus in plant seeds. The location of phytate in plant varies. In most grain, phytate is primarily found in the bran of most grains but in corn, it is found in the germ. In legumes, phytate is found in the cotyledon and endosperm (Pallauf and Rimbach, 1997).

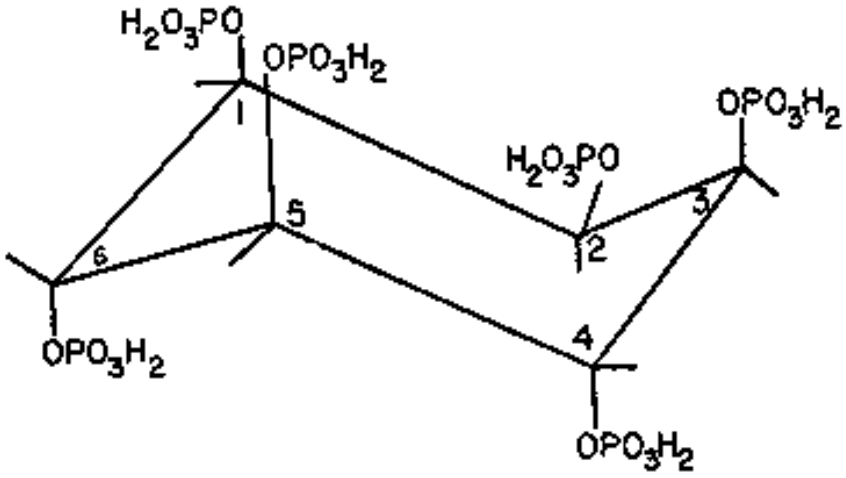

Figure 1. Phytic acid adapted from Johnson and Tate, 1968

There are several different methods that have been used to analyze phytic acid but there are no direct methods. There are no specific reagents that detect phytic acid or its various forms. Phytic acid also does not have a characteristic absorption spectrum in the UV or visible light region. This makes phytic acid difficult to analyze and most methods are based on extraction or isolation of phytic acid (Oatway et al. 2001). The AOAC anion exchange method (986.11) is one method that has been used to determine the amount of phytic acid in a material or feed. This method has its downfalls in its not capable of distinguishing phytic acid which has six phosphate groups and the other inositol phosphates with 5 or less phosphate groups attached. This method will often overestimate for the amount of phytic acid present (Oatway et al. 2001).The high-performance liquid chromatography (HPLC) method is the main way that phytic acid is analyzed. It separates phytic acid and inositol phosphates and quantifies them individually. It has the ability to measure low

concentrations in products. There are a few problems with this method. The reagents in this method must be pure and free from metals or it could cause problems in the readings (Lehrfield 1994).There are different modifications to the HPLC method which use different columns, mobile phases, flow rates, extraction solvents, and preparation techniques (Reddy et al. 1989)

Phytic acid has been shown to decrease performance in several studies (Woyengo and Nyachoti 2013). In pigs, this is due to the negative effects phytic acid has on P digestibility of minerals as well as increased gut secretion minerals and amino acids. Woyengo et al. (2012) fed 2% phytic acid in the diets of weaning pigs and found a 37% reduction in performance when phytic acid was added to the diets (Woyengo et al. 2012).

Phytic acid has been shown, *in vitro,* to precipitate divalent cations. By being poorly hydrolyzed in the gut, phytic acid forms insoluble complexes with cations at neutral pH. This is especially true with divalent cations such as calcium, zinc, copper and manganese (Woyengo and Nyachoti, 2013). Lyon (1984) added phytic acid to a mineral solution at a neutral pH and it resulted in a 99.5, 75, 83, and 62% of precipitation of zinc, iron, calcium, and magnesium in the solution respectively. Davis and Olpin (1979) also added phytic acid and it precipitated 98, 91, and 80% of zinc, copper and manganese, respectively.

Phytic acid has been known to decrease the true absorption of minerals in several species. In humans, Bronner et al. (1954) fed phytic acid to young boys at two levels, 0 and 0.5 % of the diet and found that calcium absorption was reduced by 55%. Kim et al. (2007) fed two different levels of phytic acid to young women (690 to 1,623 mg/d) and elderly

women (760 to 1,713 mg/d) and found that phytic acid reduced zinc absorption significantly in both cases (young women, 43 vs. 22%; and elderly women, 34 vs. 20%). The same has been true in rats and divalent cations. Lonnerdal et al. (1989) fed rats phytic acid different concentrations of inositol phosphates or not and measured an increase in calcium in the cecal contents. This shows that calcium was not absorbed and could have been bound to phytic acid. Kim et al. (1993) found a decrease in iron absorption by 33% when rats were fed a diet that had 0.13% phytic acid added to the diet compared to the control. The same is true with zinc absorption in rats as Rubio et al. (1994) found an 18% decrease in zinc absorption when phytic acid was added in the diet.

Phytic acid has also been shown to increase endogenous losses of minerals. In a study with piglets, phytic acid was fed at 0 or 2% of the diet and the apparent ileal digestibility of sodium and magnesium decreased by 18.2 and 3.0% respectively (Woyengo et al. 2009). The same was true with a study in rats as zinc digestibility was reduced with the addition of phytic acid (Davies and Nightingale, 1975). There are two methods that are proposed as to how phytic acid may increase endogenous secretions of minerals. Phytic acid may bind to enzyme co-factors in the gut which would then result in secretion of the minerals by negative feedback mechanism. Another mechanism would be phytic acid interacting with the positive charge on basic amino acids which would interfere with protein digestion. This would cause a secretion of pepsin which would lower the pH of the stomach. The small intestine would provide more mineral-bicarbonates as to neutralize the pH of the digesta. As seen in Woyengo, et al. (2009), sodium was the mineral which was most affected by the increased phytic acid so the second mechanism may be more plausible (Woyengo et al. 2009).

There have also been few studies conducted with pigs on the effect of phytate and endogenous amino acids and results have varied. Bohlke et al. (2005) and Liao et al. (2005) both completed studies measuring the apparent ileal digestibility of indispensable amino acids and nitrogen due to dietary phytic acid. Both studies demonstrated a significant reduction in several amino acid digestibilities due to the addition of phytic acid. In another study completed by Woyengo et al. (2009), there was no effect on endogenous losses of amino acids when phytate was added in the diet at 2%.

There are several proposed mechanisms as to why phytic acid can affect mineral digestibility and endogenous losses of amino acids. One is that phytic acid may interact with dietary protein in the stomach. It does this by possibly binding with pepsin or pepsinogen (Cowieson et al. 2006), which then reduces the activity of pepsin. There have been several studies that measured the *in vitro* activity of pepsin when phytic acid was added. Knuckles et al. (1989) added phytic acid to a solution of incubation media that contained pepsin, casein and bovine serum albumin. Phytic acid decreased the rate of pepsin digestion from 9 to 14%. Vaintraub and Bulmaga (1991) conducted a similar study and found that the rate of pepsin digestion was decreased up to 92% by phytic acid. In animal studies, Liu et al. (2009) found a 6.3% reduction in the activity of pepsin in the proventriculus of broiler chickens when phytic acid was added in the diet. In pigs, Woyengo et al. (2010) found that pepsin activity was reduced by 46% when phytic acid was added to the diet. It is thought that phytic acid binds pepsinogen in the stomach of the animal (Woyengo et al. 2013). By this occurring, it causes a negative feedback loop where more pepsinogen and hydrochloric acid is secreted into the stomach. This causes the animal to use more energy and amino acids by secreting

additional pepsinogen as well as causing a pH imbalance. More bicarbonate and sodium is secreted which also increases the loss of endogenous minerals. A detailed summary of the impact of phytic acid on digestive processes can be seen in Figure 2, which is adapted from Woyengo et al. (2013).

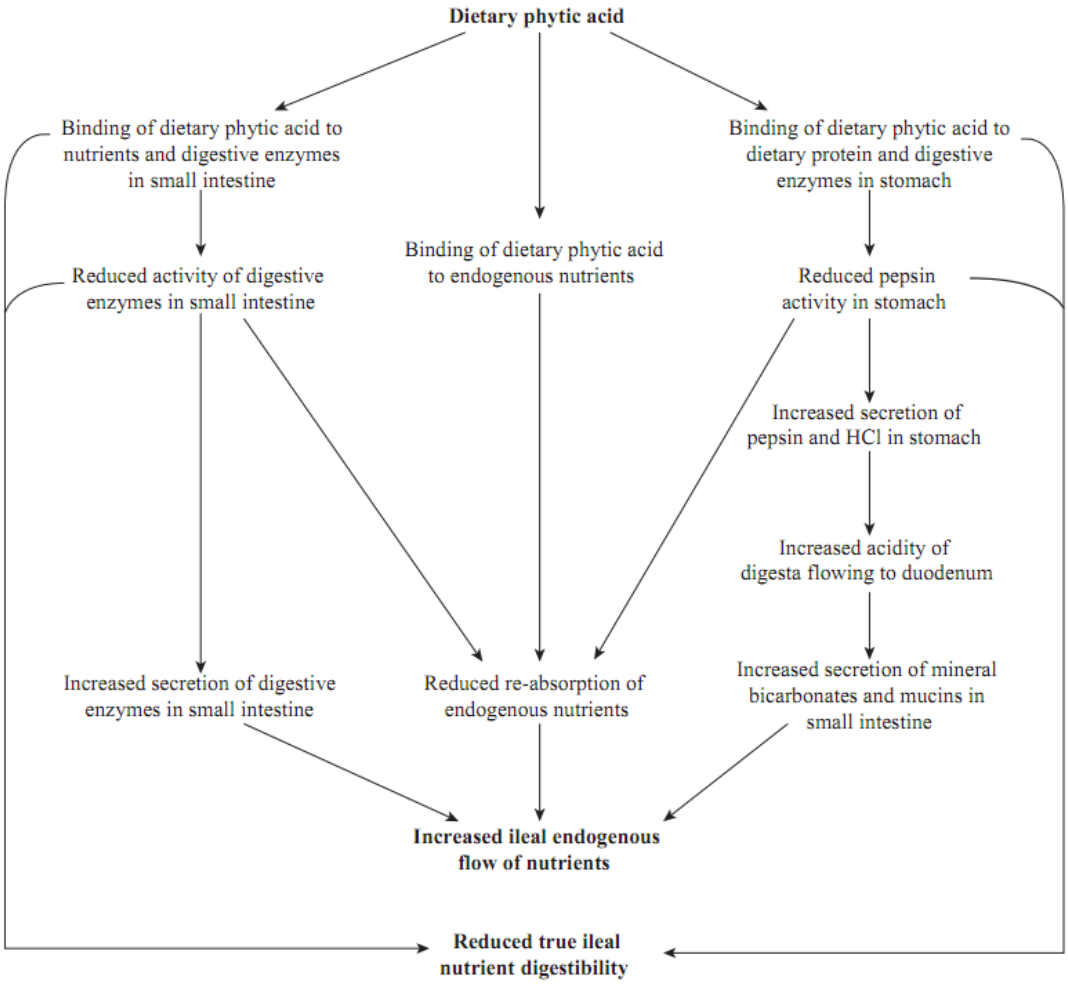

Figure 2. Proposed mechanism of action of dietary phytic acid on ileal digestibility and endogenous losses of nutrients adapted from Woyengo et al. (2013)

Phytase

The enzyme responsible for degrading the antinutritional factor, phytate, is phytase. There are four possible sources of phytate degrading enzyme activity for swine. The first is mucosal phytase activity. There is little mucosal phytase activity in pigs. Hu et al. (1996) investigated mucosal phytase activity in pigs and found phytase activity in the jejunum but not enough to cause a big difference in P digestibility. Phytase was most effective for IP_3 and declined with increasing phosphorylation of the phytate molecule. Mucosal phytase was better at complimenting exogenous phytases once the phytate molecule had already started being degraded (Selle and Ravindran 2008).

The second possible source of phytate degrading enzyme activity for swine is intestinal microfloral activity. Hind gut fermentation degrades phytate in the large intestine. Leytem et al. (2004) fed barley diets which were high in phytate. When measuring the fecal sample, small amounts of phytate along with phosphate were measured. This indicated that the animal was having some sort of hind-gut fermentation (Leytem et al. 2004). Even though phytate is degraded in the large intestine, phosphorus is of no use to the animal because it is not able to be absorbed.

The third possible source is of phytate degrading enzyme activity is intrinsic plant phytase. Phytase is present in several plant ingredients including wheat and its byproducts, barley, rye and triticale (Peers, 1953; Weremko et al., 1997, Viveros et al. 2000). The issue with this phytase is that if the diets are pelleted, the phytase will be degraded because of the steam used during pelleting which will destroy the phytase (Jongbloed and Kemme 1990). Rodehutscord et al. (1996) fed a diet with exogenous phytase and plant phytase and found

that the plant phytase limited the effect of exogenous phytase on phosphorus digestibility. Total tract digestibility of phosphorus was increased by 135% in a diet with 750 FTU of *A. niger* phytase and soybean meal with no detectable levels of phytase originating from the ingredients. When barley that had 550 FTU of intrinsic phytase activity was included, the response was a 47% increase in total tract digestibility of phosphorus. When wheat was added which contained 700 FTU, phosphorus digestibility was not different compared to the control.

The fourth possible source of phytate degrading enzyme activity is exogenous microbial phytase. Phytase was commercially developed in 1991 as an *A. niger* phytase. Commercially available phytases are derived from either fungi or bacteria. There have been many studies examining the effect of exogenous phytases and several are highlighted below.

There are three main characteristics of a phytase enzyme according to Lei and Stahl (2001). The most important quality is the ability to release phytate-P in the digestive tract. Many studies have shown the ability of phytase to release phosphorus from phytate-P which makes more phosphorus available to the animal. This leads to less inorganic phosphorus being added to the diet and less phosphorus in the waste of the animal (Kerr et al. 2010). Another important quality is to be able to withstand degradation caused by storage and feed processing (Lei and Stahl, 2001). Sulabo et al. (2011) measured the stability of six commercially available phytases for a year in different storage forms. When stored at room temperature, phytases held the majority of their activity for the entire year. When stored at 37^{o}C, phytases degraded much faster but when coated, phytases mitigated the negative

effects of high temperature and allowed for longer storage times (Sulabo et al., 2011). A third important factor is for the phytase to be able to be produced cheaply (Lei and Stahl, 2001).

The two main sources of commercially available phytase are fungal (usually *A. niger*) or bacterial (usually *E. coli*). The fungal phytases normally have a pH optima of 2.5 and show little activity above pH of 5.5. This means that there is a limited region of the digestive tract where the phytase is most effective. The fungal phytase has a specific activity of 100 U/mg of protein (U is defined as the amount of enzyme that releases 1µmol of phosphorus from phytate per minute). The bacterial phytase has a wider pH range between 2.0 and 6.0 with a pH optima of 4.5. The bacterial phytase has a specific activity of 400 U/mg of protein (Hong et al. 2004).

Phytate hydrolysis by phytase has been examined in several studies. Kemme et al. (2006) performed a study determining the stepwise degradation of phytate when phytase was added in the diet using dietary markers. Pigs were offered pelleted diets with low intrinsic phytase activity. Three different levels of *A. niger* phytase were supplemented (0, 150, and 900 FTU) and concentrations of phytate esters in digesta of the duodenum and ileum were measured. Phytase decreased the amount of phytate (IP_6) from 7% in the control to 52% in the phytase treatments in the duodenum. In the ileum phytase decreased phytate from 27 to 65% which shows that phytase degraded phytate at 45% in the stomach of pigs (Kemme et al. 2006). Schlemmer et al. (2001) performed a study examining the stepwise degradation of phytate by phytase. Phytate is hydrolyzed via a stepwise method highlighted in Figure 3. Phytate is hydrolyzed from IP_6 to IP_2 as seen in the figure. The bold lines show the path where phosphorus from phytate is most often cleaved. Bacterial phytases mostly cleave from

the 6 position of phytate while fungal phytase normally cleave from the 3 position (Schlemmer et al. 2001).

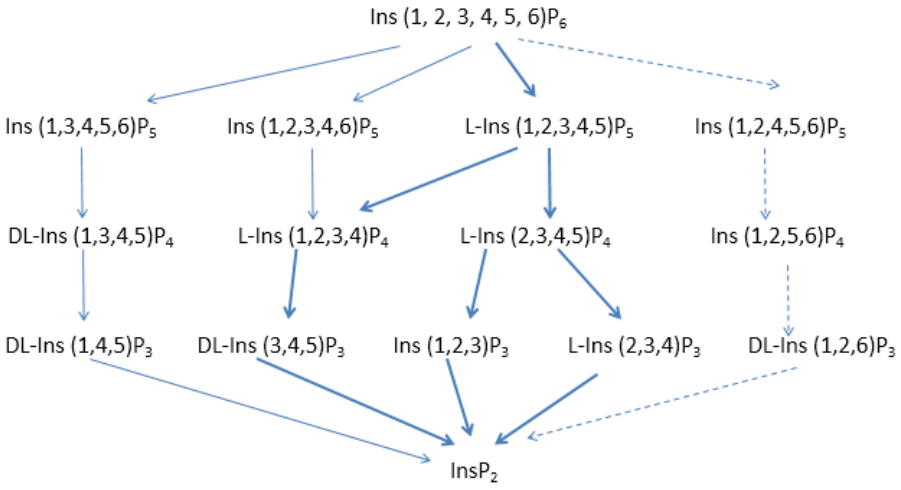

Figure 3. Hydrolysis of phytic acid adapted from Schlemmer et al. 2001

The major reason for feeding phytase to pigs is to release phosphorus from phytate. There have been many studies which demonstrate the effectiveness of phytase in releasing phosphorus. As an example, a study by Kerr et al. (2010) compared the effects of several different phytase enzymes (Natuphos, OptiPhos, RonozymeP, and Phyzyme) on the total tract digestibility of phosphorus fed to finishing pigs. Phytase was fed at 0, 200, 400, 600, 800, or 1,000 FTU. All of the phytase enzymes improved phosphorus digestibility as phytase was added in the diet. Regressions analysis to estimate phosphorus release indicated that at 500 FTU, phosphorus was released at 0.070, 0.099, 0.038 and 0.028% for Natuphos, OptiPhos, and RonozymeP, respectively (Kerr et al. 2010).

There have also been several studies measuring phosphorus equivalency of microbial phytase in pigs. In these studies, phytase is added to basal phosphorus deficient diets and measures such as bone ash, growth performances and other responses are measured to calculate the phosphorus equivalency of phytase. There are variations in many of the study's methods which lead to variable results. This is due to several differences in the basal diet in regards to calcium and total phosphorus levels. Another difference is the level of phytate phosphorus in the basal diet. Also, the source of inorganic phosphorus can cause inconsistencies in the values for phosphorus equivalency. The source of phytase is also a consideration in phosphorus equivalency as well. Early studies from Hoppe et al. (1993) and Hoppe and Schwarz (1993) found 380 to 403 FTU and 500 FTU were equivalent to 1 g/kg phosphorus as monocalcium phosphate in young pigs. Cromwell et al. (1995) found that 500 FTU phytase was equivalent to 0.425 g/kg of inorganic phosphorus in growing pigs. Auspurger et al. (2003) compared three different phytases derived from *P. lycii, A. niger,* and *E. coli* in young pigs. The pigs were offered three phosphorus deficient corn-soy diets. Using fibula ash as a measure, phytases which were fed at 400 FTU released 0.43, 0.81, and 1.08 g/kg relative to monopotassium phosphate. This corresponded to a 16.2, 30.6, and 40.8% phytate degradation rate for *P. lycii, A. niger,* and *E. coli.* In a similar study, Adeola et al. (2006) compared *P. lycii* and *E. coli* phytases and found that at 500 FTU they were equivalent to 0.572 and 0.770 g/kg respectively when using bone mineralization of metacarpal bones relative to monosodium phosphate.

In phosphorus deficient diets, adding phytase will obviously increase growth performance in pigs. One of the more interesting areas is whether or not phytase can benefit

the animal in phosphorus adequate diets. In an early example, Beers and Jongbloed (1992) reported that feeding 1450 FTU increased growth rate in young pigs by 12.8%, feed intake by 8.5% and feed efficiency by 4.4%. Campbell et al. (1995) found similar results by feeding just 500 FTU in young pigs.

There are several possible underlying mechanisms as to why feeding phytase in phosphorus adequate diets could also provide a benefit to the animal. One is that by feeding phytase, ileal digestibility of amino acids is increased. As discussed before, phytate has the ability to bind to basic amino acid residues such as arginine, histidine and lysine. This will form a protein-phytate complex under acidic conditions in the stomach, which will then cause the protein-phytate complex to precipitate, making the protein indigestible (Selle and Ravindran 2008). It makes it indigestible by making the compound refractory to pepsin digestion. By increasing pepsin secretion and hydrochloric acid secretion, mucin is stimulated which will then increase endogenous amino acid flow as has been demonstrated in broilers (Ravindran et al. 1999).

There have been several studies to determine the effect of phytase on amino acid digestibility and results have been inconsistent. Selle and Ravindran (2008) performed a meta-analysis on 24 studies and 342 observations. They found that the impact of phytase (500 or 1000 FTU) on amino acid digestibility is marginal and the overall median increase in amino acid digestibility when phytase is added is 2.4% (Selle and Ravindran, 2008). There were two studies that were completed using intact pigs (slaughtered or anesthetized) instead of using an ileal cannula. Both found that phytase increased the ileal amino acid digestibility much more than the other studies. (Officer and Batterham 1992 a, b; and Kornegay et al.

1998). It is not clear why digesta collected from slaughtered or anesthetized pigs show higher amino acid digestibility when pigs are fed phytase (Selle and Ravindran, 2000).

Other studies measuring the extra phosphoric effects of phytase have examined the effect phytase has on proteolytic enzymes. The majority of the research has measured pepsin and pepsinogen activity because phytase is most effective in the stomach and pepsin is active in the stomach. In an *in vitro* study, Yu et al. (2012) measured phytate and *myo*-inositol esters (IP_{1-5}) effects on pepsin and the effects when phytase was added. Phytate, as expected, was able to create a complex with pepsin. When phytase was added, it alleviated the pepsin inhibition by phytate. Woyengo et al. (2010) performed an in vivo study feeding 0 or 2% phytic acid in a casein-cornstarch diet. There was a third treatment where 500 FTU of phytase was added. Phytase did not have an effect on pepsin in this experiment but phytate reduced pepsin activity. Morales et al. (2012) fed 0 or 500 FTU to growing pigs and slaughtered the pigs collecting digesta from the small intestine. Phytase reduced trypsin activity in the jejunum and the ileum. It also decreased chymotrypsin activity in the ileum. This is surprising because phytate is thought to form a complex with trypsinogen which would cause a decrease in trypsin and chymotrypsin. Phytase would then be expected to increase trypsin and chymotrypsin (Pomar et al. 2008).

Super-dosing phytase is defined as supplementing phytase at a dose higher than 2,500 FTU (Cowieson et al. 2011). Super-dosing phytase in poultry has been in the literature since 1980. There has been an increase in those studies in the last 10 years (Shirley and Edwards 2003; Ausburger and Baker 2004; Cowieson et al. 2006). All of the previous studies added at least 2,500 FTU of phytase and found improvements in growth performance and phosphorus

digestibility in P deficient diets. Zier-Rush et al. (2012) added 3,750 phytase units to a diet that was adequate in phosphorus and found that it tended to improve growth performance in nursery pigs. More research needs to be completed in pigs where a high dose of phytase is fed to examine effects on the animal.

Normally, with the addition of 500 to 1000 units of phytase, which is the industry standard, phosphorus from phytate is liberated from the heavier IP6, IP5, and IP4 first, as bacterial phytases prefer heavier esters. This leaves smaller IP3, and IP2 esters which then pass through the intestine and are excreted (Adeola and Cowieson 2011). When high doses of phytases are fed, this could lead to more of the phytate being degraded resulting in myo-inositol. Adeola and Cowieson (2011) hypothesize three main mechanisms by which super-dosing phytase is beneficial. The first is by releasing more phosphorus. The second is increased degradation of insoluble antinutritional phytate esters. They are then converted to more soluble myo-inositol esters. The third is by generating more soluble myo-inositol esters which may have vitamin-like or lipogenic effects. Liu et al. (2008) fed up to 1,000 FTU of phytase to broilers and found that phytase improved the immune status. Classic studies in the literature have shown growth benefits by feeding myo-inositol to chickens in vitamin deficiency trials (Adeola and Cowieson 2011). Myo-inositol increased the amounts of vitamin A and E in the liver (Hegstad et al. 1941 and Dam 1944).

E-coli

Weaning causes a great amount of stress on piglets which then causes a wide array of changes in gastrointestinal physiology, microbiology, and immunology (Heo et al., 2012). During weaning, one of the most common occurrences is diarrhea and lower growth

performance in affected pigs. At weaning, pigs have to deal with many stresses such as a new social interaction with other pigs in pens, withdrawal of sow milk, and to have to adapt to a less digestible, plant based dry diets (Heo et al., 2012). Because of this, pigs will have a reduction in feed intake after weaning (Pluske et al. 1997). All of this leads to the possibility of the pigs being more susceptible to developing a bacterial disease.

One of the more common diseases observed in the swine industry is post-weaning diarrhea. Post-weaning diarrhea is a condition in which weaned pigs exhibit frequent discharge of watery feces during the first two weeks after weaning. It results in of the major economic problems for the pig industry (Cutler and Gardner, 1988). *E. coli* cause an estimated 50% of the deaths that occur due to post-weaning diarrhea (Cutler et al. 2007). Post-weaning diarrhea is usually associated with fecal shedding of a large number of enterotoxigenic *E. coli (*ETEC) that proliferate in the small intestine after weaning (Schierack et al. 2006).

Enterotoxigenic *E. coli* attaches to enterocytes microvilli by using surface antigens which are called fimbria. This then leads to secretion of enterotoxins which leads to diarrhea. There are several strains which are named for their fimbrial attachment such as K88, K99 and F18. The most virulent strains are usually the K88 strains. This is because they colonize the entire small intestine (Moeser and Blikslager, 2007). There are 3 types of toxins produced by the enterotoxins, large molecular-weight heat labile toxins (LT), small molecular-weight heat-stable toxins (STa and STb), and enteroaggregative *E.* coli heat-stable enterotoxin-1 (EAST1) (Moeser and Blikslager, 2007). The toxins stimulate guanylate cyclase which

increases the level of cCMP. The LT toxins will stimulate adenyl cyclase activity which will increase levels of cAMP in the villous and crypt of the small intestine. The LT toxin binds to the GM1 ganglioside receptors on cell surfaces. This activates adenylate cyclase activity which will then produce high amounts of cAMP and activation of PKA. The CFTR is phosphorylated and apical NHE isoforms (Moeser and Blickslager 2007) which leads to increase production of sodium chloride and hydrogen carbonate ions into the lumen (Kiarie et al. 2008). The STa and EAST1 enterotoxins stimulate increased secretion by binding to glyocoproteins associated with luminal membrane guanylate-cyclase receptor which will then proceed to activate guanylate cyclase. By this occurring, it will cause intracellular accumulation of cGMP, fluid secretion, and inhibition of Na^+ absorption. The mode of action of STb is less known but it is thought that it interacts with the enteric nervous system (Figure 4) (Moeser and Blikslager, 2007). This causes an increase in water and electrolyte secretion at a higher rate than the animal can absorb in the colon. Because the animal is unable to absorb water and electrolytes, this leads to diarrhea, reduced feed intake, nutrient digestibility, and growth which can lead to death. Another effect of PWD is the damage to the intestinal epithelium. This leads to mucosal and cellular barrier function that is damaged which lowers active immunity. This allows more pathogenic bacteria to bind to the mucosal layer which will cause the animal to become more ill (Pluske 2012).

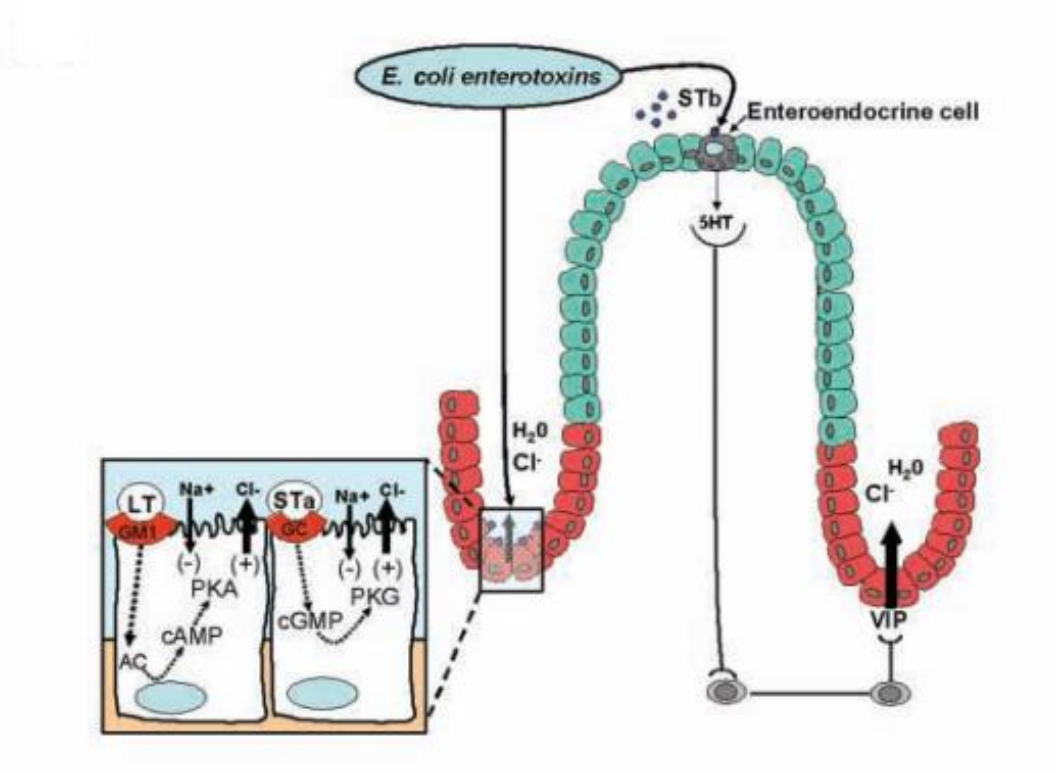

Figure 4. Illustration of the ETEC model of secretary diarrhea from Moeser and Blikslager, 2007.

One possible hypothesis as to why phytase could prevent or help a pig who has post-weaning diarrhea caused by ETEC is the effect of iron on ETEC. It has been well documented that phytase can increase iron absorption in pigs (Stahl et al. 1999; Veum et al. 2006). Karjalainen et al. (1991) performed a study determining the effect of iron on ETEC. Iron represses the expression and synthesis of the colonization factor antigens (CFA). The repressive effect of iron on the CFAs is mediated by the ferric uptake regulator protein (fur),

which requires iron as a co-factor. When the fur protein is active, it requires iron and this represses the CFA from being formed and causing adhesions to the intestinal wall. This will make the bacteria unable to colonize in the intestine which will prevent the animal from contracting ETEC (Karjalainen et al. 1991).

Summary

In summary, phytase has been shown to improve performance and phosphorus digestibility in pigs. This review has highlighted several of the research studies performed in pigs in regards to performance, but more research needs to be completed on the extra-phosphoric effects of phytase. The purpose of the following research projects is to determine the extra phosphoric effects of phytase under normal conditions and in situations where pigs are challenged with a disease.

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Chapter 2: Effect of increasing levels of an *Escherichia coli* phytase enzyme in weaned pigs on growth performance, proteolytic enzyme activity and gut morphology

Abstract

This study was designed to evaluate the effect of an *Escherichia coli* phytase on pig performance, nutritional and physiological responses in weaned pigs. Pigs (n = 40; BW = 9.96± 0.36kg) were weaned at 21 days of age and housed individually. Pigs were acclimated and fed a starter diet for 14 d before being assigned to 1 of 5 dietary treatments. Treatments consisted of a negative control diet (0.45% total P and 0.14% available P) containing 4 levels of supplemental phytase (0, 500, 1000, 2000 phytase units (FTU)) and a positive control (PC) without phytase (0.6% total P and 0.29% available P). Diets were corn and soybean meal based and were fed in mash form for 14 d.

Phytase increased bone ash % (P= 0.01) linearly with increasing levels of phytase. Pigs fed the positive control (PC) diet also had a higher bone ash % (P=0.01) than pigs fed the negative control (NC). Pigs fed phytase had a linear increase in G:F (P=0.03) as phytase was increased in the diet. Pigs supplemented with phytase tended to have an increase in the gene expression of chymotrypsinogen (P=0.10). There was no effect on the gene expression of pepsinogen, trypsinogen, gut morphology measurements, or pH of the stomach and small intestine.

Introduction

Phytic acid, which is the major storage form of P in plants, has long been known to have a negative impact on swine through reduced digestibility of minerals and increased gut secretion of minerals and amino acids. This will result in decreased growth performance of

the animal (Woyengo et al. 2013). The impact of phytase supplementation on improving P digestibility and pig performance by liberating P from phytic acid has been well established (Selle and Ravindran, 2008). In the past few years there has been more research examining the extra-phosphoric effects of phytase related to its effects on amino acid and mineral digestibility (Woyengo et al. 2009).

Little research has been completed examining the effects of phytase on the expression and activity of proteolytic enzymes. Phytic acid which is negatively charged, could possibly bind positively charged amino acids in pepsinogen in the stomach and trypsinogen in the small intestine. Phytate has been shown to inhibit pepsin (Vaintraub and Bulmaga, 1991; Liu and Cowieson, 2011; Yu et al., 2012) and trypsin activity (Singh and Krikorian, 1982) *in vitro*. In poultry, Liu et al. (2009) found that phytase increased the activity of pepsin and trypsin. There have been very few research studies completed in pigs. Woyengo et al. (2010) did not find any response on proteolytic enzymes as phytase was added in the diet.

Therefore, the primary objective of this study was to assess how supplementation of phytase affects the nutritional, physiological and morphological responses in the gastrointestinal tract of weanling pigs.

Materials and Methods

Animal use protocols were reviewed and approved by the North Carolina State University Institutional Animal Care and Use Committee.

Pig performance and Sampling

A total of 40 barrows (BW was 9.96 ± 0.36 kg) were weaned at approximately 21 d into a temperature controlled raised-deck nursery at the Swine Education Unit, Raleigh, NC, and fed a common starter diet (Renaissance Stage 3 Pellet 5820, Renaissance Nutrition, Inc.

Roaring Spring, Pa) for 14 d. At 35 d of age, pigs were transferred to individual pens. The common starter diet was corn and soybean meal based with no added phytase. Pigs were weighed and assigned within BW to 1 of 5 dietary treatments resulting in 8 pens per treatment. Each pen had 1 nipple water drinker and a double-spaced feeder. Pigs were allowed ad-libitum feed and water access throughout the experiment. Dietary treatments (Table 1) consisted of a 1) positive control diet with 0.6% total P and 0.29% available P, 2) a negative control diet with 0.45% total P and 0.15% available P, 3) negative control diet plus 500 FTU of an *E. coli* phytase (Phyzyme XP 5000G, Dupont Industrial Biosciences, Copenhagen, Denmark), 4) negative control diet plus 1000 FTU of an *E. coli* phytase, and 5) negative control diet plus 2000 FTU of an *E. coli* phytase.

Feed was manufactured at the North Carolina State University Feed Mill Educational Unit. Corn was ground with a hammer mill (model 1522, Roskamp Champion, Waterloo, IA) equipped with a 2.2-mm screen. Dry ingredients were blended in a double-ribbon mixer (model TRDB126-0604, Hayes and Stolz, Ft. Worth, TX), and poultry fat was added after dry mixing was complete. Diets were manufactured by creating a basal diet first that contained all ingredients with the exception of phytase. A premix was added for each diet containing added corn, monocalcium phosphate, limestone, and phytase. The basal was divided into 5 portions, to which corn, monocalcium phosphate, limestone, and phytase were added to create the final dietary treatments. Phytase was added to the diets after the diets were first mixed for 90 sec. Diets were then mixed for another 90 sec to ensure a proper mix. Phytase in the diets was measured using the AOAC method 2000.12. The levels in each diet can be found in Table 2.

Pigs were fed one diet for the entire 14 d experiment and pigs were weighed at the end of 14 d. Feed added to the feeders was recorded as was feed left in the feeders to measure feed disappearance on d 14 and pigs were weighed to measure growth performance. On d 14, pigs were harvested for tissue collection following an 8 h fast and 8 h re-feed. This was done to ensure each animal would have 8 h to consume feed which would then be in the digestive tract at the time of sampling. To properly maintain a harvest order, each replication was assigned to a certain hour for the fast and re-feed. The first replication was assigned to the first fast and re-feed hour and the second to the second hour and so on. Pigs were humanely euthanized by captive bolt one at a time. After euthanizing, the abdominal cavity was opened. Two samples of the stomach were collected. A scraping of the pars oesophagea was taken and placed in RNAlater (Invitrogen, Carlsbad, CA) to be analyzed later. A second 1 cm sample was taken for measurement of mucosal histology. The stomach lining was evaluated for gastric ulceration, particularly the pars oesophagea, which is prone to ulceration in the pig. Presence of lesions was evaluated visually using a 7 scale scoring system (Eisemann and Argenzio 1999) as follows: (1) smooth tissue, (2) some cornification, (3) intermediate cornification and papillae elongation, (4) completely cornified with extensive papillae development, (5) some tissue erosion, (6) extensive erosion and small ulcerations, and (7) completely ulcerated. A section of duodenum at approximately 60 cm from the pyloric valve was removed for the duodenal samples. Also, 60 cm proximal to the ileal-cecal junction was collected and represented the ileum. Scrapings from duodenum and ileum were taken and placed in RNAlater for subsequent analysis. A 1 cm section was taken from the duodenum and ileum for measurement of mucosal histology. Samples were fixed in 10% formalin for 24

h and then embedded in paraffin and subjected to hematoxylin and eosin staining. Histology measurements of villus height, crypt depth, and villus width, were examined via a light microscope and measurements were obtained using the Spot Advance software (Diagnostic Instruments, Inc., Sterling Heights, MI). Approximately 10 villus height, crypt depth, and villus width measurements that were well positioned were measured randomly and the data were averaged to provide equal morphological representation. A 1 cm section of the pancreas was also taken and placed in RNAlater for gene expression analysis. Digesta from the stomach, duodenum, and ileum was taken and placed in 15 ml conical tubes and frozen at -20^{o} for enzyme activity analysis. The left foreleg was removed and frozen for later analysis.

RT-PCR analysis

Total RNA was isolated from pancreas, stomach, duodenum, and ileum using RNeasy Mini Kits (Qiagen, Valencia, CA) according to manufacturer's instructions. Dexoyribonucleasewas used as a treatment to remove genomic DNA contamination. (Ambion DNA free-kit, Austin, TX), and the RNA was then reverse transcribed with Superscript III (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The resulting cDNA samples were then treated with RNase H (Invitrogen, Carlsbad, CA) to ensure the removal of residual RNA. Primer sets were designed using software (Integrated DNA Technologies, Coralville, IA) for the examination of trypsinogen and chymotrypsinogen in pancreas and pepsinogen in stomach tissue (Table 3).

Primer sets were validated according to the specifications set forth by Livak and Schmittgen (2001). Relative quantities of the transcripts of interest were determined by semi-quantitative real-time PCR (MyiQ Single Color Real-Time PCR Detection System and

SybrGreen Supermix, Bio-Rad Laboratories, Hercules, CA). Thermocycling conditions included 40 cycles of 20 s of melting at 95ºC followed by 20 s of annealing and extension at 60ºC. After amplification, all samples were subjected to a melt curve analysis. Gene expression was normalized to cDNA concentration, determined using a fluorescence-based quantification kit (Oligogreen, Invitrogen Life Technologies), using a modification of the 2-ΔCT method (Livak and Schmittgen2001).

Enzyme Activity Analysis

Digesta samples from the duodenum and ileum were thawed and supernatant was removed by centrifugation at 2,500 g for 15 min. Pepsin activity analysis was measured using the method of Anson et al. (1938). Samples of 0.1 ml of digesta were incubated with 5 ml of hemoglobin solution (Worthington Biochemical Corporation, Freehold, NJ; 25 g/L hydrochloric acid, pH 2) at 37º for 10 min. After 10 min, the reaction was stopped by the addition of 10 mL trichloroacetic acid (100 g/L). Contents of the tube was then filtered and absorbance of the liquid was measured at 280 nm (Beckman DU 640, Corona, CA). Sample activity was expressive relative to the activity of purified porcine pepsin with an activity of 2,500 units/mg protein (Sigma-Aldrich, St Louis, MO). One unit of activity was defined as an absorbance increase of 0.001 at 280 nm of trichloroacetic acid-soluble hydrolysis products.

Both trypsin and chymotrypsin concentrations were measured using a modified method of Hummel et al. (1959). Samples were diluted at 1:9 with 0.001 M-HCl before the assay. The substrate that was used to determine chymotrypsin activity was n-benzoyl-L-tyrosine ethyl ester (BTEE) as a substrate (Sigma-Aldrich, St Louis, MO) and trypsin used

alpha-N-tolune-p-sulphonyl-L-arginine methyl ester hydrochloride (TAME) (Sigma-Aldrich, St Louis, MO. For trypsin, 6.67 µL diluted sample, 173 µL of a 0.01 M Tris buffer, pH 8.1, containing 0.0115 M-CaCl, 20 µl 0.01 M TAME were combined and shaken for 1 min in a 96 well plate in a microplate reader (Biotek Synergy HT, Winooski, VT) and then measured at 247 nm for 3 minutes at 25^{o}C. The activity of the samples was expressed relative to that of purified trypsin (Sigma-Aldrich, St Louis, MO). One unit of activity is defined as the hydrolysis of 1 µmol substrate in 1 min at 25^{o}C and pH 8.1. For chymotrypsin, 6.67 µl of diluted sample, 140 µl BTEE (made up in a water-methanol (50:50 weight/weight)), and 100 µl 0.08 M Tris buffer, pH 7.8, containing 0.1 M CaCl were combined at gently shaken for 1 min and then measured 256 nm for 3 min. The activity of the samples was expressed relative to that of purified chymotrypsin (Sigma-Aldrich, St Louis, MO). One unit of activity of the samples is defined as the hydrolysis of 1 µmol substrate in 1 min at 25^{o}C and pH 7.8. All enzymes were normalized to digesta protein by BCA (ThermoScientific Pierce BCA Protein Assay Kit, Logan, UT).

Bone ash analysis

The third and fourth metacarpal bones from the left foreleg were cleaned by removing the soft tissue. The bones were dried at 100^{o}C for 24 h. The bones were ashed for 24 h at 550^{o}C.

Statistical analysis

Statistical analysis was performed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The model included replication and phytase level. Orthogonal contrast comparisons were made to determine linear and quadratic effects of phytase addition. In

addition, single degree of freedom contrasts comparisons were conducted to determine if there were differences between the negative and positive control diets.

Results and Discussion

There have been very few studies in pigs measuring the extra phosphoric effects of phytase. It is thought that phytate will bind positively charged amino acids in the precursors to several enzymes (Selle and Ravindran 2008). Many of the experiments completed have been *in vitro* or with purified diets. In the current experiment, our diets were similar to what would be found in a commercial swine operation

Pigs fed the PC diet (30.37%) had a higher bone ash percentage ($P = 0.01$) then the NC diet (27.54%). The PC diet had a higher concentration of available P so a higher bone ash % was expected (Braña et al. 2005). When pigs were fed phytase, they had higher bone ash % (P=0.01; 27.54, 28.36, 28.13, and 30.55% for NC, 500, 1000, and 2,000 FTU respectively) (Figure 1). Measuring bone ash has been found to be more reliable as a measure of P bioavailability than growth performance (Koch and Mahan 1985).This is because the storage of Ca and P continues after the dietary need of P for other bodily functions have been met (Vipperman et al., 1974; Mahan, 1982). The current study's results are similar to findings of Braña et al. (2005) who fed phytase at 250, 500, 750, and 1000 FTU and reported a linear increase in bone ash% as phytase was added in the diet. Ausperger et al. (2003) also found an increased tibia ash % when pigs were fed phytase in a phytase titration experiment. Bone as is also a good way to confirm that the enzyme is active when compared to the amount of phytase in the diet (Table 2).

In this study, supplemental phytase tended to increase ADG (P=0.12; 0.38, 0.41, 0.44, and 0.53 for NC, 500, 1000, 2000 FTU respectively). It also tended to decrease ADFI (P=0.11; 0.79, 0.73, 0.72, and 0.67 for NC, 500, 1000, 2000 FTU respectively). Lastly, supplementation of phytase increased G:F (P=0.03; 0.54, 0.57, 0.60, and 0.79 for NC, 500, 1000, 2000 FTU respectively). When pigs were fed more P as in the PC diet, they numerically increased ADG (P=0.12; 0.49 g/d), and G:F (P=0.15; 0.70 g) than pigs fed NC (0.47 g/d and 0.54 g) (Table 4). There have been several studies showing that including phytase in nursery pig diets will increase performance (Selle and Ravindran 2008).

The majority of the research has measured pepsin and pepsinogen activity because phytase is most effective in the stomach and pepsin is active in the stomach. In the current study, there was no effect on the activity of pepsin with the addition of phytase. Yu et al. (2012) found that phytase alleviated the bond of pepsinogen and phytic acid *in vitro*. In animal trials, Woyengo et al. (2010) found adding phytic acid to a purified diet decreased pepsin activity. Phytase was added at 500 FTU in that study and there was no impact.

With duodenal trypsin, phytase supplementation numerically increased the activity (P= 0.17; 81, 97, 133, and 108 trypsin units/mg protein for 0, 500, 1000, 2000 FTU respectively) (Table 5). It is possible that phytase could be alleviating the bond of trypsin and phytate as seen *in vitro* by Singh and Krikorian (1982) who measured trypsin activity when phytate was added in the diet and phytate inhibited the activity. There have been very few animal studies that measured the impact of phytase on trypsin and chymotrypsin. The current study's results conflict with a study by Morales et al. (2012) who fed 0 or 500 FTU to growing pigs and slaughtered the pigs collecting digesta from the small intestine. Phytase

reduced trypsin activity in the jejunum and the ileum. It also decreased chymotrypsin activity in the ileum. In other proteolytic enzyme measures in the current study, supplemental phytase did not affect duodenal chymotrypsin, ileal trypsin or ileal chymotrypsin activity (Table 5).

Phytase supplementation tended to linearly increase the gene expression of chymotrypsinogen (1.07, 1.5, 3.44, 1.79 fold change for NC, 500, 1000, 2000 FTU respectively). Because phytate and phytase can affect proteolytic enzymes, it was interesting to measure the gene expression of the enzyme precursors. Yu et al. (2012) hypothesized that phytate may bind pepsinogen which would then inhibit pepsin production. The same could be true for trypsinogen and chymotrypsinogen. By phytate binding the precursors of the enzymes, there could be a correlation with the gene expression of the enzyme and the enzyme activity. This was not the case in the current study as there was no response to chymotrypsin even though there was an increase in the gene expression of chymotrypsinogen. The same was true for trypsinogen as the current study found a numerical increase in trypsin activity but no differences in the gene expression of trypsinogen (Figure 2).

Phytate has also been shown to decrease pH along the digestive tract presumably by binding pepsinogen (Liu and Cowieson 2011) which will increase pepsin and hydrochloric acid production. This would result in a lower pH in the stomach and upper small intestine (Woyengo et al. 2010). There were no differences in pH between treatments but pH was measured at 2.47 in the stomach, 5.75 in the duodenum and 6.86 in the ileum which is normal for pigs of this age (Table 6).

In poultry it has been found that phytase can increase villus height in the duodenum (Wu et al. 2004). By improving the surface area and increasing the villus height, this could lead to a better growth performance in the animal by allowing the animal to absorb more nutrients. There have been no studies reporting the effect of phytase on gut morphology in pigs. In the present study, the addition of phytase did not have any effect on gut morphology (Table 7)

In conclusion, phytase numerically increased trypsin, ADG, and the gene expression of chymotrypsinogen. As expected it increased bone ash % and G:F. It is possible that adding phytase could have an extra-phosphoric effect on the animal, but as shown in this study, in a commercial-style diet, it may be more difficult to detect difference in proteolytic enzymes, gene expression, pH, and gut morphology. More research is needed with a diet that is higher in phytate which could show more of a response in extra-phosphoric effects of phytase.

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Table 1.Composition of the experimental diets, as-fed basis[1]

	Negative Control[2]	Positive Control
Ingredient, %		
Corn, yellow dent	66.42	65.63
Soybean meal, 47.5% CP	29.15	29.15
Poultry fat	1.50	1.50
L-lysine HCl	0.35	0.35
DL-methionine	0.12	0.12
L-threonine	0.12	0.12
Monocalcium phosphate 21%	0.31	1.02
Limestone	0.99	1.07
Salt	0.35	0.35
Titanium dioxide	0.50	0.50
Trace mineral premix[3]	0.15	0.15
Vitamin premix[4]	0.04	0.04
Chemical composition, %		
DM	89.85	89.88
CP	20.28	20.25
Ca	0.73	0.88
Total P	0.42	0.57
Available P	0.14	0.29
Standardized ileal digestible amino acids, %		
Lys	1.20	1.20
Met	0.48	0.48
Thr	0.83	0.83
Trp	0.23	0.23

[1]Formulated to contain 1.2% SID lysine
[2]Phytase was added at 500, 1000, and 2,000 FTU to the negative control diet
[3]Supplied per kg of complete diet: 16.5 mg of copper as copper sulfate, 0.3 mg of iodine as ethylenediaminedihydroiodide, 165 mg of iron as ferrous sulfate, 40 mg of manganese as manganous oxide, 0.3 mg of selenium as sodium selenite, and 165 mg of zinc as zinc sulfate.
[4]Supplied per kg of complete diet: 18,500 IU of vitamin A, 2,640 IU of vitamin D3, 106 IU of vitamin E, 8.7 mg of menadione, 13.2 mg of riboflavin, 79 mg of niacin, 53 mg of d-pantothenic acid, 0.07 mg of vitamin B12, 0.53 mg of biotin, and 4.0 mg of folic acid.

Table 2.Analyzed concentrations of phytase in experimental diets[1, 2]

Diet	Phytase units
Positive Control	<50
Negative Control	<50
500 FTU	754
1000 FTU	1,031
2,000 FTU	2,587

[1]Phytase determination was performed by Eurofins Scientific Inc (Des Moines, IA) using AOAC method 2000.12

[2]Phytase was an *E. coli* phytase (Phyzyme XP 5000G, Dupont Industrial Biosciences, Copenhagen, Denmark)

Table 3. Primers used for quantification of gene expression by real-time PCR

Gene Name	Accession Number	Primer Sequence
Pepsinogen	NM_213873	F: 5’ TGCCTGACATCGTCTTCACCATCA 3’ R: 5’ TTGTTGTTGCGGCTGTCAAAGACG 3’
Trypsinogen	NM_001162891	F: 5’ ACACCTGTGCAGCAAATTCCGTTC 3’ R: 5’ ATTCGGGACTTGTAGCAGTGAGCA 3’
Chymotrypsinogen	NM_003355749	F: 5’ CGTGAACAATGACATCACCCTGCT 3’ R: 5’ TGTTGGCGTTGTACTTGGTCTTGC 3’

Table 4. Effect of the addition of phytase in starter diets for nursery pigs on growth performance[1]

	Treatments						P-values		
	PC[2]	NC[3]	500	1000	2000	SEM	Lin	Quad	PC vs NC
ADG, kg/d	0.49	0.38	0.41	0.44	0.53	0.06	0.12	0.88	0.24
ADFI, kg/d	0.70	0.79	0.73	0.72	0.67	0.05	0.11	0.78	0.25
G:F, kg/kg	0.70	0.54	0.57	0.60	0.78	0.08	0.03	0.57	0.15

[1]Each value represents the mean of 8 replicates with 1 pig per pen
[2]PC is positive control with 0.6% total P and 0.29% available P
[3]NC is negative control with 0.45 % total P and 0.15% available P
[4] Lin is the linear effect of phytase supplementation; Quad is the quadratic effect of phytase supplementation; PC vs NC is the contrast between the positive control treatment and the negative control treatment

Table 5. Effect of phytase supplementation on enzyme activity[1]

	Treatment					
	PC[2]	NC[3]	500	1000	2000	SEM
Units/mg protein[4]						
Pepsin	432	819	609	473	730	115
Duodenum trypsin[5]	89	81	97	133	108	22.76
Duodenum chymotrypsin	263	282	321	249	230	51.78
Ileum trypsin	1,199	1,101	1,122	1,119	1,176	452
Ileum chymotrypsin	183	174	181	158	174	19.02

[1]Each value represents the mean of 8 pigs
[2]PC is positive control with 0.6% total P and 0.29% available P
[3]NC is negative control with 0.45 % total P and 0.15% available P
[4]Units/mg P were calculated by using the formula of units/ mgP = (units/mg dw)/(mgP/ml) for pepsin (Anson et al. 1938), trypsin, and chymotrypsin (Hummel et al. 1959)
[5]Quadratic P = 0.10

Table 6. Effect of phytase addition on pH in the digestive tract[1]

	Treatment						P-value[4]		
	PC[2]	NC[3]	500	1000	2000	SEM	Lin	Quad	PC vs NC
pH									
Stomach	2.58	2.66	2.46	2.41	2.23	0.35	0.39	0.86	0.87
Duodenum	5.92	5.64	5.65	5.76	5.80	5.67	0.99	0.28	0.08
Ileum	6.89	6.82	6.97	6.66	6.97	0.19	0.77	0.45	0.72

[1]Each value represents the mean of 8 replicates with 1 pig per pen
[2]PC is positive control with 0.6% total P and 0.29% available P
[3]NC is negative control with 0.45 % total P and 0.15% available P
[4] Lin is the linear effect of phytase supplementation; Quad is the quadratic effect of phytase supplementation; PC vs NC is the contrast between the positive control treatment and the negative control treatment

Table 7: Effect of phytase supplementation on intestinal morphology[1]

	Treatment					
	PC[2]	NC[3]	500	1000	2000	SEM
Duodenum						
Villi height, µm	572.10	537.41	514.51	543.31	495.43	78.14
Villi width, µm	157.03	146.60	151.64	156.56	155.46	7.89
Crypt Depth, µm	2267.02	260.74	268.71	267.11	250.27	12.43
Ileum						
Villi Height, µm	378.97	392.32	389.75	367.55	356.12	24.80
Villi Width, µm	148.19	145.04	151.08	148.92	156.10	4.96
Crypt Depth, µm	218.29	232.45	220.87	221.04	225.37	26.01

[1]Each value represents the mean of 8 replicates with 1 pig per pen
[2]PC is positive control with 0.6% total P and 0.29% available P
[3]NC is negative control with 0.45 % total P and 0.15% available P

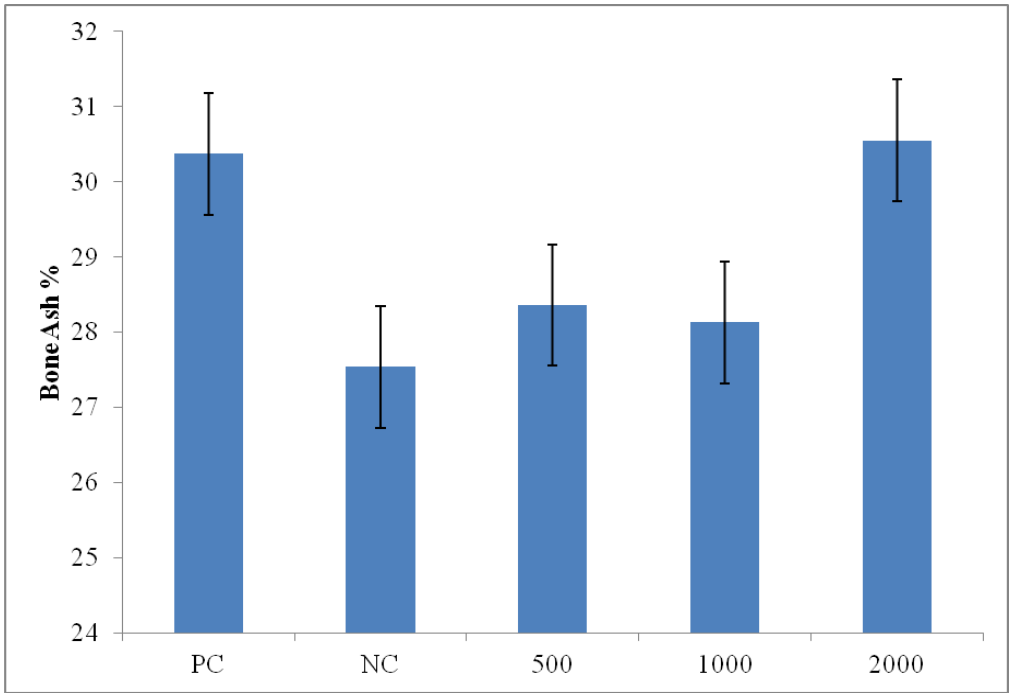

Figure 1. Effect of phytase supplementation on bone ash % of nursery pigs

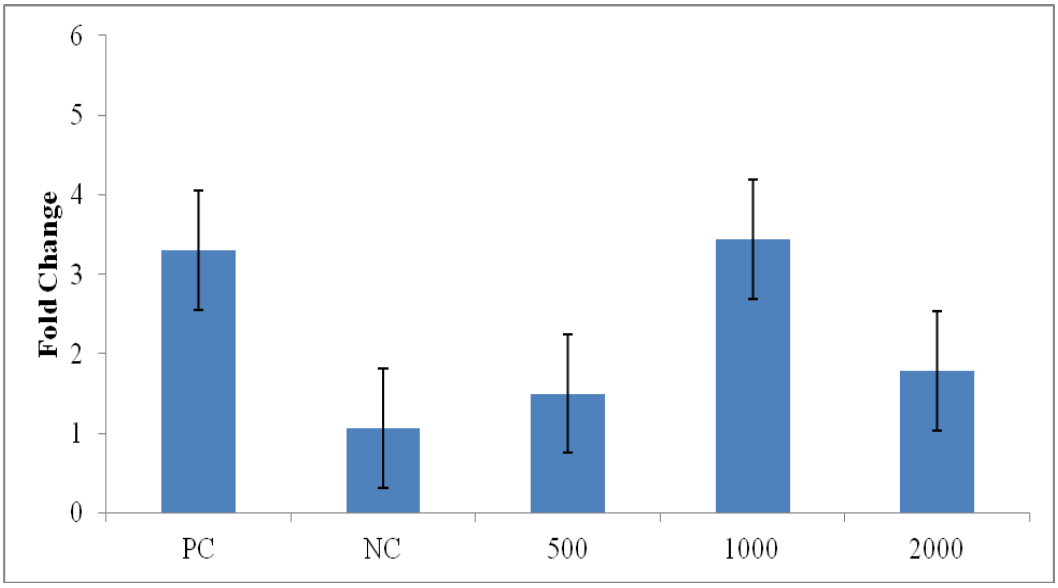

Figure 2. Effect of phytase supplementation on the gene expression of chymotrypsinogen[1]

Chapter 3: Comparing effects of a fungal phytase and an E. coli phytase on growth performance, proteolytic enzyme activity and gut morphology in weanling pigs

Abstract

The objective of this study was to assess how phytase affects the nutritional, physiological and immune responses in the gastrointestinal tract of weanling pigs. A second objective is to see how this response can be affected by two different phytase enzymes. Pigs (n = 56; BW = 11.0 ± 1.6 kg) were weaned at 21 days of age and housed individually. Pigs were acclimated and fed a starter diet for 19 d before assigned to 1 of 7 dietary treatments. . Dietary treatments consisted of a 1) positive control diet with 0.6% total P and 0.29% available P, 2) a negative control diet with 0.45% total P and 0.15% available P, 3) negative control diet plus 500 FTU of an *E. coli* phytase 4) negative control diet plus 1000 FTU of an *E. coli* phytase, 5) negative control diet plus 2000 FTU of an *E. coli* phytase, 6) negative control diet plus 500 FTU of an *A. niger* and 7) negative control diet plus 1000 FTU of an *A. niger* phytase. Diets were based on corn, soybean meal, corn gluten meal, rice bran and soy protein isolate and contained 1.25% SID lysine and 72% phytic acid. Diets were fed in mash form for 14 d.

Pigs fed the PC diet had a higher bone ash % than pigs fed the negative control diet (P=0.01). There was also a linear increase of the *E. coli* phytase (P=0.03) and the *A. niger* phytase (P=0.02). The *A.niger* phytase had a quadratic increase on ADG (P=0.04) and G:F (P=0.02) as phytase was added in the diet. The *E. coli* phytase did not have an effect on growth performance nor was it different in the *A. niger* phytase. When comparing the positive and negative control, the positive control tended to increase ADG (P=0.09) and

G:F(P=0.09). There was a numerical linear increase in duodenal (P=0.11) and a quadratic effect in ileal (P=0.11) trypsin activity as an *E.coli* phytase was added in the diet. The positive control diet tended to have a higher level of trypsin activity (P=0.08) compared to the negative control. There was no effect on proteolytic enzymes in pigs fed the *A. niger* phytase. Pigs fed *E. coli* phytase had a lower stomach pH (p=0.04) than pigs fed *A. niger* phytase. There was no effect of phytase on gut morphology or the gene expression of pepsinogen, chymotrypsinogen, or trypsinogen. In summary, *E. coli* phytase supplementation had a numerical increase in trypsin concentration while *A. niger* phytase did not. In regards to growth performance, *A. niger* phytase increased ADG and G:F while the *E. coli* phytase did not have an effect on growth performance. There were no key differences in either phytase throughout the study.

Introduction

Phytic acid or *myo*-inositol hexakisphosphate (IP_6) is the major storage form of P in grain which has long been known to be indigestible in pigs (Woyengo et al. 2010). Phytase supplementation has been shown to improve phosphorus digestibility and pig performance. There are several different kinds of phytase enzymes on the market. The 2 most common types are derived either from bacteria, *E. coli,* or fungi, *A. niger*. Kerr et al. (2010) performed a study comparing the effectiveness of each of these enzymes in nursery and finishing pigs finding that both sources of phytase improved P digestibility and growth performance but did not observe a difference in response between the 2 phytases.

Recently, several studies in poultry and swine suggest that phytase may have extra-phosphoric effects, including improved amino acid digestibility and increased activity of

proteolytic enzymes (Cowieson et al. 2011). In a previous study by our research group, it was found that adding an *E.* coli phytase could increase trypsin activity and the gene expression of chymotrypsinogen (Shields et al. unpublished). There have been no published studies in which the extra-phosphoric effects of *E. coli* and *A. niger* phytase enzymes were compared.

Therefore, the objectives of this study were to assess how supplementation of phytase affects the nutritional and physiological in the gastrointestinal tract of a weanling pig. A second objective was to determine how this response can be affected by two different phytase enzymes.

Materials and Methods

Animal use protocols were reviewed and approved by the North Carolina State University Institutional Animal Care and Use Committee.

Pig performance and sampling

A total of 56 barrows were weaned at approximately 21 d into a temperature controlled raised-deck nursery at the Swine Education Unit, Raleigh, NC, and fed a common starter diet for 19 d. At 40 d of age, pigs (BW 11.00±1.60 kg) were transferred to individual pens. Pigs were weighed and assigned by BW to 1 of 7 dietary treatments resulting in 8 pens per treatment. Each pen had 1 nipple water drinker and a double-spaced feeder. Pigs were allowed ad-libitum feed and water access throughout the experiment. Dietary treatments (Table 1) consisted of a 1) positive control diet with 0.6% total P and 0.29% available P, 2) a negative control diet with 0.45% total P and 0.15% available P, 3) negative control diet plus 500 FTU of an *E. coli* phytase (Phyzyme XP 10,000G, Dupont Industrial Biosciences, Copenhagen, Denmark) 4) negative control diet plus 1000 FTU of an *E. coli* phytase, 5)

negative control diet plus 2000 FTU of an *E. coli* phytase, 6) negative control diet plus 500 FTU of an *A. niger* phytase (Natuphos 10,000L, BASF, Florham Park, New Jersey) and 7) negative control diet plus 1,000 FTU of an *A. niger* phytase. Feed was manufactured at the North Carolina State University Feed Mill Educational Unit. Corn was ground with a hammer mill (model 1522, Roskamp Champion, Waterloo, IA) equipped with a 2.2-mm screen. Dry ingredients were blended in a double-ribbon mixer (model TRDB126-0604, Hayes and Stolz, Ft. Worth, TX), and poultry fat was applied after dry mixing was complete. Phytase was added to the diets after the diets were first mixed for 90 seconds. Diets were manufactured by creating a basal diet first that contained all ingredients with the exception of phytase. A premix was added for each diet containing added corn, monocalcium phosphate, limestone, and phytase. The basal was divided into 7 portions, to which corn, monocalcium phosphate, limestone, and phytase were added to create the final dietary treatments. Diets were then mixed for another 90 seconds to ensure a proper mix. Enzyme levels in each diet can be seen in Table 2.

Pigs were fed one phase for the entire 18 d experiment. Pigs were weighed at the end of 18 d. Feed added to the feeders was recorded as was feed left in the feeders to measure feed disappearance. On d 18 pigs were harvested for tissue collection following an 8 h fast and an 8 h re-feed. To properly maintain a harvest order, each replication was assigned to a certain hour for the fast and re-feed. The first replication was assigned to the first fast and re-feed hour and the second to the second hour and so on. Pigs were humanely euthanized by captive bolt one at a time. After pigs were euthanized, the abdominal cavity was opened. Two samples of the stomach were collected. A scraping of the pars oesophagea were taken

and placed in RNAlater (Invitrogen, Carlsbad, CA) to be analyzed later. A second 1 cm sample was taken for measurement of mucosal histology. The stomach lining was evaluated for gastric ulceration, particularly the pars oesophagea, which is prone to ulceration in the pig. Presence of lesions was evaluated visually using a 7 scale scoring system (Eisemann and Argenzio (1999) as follows: (1) smooth tissue, (2) some cornification, (3) intermediate cornification and papillae elongation, (4) completely cornified with extensive papillae development, (5) some tissue erosion, (6) extensive erosion and small ulcerations, and (7) completely ulcerated. Approximately 60 cm from the pyloric valve was removed for the duodenal samples. Also, 60 cm proximal to the ileal-cecal junction was collected and represented the ileum. Scrapings from duodenum and ileum were taken and placed in RNAlater for subsequent analysis. A 1 cm section was taken from the duodenum and ileum for measurement of mucosal histology. Samples were fixed in 10% formalin for 24 h and then embedded in paraffin and hematoxylin and eosin staining. Histology measurements of villus height, crypt depth, and villus width, were examined via a light microscope and measurements were obtained using the Spot Advance software (Diagnostic Instruments, Inc., Sterling Heights, MI). Approximately 10 villus height, crypt depth, and villus width measurements in villi that were well positioned were measured randomly and the data were averaged to provide equal morphological representation. A 1 cm section of the pancreas was also taken and placed in RNAlater for gene expression analysis. Digesta from the stomach, duodenum, and ileum was taken and placed in 15 ml conical tubes and frozen at -20^{o} for enzyme activity analysis. The left foreleg was removed and frozen for later analysis.

Total RNA was isolated from pancreas, stomach, duodenum, and ileum using RNeasy Mini Kits (Qiagen, Valencia, CA) according to manufacturer's instructions. Dexoyribonucleasewas used as a treatment to remove genomic DNA contamination. (Ambion DNA free-kit, Austin, TX), and the RNA was then reverse transcribed with Superscript III (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The resulting cDNA samples were then treated with RNase H (Invitrogen, Carlsbad, CA) to ensure the removal of residual RNA. Primer sets were designed using software (Integrated DNA Technologies, Coralville, IA) for the examination of trypsinogen and chymotrypsinogen in pancreas; pepsinogen in stomach tissue (Table 3).

Primer sets were validated according to the specifications set forth by Livak and Schmittgen (2001). Relative quantities of the transcripts of interest were determined by semi-quantitative real-time PCR (MyiQ Single Color Real-Time PCR Detection System and SybrGreen Supermix, Bio-Rad Laboratories, Hercules, CA). Thermocycling conditions included 40 cycles of 20 s of melting at 95ºC followed by 20 s of annealing and extension at 60ºC. After amplification, all samples were subjected to a melt curve analysis. Gene expression was normalized to cDNA concentration, determined using a fluorescence-based quantification kit (Oligogreen, Invitrogen Life Technologies), using a modification of the 2-ΔCT method (Livak and Schmittgen2001).

Enzyme activity analysis

Digesta samples from the duodenum and ileum were thawed and supernatant was removed by centrifugation at 2500 g for 15 min. Pepsin activity analysis was measuring by using the method of Anson et al. (1938). Samples of 0.1 ml of digesta were incubated with 5

ml of hemoglobin solution (Worthington Biochemical Corporation, Freehold, NJ; 25g/l hydrochloric acid, pH 2) at 37^{o} for 10 min. After 10 min, the reaction was stopped by the addition of 10 ml trichloroacetic acid (100 g/l). Contents of the tube are then filtered and the liquid was measured at 280 nm (Beckman DU 640, Corona, CA). Sample activity was expressed relative to the activity of purified porcine pepsin with an activity of 2500 units/mg protein (Sigma-Aldrich, St Louis, MO). One unit of activity was defined as an absorbance increase of 0.001 at 280 nm of trichloroacetic acid-soluble hydrolysis products.

Both trypsin and chymotrypsin concentrations were measured using a modified method of Hummel et al. (1959). Samples were diluted at 1:9 with 0.001 M-HCl before the assay. The substrate that was used to determine chymotrypsin activity was n-benzoyl-L-tyrosine ethyl ester (BTEE) as a substrate (Sigma-Aldrich, St Louis, MO) and trypsin used alpha-N-tolune-p-sulphonyl-L-arginine methyl ester hydrochloride (TAME) (Sigma-Aldrich, St Louis, MO. For trypsin, 6.67 µl diluted sample, 173 µl of a 0.01 M Tris buffer, pH 8.1, containing 0.0115 M-CaCl, 20 µl 0.01 M TAME were combined and shaken for 1 min in a 96 well plate in a microplate reader (Biotek Synergy HT, Winooski, VT) and then measured at 247 nm for 3 minutes at 25^{o}C. The activity of the samples was expressed relative to that of purified trypsin (Sigma-Aldrich, St Louis, MO). One unit of activity is defined as the hydrolysis of 1 µmol substrate in 1 min at 25^{o}C and pH 8.1. For chymotrypsin, 6.67 µl of diluted sample, 140 µl BTEE (made up in a water-methanol (50:50 weight/weight)), and 100 µl 0.08 M Tris buffer, pH 7.8, containing 0.1 M CaCl were combined at gently shaken for 1 min and then measured 256 nm for 3 min. The activity of the samples was expressed relative to that of purified chymotrypsin (Sigma-Aldrich, St Louis, MO). One unit of activity of the

samples is defined as the hydrolysis of 1µmol substrate in 1 min at25^{o}C and pH 7.8. All enzymes were normalized to digesta protein by BCA (ThermoScientific Pierce BCA Protein Assay Kit, Logan, UT).

Bone ash analysis

The third and fourth metacarpal bones from the left foreleg were cleaned by removing the soft tissue. The bones were dried at 100^{o}C for 24 hours. The bones were ashed for 24 hours at 550^{o}C.

Statistical analysis

Statistical analysis was performed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The model included replication, phytase level and phytase source. Orthogonal contrast comparisons were made to determine linear and quadratic effects of phytase addition. In addition, single degree of freedom contrasts comparisons were conducted to determine if there were differences between the negative and positive control diets as well as differences between the two phytases.

Results and Discussion

There have been very few studies that measured the extra phosphoric effects of phytase in pigs. Several have measured effects in purified diets with added phytate. In a previous study by Shields et al, (unpublished) a corn-soybean meal based diet containing a calculated concentration of 1.5% phytate and added phytase to measure extra-phosphoric effects. In the current study, we used high-phytate ingredients, including corn gluten meal, rice bran, and soy protein isolate and increased dietary phytate concentration to 2.2%. This

was to determine the effects of added phytase in a diet that contained more phytate. Phytate, which is negatively charged, has the ability to bind positively charged amino acids which will have a negative impact on protein digestion (Selle and Ravindran, 2008).

Pigs fed the positive control diet (32.24%) had a higher bone ash % than pigs fed the negative control diet (27.71%; $P = 0.01$). When pigs are fed a higher concentration of P, it is expected that they would have a higher bone ash (Braña et al. 2005). There was also a linear increase in bone ash with increasing concentrations of the *E. coli* phytase (27.54, 30.47, 30.42, 32.41% for negative control, 500, 1,000, and 2,000 FTU respectively; P=0.03) and the *A. niger* phytase (27.54, 33.33, and 33.32% for negative control, 500, and 1,000 FTU $P = 0.02$; Figure 1). This is concurrent with previous research by our group in which added *E. coli* phytase at 500, 1,000, and 2,000 FTU increased bone ash % (Shields et al. unpublished). Braña et al. (2005) fed 0, 250, 750, and 1,000 FTU of an *A. niger* phytase and calculated an increase in bone ash which is also in agreement with the current study. The increase in bone ash is caused by enhanced storage of Ca and P after the dietary needs for those minerals have been met. (Vipperman et al. 1974; Mahan 1982)

There have been several studies that measured growth performance when phytase was added to the diet and many have shown increased performance (Selle and Ravindran 2008). In the current study, feeding the *A.niger* phytase resulted in a quadratic increase in ADG (0.47, 0.64, 0.55, g/d for 0, 500, and 1000 FTU/kg; P=0.04) and G:F (0.58, 0.74, and 0.66 for 0, 500, and 1000 FTU/kg; P=0.02). The *E. coli* phytase did not have an effect on growth performance, nor was it different from the *A. niger* phytase. When comparing the positive and negative control, the positive control tended to increase ADG (0.61 and 0.47g/d; P=0.09)

and G:F (0.69 vs. 0.58; P=0.09) (Table 6) which was expected as the negative control diet was deficient in phosphorus.

Of the measurements used to evaluate the effect of phytase on proteolytic digestion, pepsin and pepsinogen are most common. The majority of the trials have been *in vitro* or with a purified diet. Yu et al. (2012) added phytase to a solution containing pepsinogen and phytic acid and found that phytase broke the bond between phytic acid and pepsinogen. Woyengo et al. (2010) added phytase to a purified diet containing pure phytic acid and found that adding 500FTU/kg phytase increased pepsin activity. In the current study, supplementing either type of phytase did not have an effect on pepsin activity (Table 4) or pepsinogen gene expression which is similar to the previous study completed by Shields et al. (unpublished).

The other proteolytic enzyme that is commonly measured is trypsin. The current study had a numerical linear increase in duodenal (P=0.11) and a quadratic effect in ileal (P=0.11) trypsin activity as *E .coli* phytase was added in the diet. The positive control diet tended to have a higher level of trypsin activity (P=0.08) compared to the negative control (Table 4). Phytic acid has been shown to inhibit trypsin *in vitro* (Singh and Krikorian, 1982). It was hypothesized that phytic acid binds to trypsinogen and it is not able to form trypsin. Shields et al. (unpublished) measured an increase in duodenal trypsin concentration when phytase was added in the diet which correlates with the hypothesis by Sing and Krikorian, (1982) who found that phytate inhibited trypsin activity *in vitro.* Morales et al. (2012) fed 0 or 500 FTU and measured trypsin activity and when phytase was added in the diet trypsin activity was decreased. There was no effect on the *A. niger* phytase on any of the proteolytic

enzyme activities. There was also no difference in the gene expression of the precursors of trypsin and chymotrypsin.

Phytate has been known to decrease pH throughout the digestive tract. It does this by binding pepsinogen which in-turn causes increased secretion of pepsin and hydrochloric acid (Liu and Cowieson, 2011). In the current study, pigs fed *E. coli* phytase (2.82) had a lower stomach pH (p=0.04) than pigs fed *A. niger* phytase (3.5). It is not clear why the pH was lower. There were no differences in pH in the duodenum or ileum between the two phytases. In the previous study by Shields et al. (unpublished), the pH of the stomach when *E. coli* phytase was fed was 2.47.

One way that phytase could possibly increase performance is by increasing villi height throughout the small intestine, which has been shown in poultry (Wu et al. 2004). In the current study, there was no effect on phytase supplementation on gut morphology, which is similar to the previous study by Shields et al. (unpublished).

In summary, *E. coli* phytase supplementation resulted in a numerical increase in trypsin concentration while *A. niger* phytase did not. *A. niger* phytase increased ADG and G:F while the *E. coli* phytase did not have an effect on growth performance. There were no key differences in either phytase throughout the study.

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Table 1. Composition of experimental diets, as-fed basis[1]

	Negative Control	Positive Control
Ingredient, %		
Corn, yellow dent	56.13	55.34
Soybean meal, 47.5% CP	23.02	23.02
Corn gluten meal	7	7
Rice bran	5	5
Soy protein isolate	5	5
Poultry fat	1.5	1.5
L-lysine HCl	0.3	0.3
DL-methionine	0.2	0.2
Monocalcium phosphate 21%	0.3	1.02
Limestone	1.01	1.08
Salt	0.35	0.35
Trace Mineral Premix[3]	0.15	0.15
Vitamin Premix[4]	0.04	0.04
Chemical Composition, %		
DM	89.78	89.85
CP	24.07	24.01
Calcium	0.55	0.7
Total P	0.45	0.6
Available P	0.14	0.29
Standardized ileal digestible amino acids, %		
Lysine	1.25	1.25
Methionine	0.57	0.57
Threonine	0.78	0.78
Tryptophan	0.22	0.22

[1]Formulated to contain 1.25% SID lysine
[2]Phytase was added at 500, 1,000, and 2,000 FTU (*E-coli)* or 500 and 1000 (*A. niger)* to the negative control diet
[3]Supplied per kg of complete diet: 16.5 mg of copper as copper sulfate, 0.3 mg of iodine as ethylenediaminedihydroiodide, 165 mg of iron as ferrous sulfate, 40 mg of manganese as manganous oxide, 0.3 mg of selenium as sodium selenite, and 165 mg of zinc as zinc sulfate.
[4]Supplied per kg of complete diet: 18,500 IU of vitamin A, 2640 IU of vitamin D3, 106 IU of vitamin E, 8.7 mg of menadione, 13.2 mg of riboflavin, 79 mg of niacin, 53 mg of d-pantothenic acid, 0.07 mg of vitamin B12, 0.53 mg of biotin, and 4.0 mg of folic acid.

Table 2: Analyzed concentrations of phytase in experimental diets[1]

Diet	Phytase units
Positive Control	<50
Negative Control	<50
E-coli phytase 500[2]	752
E-coli phytase 1000[2]	918
E-coli phytase 2000[2]	1,930
Fungal Phytase 500[3]	471
Fungal Phytase 1000[3]	880

[1]Phytase determination was performed by Eurofins Scientific Inc (Des Moines, IA) using AOAC method 2000.12

[2]Phytase was an *E. coli* phytase (Phyzyme XP 10,000G, Dupont Industrial Biosciences, Copenhagen, Denmark)

[3]Phytase was an *A. niger* phytase (Natuphos 10,000L, BASF, Florham Park, New Jersey, USA)

Table 3. Primers used for quantification of gene expression by real-time PCR

Gene Name	Accession Number	Primer Sequence
Pepsinogen	NM_213873	F: 5’ TGCCTGACATCGTCTTCACCATCA 3’ R: 5’ TTGTTGTTGCGGCTGTCAAAGACG 3’
Trypsinogen	NM_001162891	F: 5’ ACACCTGTGCAGCAAATTCCGTTC 3’ R: 5’ ATTCGGGACTTGTAGCAGTGAGCA 3’
Chymotrypsinogen	NM_003355749	F: 5’ CGTGAACAATGACATCACCCTGCT 3’ R: 5’ TGTTGGCGTTGTACTTGGTCTTGC 3’

Table 4. Effect of two different phytases addition in nursery diets on growth performance[1]

	Treatments							
	Control Diets		*E-coli*			*A.Niger*		
	PC[2]	NC[3]	500	1000	2000	500	1000	SEM
ADG, kg/d[4,6]	0.61	0.47	0.59	0.57	0.53	0.64	0.55	0.05
ADFI, kg/d	0.87	0.82	0.91	0.89	0.88	0.85	0.85	0.05
GF, kg/kg[5,7]	0.69	0.58	0.65	0.62	0.61	0.74	0.66	0.05

[1]Each value represents the mean of 8 replicates with 1 pig per pen
[2]PC is positive control with 0.6% total phosphorus and 0.29% available phosphorus
[3]NC is negative control with 0.45 % total phosphorus and 0.15% available phosphorus
[4]*A. niger* quadratic P=0.04
[5]*A. niger* quadratic P=0.02
[6]Positive control versus negative control P=0.09
[7]Positive control versus negative control P=0.09

Table. 5. Effect of phytase supplementation on enzyme activity[1]

	Treatments							
	Control Diets		*E-coli*			*A.Niger*		
	PC[2]	NC[3]	500	1000	2000	500	1000	SEM
Units/mg Protein[4]								
Pepsin	315	3237	715	1106	619	1045	2358	884
Duodenum Trypsin[5]	370	227	291	174	381	176	270	112
Duodenum chymotrypsin	518	360	393	318	514	347	363	133
Ileum Trypsin[6,7]	436	245	433	395	369	312	461	72
Ileum Chymotrypsin	312	301	353	350	305	241	361	55

[1]Each value represents the mean of 8 replicates with 1 pig per pen
[2]PC is positive control with 0.6% total phosphorus and 0.29% available phosphorus
[3]NC is negative control with 0.45 % total phosphorus and 0.15% available phosphorus
[4]Units/mg P were calculated by using the formula of units/ mgP = (units/mg dw)/(mgP/ml) for pepsin (Anson et al. 1938), trypsin, and chymotrypsin (Hummel et al. 1959)
[5]*E-coli* lin effect P=0.11
[6]*E-coli* quad effect P=0.11
[7]Postive control versus negative control contrast P=0.08

Table 6. Effect of phytase addition on pH in the digestive tract[1]

	Control Diets		*E-coli*			*A.Niger*		
	PC[2]	NC[3]	500	1000	2000	500	1000	SEM
pH								
Stomach[4]	3.42	3.09	2.99	2.80	2.69	3.74	3.57	0.36
Duodenum	5.73	5.75	5.64	5.89	5.79	6.05	5.82	0.13
Ileum	6.72	6.64	6.68	6.18	6.75	6.57	6.44	0.15

[1]Each value represents the mean of 8 replicates with 1 pig per pen
[2]PC is positive control with 0.6% total phosphorus and 0.29% available phosphorus
[3]NC is negative control with 0.45 % total phosphorus and 0.15% available phosphorus
[4]*E-coli* versus *A. niger* P=0.04

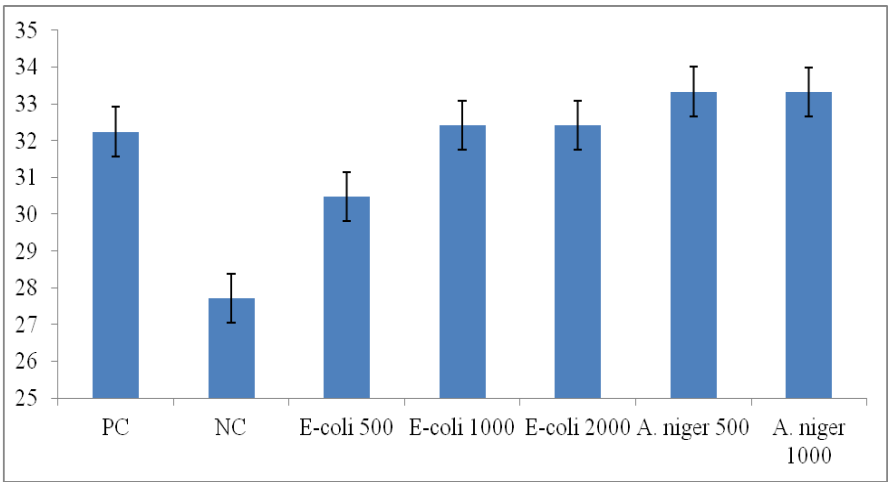

Figure 1. Effect of phytase supplementation on bone ash % of nursery pigs

Chapter 4: Supplementation of an *Escherichia coli* phytase in weaned pigs to ameliorate the effect of post weaning diarrhea

Abstract

This study was designed to determine the effect of high levels of phytase on performance and intestinal health of weanling pigs that were challenged with *Escherichia coli* F18 or a control group that was not challenged. Pigs (270 pigs) were weaned at 21 d and were assigned to one of six dietary treatments (36 pens, 6 replicates) arranged in a 2 x 3 factorial randomized complete block design at the Swine Evaluation Station in Clayton, NC. The 36 pens were held in two separate barns, one for the control group and one for the challenged group. A third barn was used for seeder pigs. Factors consisted of: 1) disease challenge status (challenged orally with *Escherichia coli* F18 strain or not) and 2) dietary treatments (a control diet with adequate phosphorus at 0.60% total P, 0.29% available P and no phytase, a diet with 0.45% total P and 0.14% available P and 500 phytase units, and the negative control diet with 2,500 phytase units). Pigs were fed experimental diets for two weeks prior to being challenged. After two weeks, seeder pigs were either challenged with E-coli or not and placed in experimental pens. Because of contamination, 22 pens were used as "challenged" pens and 14 were used as control pens. One week after the seeder pig was placed in the pen, 1 pig per pen was randomly selected and slaughtered for further analysis. Pigs were weighed weekly for 5 weeks throughout the experiment.

During the third weigh period (d10-d17) there was an interaction between disease and phytase (P=0.01) with healthy pigs fed 500 FTU/ kg growing the least. There were no other effects on growth performance as phytase was added in the diet. As expected, there was no

effect in bone ash as phytase was added to P adequate diets. There was also no effect on intestinal absorptive capacity or gut morphology.

Introduction

Phytase has been fed for many years to livestock as a method to release phosphorus from phytate, which is the major storage form of phosphorus in plants. Recently, it has been shown in poultry and pigs that phytase may have other benefits in addition to liberating phosphorus, especially when super-dosing phytase. Super-dosing phytase is defined as supplementing phytase at a dose higher than 2,500 FTU (Cowieson et al., 2011). Super-dosing phytase in poultry has been in the literature since 1980. There have been an increase in those studies in the last 10 years (Shirley and Edwards, 2003; Ausperger and Baker, 2004; Cowieson et al., 2006). All of the previous studies added at least 2,500 FTU of phytase and found improvements in growth performance and phosphorus digestibility in P deficient diets. There has not been as much research completed in pigs. Zier-Rush et al. (2012) added 3,750 phytase units to a diet that was adequate in phosphorus and found that it tended to improve growth performance in nursery pigs. More research needs to be completed in pigs where a high dose of phytase is fed to examine effects on the animal.

One of the more common diseases observed in the swine industry is post-weaning diarrhea. The effects *Escherichia coli* cause an estimated 50% of the deaths that occur because of post-weaning diarrhea *(*Cutler et al. 2007). When a pig has post-weaning diarrhea, the *Escherichia coli* binds to receptors for F18 fimbrae on the enterocyte which results in colonization of the small intestine. Enterotoxins which are produced by the F18 fimbrae can be either heat labile or stabile are then produced. Then, activation of cyclic guanosine

monophosphate and cyclic adenosine monophosphate pathways by heat labile toxins increase the secretion of sodium, chloride, and hydrogen carbonate ions in the lumen causing secretory diarrhea. Heat stabile toxins decrease the absorption of water and other nutrients which increases the flow through the lumen, causing diarrhea (Pluske, 2012). Another negative aspect of post-weaning diarrhea is the alteration of gut permeability. Kiarie et al. (2008) challenged pigs with *Escherichia coli* and found an increase in gut permeability which decreased the pig's barrier function because of the diarrhea.

There have been no studies that measured the response of pigs to a disease challenge when phytase was supplemented. Metzler et al. (2008) fed 1,000 FTU of phytase and compared fecal bacteria mass in diets supplemented with P or not. Pigs fed the low-P diets with phytase had a lower fecal bacterial mass than pigs fed the pigs supplemented with P. This could mean that in a disease situation, a diet that contains lower inorganic P with added phytase could allow the animal to utilize the P from phytic acid leaving less for the bacteria to use for cellular functions, which could possibly alleviate the symptoms of an *E-coli* infection in pigs.

Our objective of the study was to determine the effect of high levels of phytase on performance and intestinal health of weanling pigs that are challenged with *Escherichia coli* F18 or a control group that is not challenged. We hypothesized that feeding phytase will alleviate the negative effects that *E-coli* F18 has by improving performance and intestinal health of the weanling pig.

Materials and Methods

Pig performance and Sampling

In this experiment, 270 pigs (21 days of age) were assigned to one of six dietary treatments (36 pens, 6 replicates) arranged in a 2 x 3 factorial randomized complete block design at the Swine Evaluation Station in Clayton, NC. Factors consisted of 1) disease challenge status (challenged orally with *Escherichia coli* F18 strain or not) and 2) three different diets (a control diet with adequate phosphorus containing 0.70% Ca, 0.60% total P, 0.29 available P, and no supplemental phytase; a negative control diet with 0.55% Ca, 0.45% total P, 0.14% available P, and 500 FTU; and the negative control diet with 2,500 FTU) (Table 1). The source of phytase used was Phyzyme (Danisco). Diets were manufactured at the North Carolina State Feed Mill Educational Unit. Diets were manufactured by first creating a basal diet that contained all ingredients except added limestone, monocalcium phosphate and phytase. The basal diet was split into three portions. For the control, additional limestone and monocalcium phosphate were mixed with the basal diet to increase available P by 0.15% and Ca by 0.15%. The two remaining diets were manufactured from the basal diet by the addition of the appropriate concentration of phytase. Pigs were fed experimental diets for approximately 14d before the challenge.

Two *Escherichia coli* F18 producing strains (O147:NM and S1191 (O139)) were used as challenge strains. Both strains were grown overnight in Luria-Bertani (LB) medium at 37^{o}C with shaking. They were then diluted to an optical density of 600 nm (OD_{600}) of 0.1 LB medium and allowed to grow to an $OD_{600}$of 1. The cultures were then centrifuged at 4,000 x g for 10 min at 4^{o}C. Then, the bacterial pellets were resuspended in 20% dextrose

and 5% nonfat dry milk in water. The challenge dose consisted of an equal amount of each strain, O147:NM and S1191 (O139), and were determined by serial dilution and plating to provide a total of 2 x 10^9 CFU/0.5 mL oral dose. Nalidixic acid (NA) resistant *E. coli* was used so that we could enumerate the bacteria used as the challenged strain versus total *E. coli* (Cutler et al. 2007).

The barn that the pigs were housed in was separated into three sections. One section was used for pigs that were not challenged with *E. coli* that held 18 pens. A second section was used to house that were challenged with *E. coli* also containing 16 pens. The third section contained thirty pigs that were held in two groups as seeder pigs. One of these groups was used as "challenge pigs" which were infected with *Escherichia coli* F18. Because not every pig is susceptible to infection, 30 pigs were infected to ensure that there were at least 18 infected pigs. The other group of pigs was not infected with *E. coli* F18. After the pigs exhibited signs of the disease, 18 pigs which were in the same state of progression of the disease were selected. One pig was placed in each of the pens designated as challenge pens. At the same time, pigs from the group of pigs that were not challenged were placed in the control pens at one pig per pen. This was done to keep the number of pigs in each pen the same.

Contamination during the process where pigs were infected caused us to alter the design of the study. This was determined as pigs became sick with diarrhea and lethargy throughout all three barns. By measuring nalidixic acid resistant *E-coli* counts, new disease pens and control pens were assigned. Pens that had nalidixic acid resistant *E-coli* in their feces were deemed to be "diseased." Pens that did not have nalidixic acid resistant *E-coli*

were deemed to be "control" pens. In total, there were 22 pens infected with *E-coli* and 14 pens that were the new control group.

Pigs were weighed weekly throughout the study and at the end of the study. Feed added to the feeders was recorded and feeders with remaining feed were weighed to determine feed disappearance. Two days prior to and on d 4 and 7 post challenge, a fecal sample from 1 pig per pen was taken by inserting a 10-µl fecal loop into the rectum. The samples were diluted in 5 ml of sterile phosphate-buffered saline. Serial 10-fold dilutions were plated onto MacConkey agar plates for CFU determination (Cutler 2007). Scour scores were measured based on the methods of Marquardt et al. (1999). Briefly, each pen was scored daily based on the severity of the scours (0-no scours, 1-soft feces, 2-mild diarrhea, and 3-severe diarrhea).

One week after the seeder pig was placed into the pen, 1 pig exhibiting clear signs of disease was selected from each pen and euthanized by captive bolt. Next, the GI tract was removed. A 1 cm section of the duodenum was cleaned and was fixed in 10% formalin and was later measured for mucosal morphology. The left foreleg was removed and frozen for later analysis.

Intestinal absorptive capacity

With the remaining pigs, after the final weigh date, pigs were withheld from feed for eight hours. Then, one animal per pen was selected for *in vivo* intestinal absorptive capacity (Kim et al. 2009). Briefly, pigs were given 0.6 g/kg BW of D-mannitol in a total volume of 13 ml/kg BW. After infusion, blood samples were collected in heparinized tubes from pigs at 60, and 120 min. Plasma was then be removed after centrifugation and stored until later

analysis. D-mannitol concentrations were measured using a spectrophotometer as described in Kim et al. (2009).

Bone ash analysis

The third and fourth metacarpal bones were cleaned by removing the soft tissue. The bones were dried at 100^{o}C for 24 hours. The bones were ashed for 24 hours at 550^{o}C. Calcium and phosphorus content were then measured using atomic absorption.

Statistical analysis

Statistical analysis was performed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The model for bacterial concentration, body weight, bone ash, intestinal absorptive capacity, diarrhea scores, and gut morphology included disease and phytase level and the interaction between disease and phytase.

Results and Discussion

Ambient temperature is extremely important to weaned pigs. During the first week after weaning, activity level is high and food intake is low; therefore the newly weaned pig is in a negative energy balance (McCracken and Caldwell, 1980). This negative energy balance leads to a loss of fat and a lower growth rate for the pig (Whitmore et al. 1978). Another effect of fat loss is the decrease of insulation of heat from fat, as much as 30% in the first week after weaning (Fenton et al. 1985). With these data, Le Dividich and Herpin (1994) suggested that the temperature in the barn during the first two weeks should be between 26 to 28^{o}C and decrease 2-4^{o}C each two weeks after that. This trial was conducted in an older barn that is not insulated well and temperatures are harder to control. Extra heaters were brought

in at the beginning of the study but the barn still remained lower in temperature than the proposed temperatures by Le Dividich and Herpin (1994). During the first week after weaning temperatures ranged from 21 to 33^{o}C in the control barn and 17 to 32^{o}C in the challenged barn (Figure 1). Pigs in this study exhibited high rates of scours throughout the study and low growth performance. These lower temperatures at night could have been the reason more pigs had scours throughout the barn which lowered growth performance.

There have been no studies in the literature where a disease challenge was tested in combination with super-dosing phytase. Metzler et al. (2008) fed 1,000 units of phytase to pigs with in adequate P and low P diets. Low P diets, there were fewer bacteria in the ileal digesta. They believed that this was due to the fact that the animal was able to absorb the phosphorus leaving less for the bacteria. In an *E-coli* challenge, this would be important as the phytase diets were deficient in phosphorus and the majority of the phosphorus would be obtained from cleaved phytate by phytase addition. Bacterial counts are found in Table 3. There was a disease effect (P = 0.02) in the first collection post challenge, which occurred on d 4. Diseased pigs had a higher *E-coli* bacterial count than non challenged pigs. There was a higher amount of gram negative bacteria in the d 7 collection in pigs with phytase. There is no explanation for why this occurred.

Diarrhea scores were measured for 7 d post *E-coli* challenge. On d 1 there was an increase in diarrhea score with challenge pigs (1.72 versus 1.23; P=0.04). There was a numerical increase in score throughout the entire 7 d but was more pronounced on d 3 (1.86 versus 1.30; P=0.11) and d 5 (1.77 versus 1.30; P = 0.16) (Table 4).

According to the NRC (2012), pigs should gain 335 g/day when their weight range is from 7-11 kg and 585 g/day for 11-25 kg. The pigs in the current study had an average starting weight of 6.98 kg and an average 36 d weight of 10.30 kg. It is thought that because of the colder temperatures in the barn, pigs had poor growth performance over the 36 d study (ADG 0.11, ADFI 0.18, and G:F 0.73). Even though phytase has been shown in several studies to improve growth performance when added in the diet (Selle and Ravindran 2008), there was no effect of phytase on growth performance in this study, except during the third weigh period on G:F where pigs fed 2500 FTU had a higher G:F than the 500 FTU and the control diet (P=0.01) (Table 5).

Bone ash is a good measure of P availability and more reliable than performance (Koch and Mahan 1985). This is because the storage of calcium and phosphorus continues even after the needs for the minerals have been met (Vipperman et al., 1974; Mahan, 1982). The control diet in this study was P-adequate and the phytase diets were P-deficient. There were no differences in bone ash when phytase was added in the diet. Because there was no difference between the phytase treatments and the control, this shows that P was adequate in all treatments

There was no effect of phytase or challenge status (Table 6 and Figure 3) on intestinal absorptive capacity or morphology. This was surprising because pigs challenged with *E-coli* should have increased intestinal absorptive capacity and a decreased villus height (Kiare et a. 2008). This could have been due to the fact of the health status of the barn. Even though some of the pigs were not exhibiting signs of the strain of *E-coli* that was used in this study,

many pigs were still exhibiting signs of scours. This could have caused no differences in gut morphology or intestinal absorptive capacity.

In conclusion, pigs fed phytase, as expected, had the same amount of bone ash as the P-adequate control diet. Phytase did not impact growth performance or have any interaction with the disease challenge. The challenged pigs had a higher amount of bacteria and scour scores than the control pigs. There was no effect of phytase or disease challenge on intestinal absorptive capacity or morphology. More research needs to be completed in a more controlled environment where temperature and the disease can be better contained.

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Table 1. Composition of experimental diets, as-fed basis

	Phase 1[1]		Phase 2[2]	
Ingredient	Diet w/ phytase % in Diet[3]	Control Diet % in Diet	Diet w/ phytase % in Diet[3]	Control Diet % in Diet
Corn, yellow dent	55.12	54.35	63.74	62.95
Soybean meal, 47.5% CP	25.00	24.99	27.73	27.73
Soy protein isolate	5.00	5.00	0.00	0.00
Whey, permeate	10.00	10.00	0.00	0.00
Rice Bran	1.50	1.50	5.00	5.00
Poultry fat	1.50	1.50	1.50	1.50
L-lysine HCl	0.36	0.36	0.18	0.18
Monocalcium phosphate 21%	0.06	0.78	0.34	1.05
Limestone	0.92	0.98	0.98	1.06
Salt	0.35	0.35	0.35	0.35
Trace Mineral Premix[4]	0.15	0.15	0.14	0.14
Vitamin Premix[5]	0.04	0.04	0.04	0.04

[1]Formulated to contain 1.4% SID lysine
[2]Formulated to contain 1.25% SID lysine
[3]Phytase was added at 500 and 2,500 FTU
[4]Supplied per kg of complete diet: 16.5 mg of copper as copper sulfate, 0.3 mg of iodine as ethylenediaminedihydroiodide, 165 mg of iron as ferrous sulfate, 40 mg of manganese as manganous oxide, 0.3 mg of selenium as sodium selenite, and 165 mg of zinc as zinc sulfate.
[5]Supplied per kg of complete diet: 18,500 IU of vitamin A, 2640 IU of vitamin D3, 106 IU of vitamin E, 8.7 mg of menadione, 13.2 mg of riboflavin, 79 mg of niacin, 53 mg of d-pantothenic acid, 0.07 mg of vitamin B12, 0.53 mg of biotin, and 4.0 mg of folic acid.

Table 2. Analyzed values of phytase in experimental diets[1]

Diet	Phytase Units
Phase 1 Control	190
Phase 1 500 FTU	670
Phase 1 2,500 FTU	2,300
Phase 2 Control	94
Phase 2 500 FTU	540
Phase 2 2,500 FTU	2,200

[1]Phytase determination was performed by Eurofins Scientific Inc (Des Moines, IA) using AOAC method 2000.12

Table 3. Effect of phytase on bacterial concentration in fecal samples of nursey pigs.[1]

	Healthy			Challenged			
Treatment	Control	500	2500	Control	500	2500	SEM
Pre Challenge							
Mac[2]	6.57E+05	2.54E+06	4.99E+06	1.13E+06	1.61E+06	1.88E+06	1.47E+06
Mac+NA[2]	0	0	3.62E+05	1.61E+05	2.12E+05	3.17E+05	2.27E+05
Post Challenge							
d 4							
Mac[3]	3.25E+06	3.30E+06	1.91E+06	2.40E+06	2.77E+06	2.83E+06	8.86E+05
Mac+NA[3]	1.42E+05	0	0	1.06E+06	1.27E+06	2.93E+06	6.88E+05
d 7							
Mac[4, 5]	4.58E+05	3.76E+06	7.29E+05	1.32E+06	1.22E+06	8.20E+05	5.78E+05
Mac+NA[4, 6]	0	0	1.62E+04	8.09E+03	1.70E+04	2.00E-01	1.21E+04

[1]Mac is the concentration of gram negative bacteria which was measured on Mckonkey agar plates. Mac+NA is the concentration of the specific *e-coli* used in this challenge
[2]Fecal samples collected one day before challenge
[3]Fecal samples collected 4 days post challenge
[4]Fecal Samples collected 7 days post challenge
[5]Disease effect P=0.01
[6]Phytase P = 0.02 and Interaction between phytase and disease P=0.03

Table 4. Effect of an *E-coli* challenge on scour scores in nursey pigs for 7 d post challenge[1]

	Healthy			Challenged				P-Values		
Treatment	Control	500	2500	Control	500	2500	SEM	Phytase	Disease	Interaction
Day 1	1.00	1.00	0.42	2.00	1.60	1.20	0.42	0.22	0.04	0.90
Day 2	1.40	1.00	1.30	1.80	1.90	1.40	0.38	0.45	0.18	0.63
Day 3	1.60	1.00	1.30	1.57	2.20	1.80	0.41	0.99	0.11	0.39
Day 4	1.80	0.50	1.57	1.85	1.80	1.60	0.42	0.34	0.20	0.33
Day 5	2.00	0.50	1.40	1.71	1.80	1.80	0.38	0.25	0.16	0.18
Day 6	2.00	1.00	1.70	1.86	1.90	1.60	0.32	0.40	0.44	0.30
Day 7	2.00	1.00	1.57	2.00	1.80	2.00	0.32	0.24	0.14	0.50

[1]Each pen was scored daily based on the severity of the scours (0-no scours, 1-soft feces, 2-mild diarrhea, and 3-severe diarrhea)

Table 5. Effect of phytase supplementation in an *E-coli* challenge on nursery pigs[1]

	Healthy			Challenged			
Treatment	Control	500	2500	Control	500	2500	SEM
Average daily gain, kg							
d 0 to 7	0.05	0.04	0.06	0.06	0.06	0.06	0.01
d 7 to 10	0.09	-0.03	-0.04	0.01	0.03	0.03	0.04
d 10 to 17	0.07	0.00	0.06	0.01	0.04	0.05	0.02
d 17 to 24	0.17	0.17	0.17	0.17	0.20	0.13	0.03
d 24 to 36	0.13	0.09	0.11	0.11	0.12	0.11	0.04
Total ADG	0.13	0.09	0.11	0.11	0.12	0.11	0.01
Average daily feed intake, kg							
d 0 to 7	0.13	0.11	0.12	0.14	0.12	0.14	0.01
d 7 to 10	0.16	0.12	0.14	0.17	0.17	0.18	0.01
d 10 to 17	0.14	0.03	0.10	0.07	0.10	0.07	0.01
d 17 to 24	0.27	0.18	0.24	0.28	0.25	0.21	0.03
d 24 to 36	0.27	0.15	0.20	0.20	0.20	0.19	0.06
Total ADFI	0.21	0.11	0.17	0.16	0.17	0.16	0.02
Gain: Feed, kg/kg							
d 0 to 7	0.41	0.32	0.45	0.45	0.48	0.43	0.07
d 7 to 10	0.61	0.13	0.21	0.05	0.17	0.17	0.26
d 10 to 17	0.44	0.00	0.80	0.14	0.40	0.46	0.13
d 17 to 24	0.64	0.80	0.78	0.89	0.86	0.68	0.15
d 24 to 36	0.48	0.60	0.55	0.55	0.60	0.58	0.14
Total G:F	0.62	0.75	0.73	0.80	0.75	0.70	0.12

[1]Each value represents the mean of 8 replicates with 7 pigs per pen

Table 6. Effect of phytase supplementation on gut morphology in an *E-coli* challenge on nursery pigs

			Healthy			Challenged		P values		
Treatment	Control	500	2500	Control	500	2500	SEM	Phytase	Disease	Interaction
Duodenum										
Villus Height	249.42	235.56	210.74	235.24	241.01	227.54	19.81	0.40	0.88	0.70
Villus Width	76.73	56.19	84.16	80.16	74.64	74.34	6.45	0.15	0.48	0.15
Crypt Depth	116.16	106.42	120.70	130.55	124.65	117.54	8.78	0.75	0.22	0.41
Ileum										
Villus Height	190.39	198.14	184.03	192.94	187.09	196.62	11.98	0.98	0.90	0.70
Villus Width	67.20	63.41	65.94	74.49	70.53	62.95	6.00	0.45	0.48	0.56
Crypt Depth	83.97	104.95	106.22	107.66	104.09	90.21	6.69	0.56	0.71	0.01

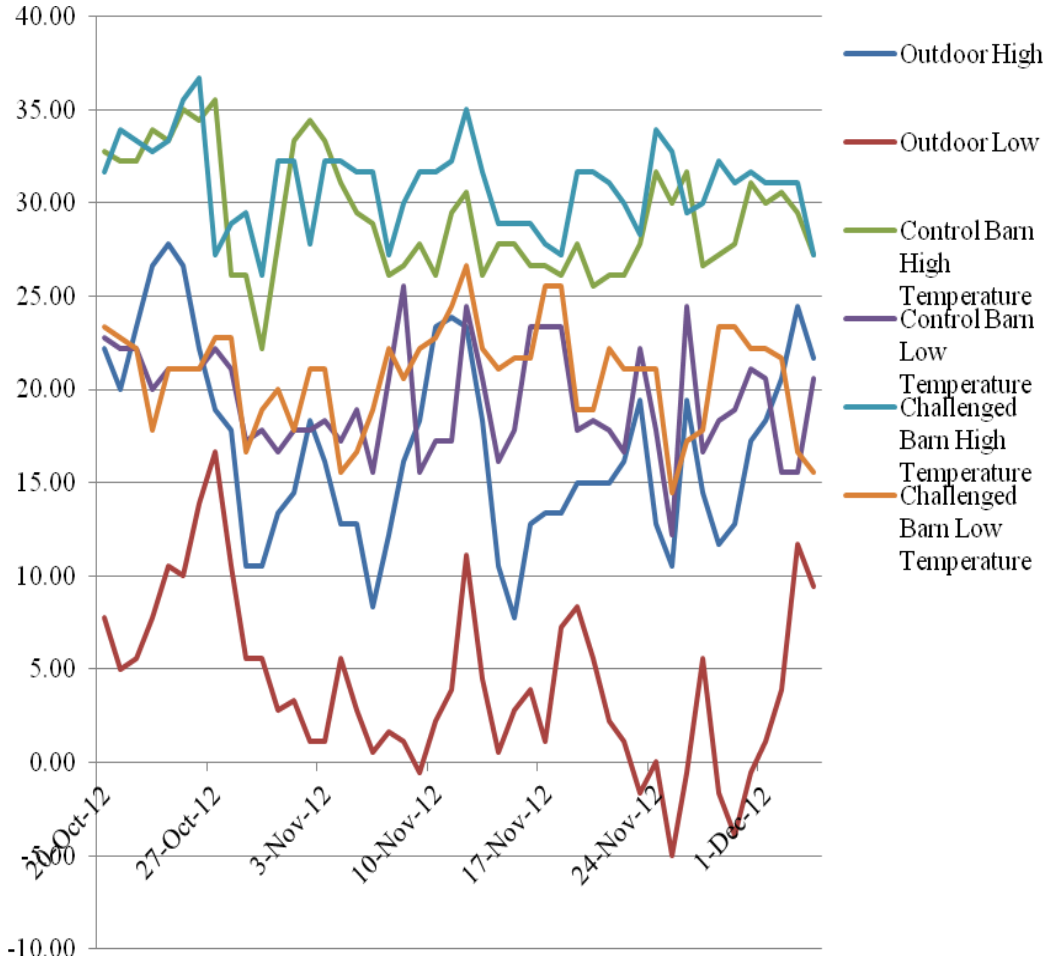

Figure 1. Outdoor and barn temperatures from October 20, 2012-December 3, 2012

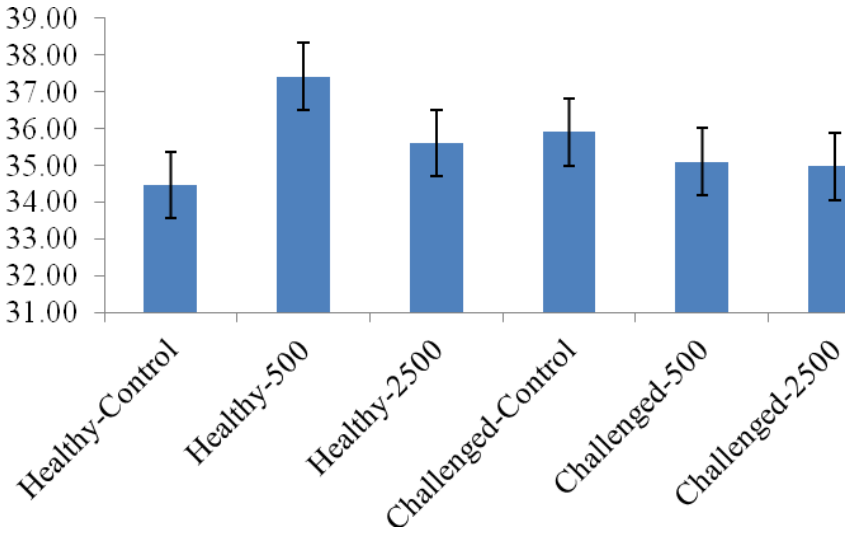

Figure 2. Effect of phytase supplementation on bone ash % in an *E-coli* challenge on nursery pigs

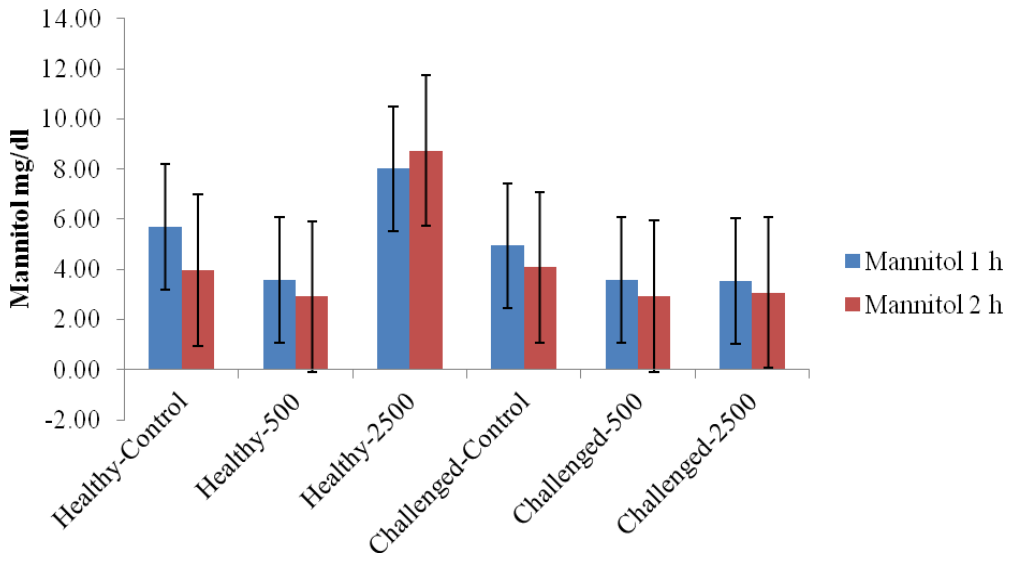

Figure 3: Effect of phytase supplementation on intestinal absorptive capacity n an *E-coli* challenge on nursery pigs

Chapter 5. Improving the bioavailability of P using an *E. coli* phytase

Abstract

This study was designed to measure the ability of an *E. coli* phytase to improve the bioavailability of P using a slope ratio assay in nursery pigs. Pigs (n = 60; BW was 8.50 ±0.76 kg) were weaned at 21 days and fed a common diet for 14 days before being individually housed and placed on experimental diets. Dietary treatments consisted of 1) a negative control diet which was deficient in P (0.55% and 0.17% Ca and available P respectively), 2) negative control diet plus 0.075% additional P which was added from monosodium phosphate, 3) negative control diet plus 0.15% additional P which was added from monosodium phosphate, 4) negative control diet with 250 FTU of phytase, 5) negative control diet with 500 FTU of phytase, 6) negative control diet plus 1000 FTU of phytase.

When phytase was added in the diet at 250, 500, and 1,000 ftu/g bone ash increased 7.2, 9.8, and 15.4% respectively ($P < 0.001$). Bone ash also had an estimated available P equivalent of 0.051, 0.071, and 0.117 %for 250, 500, and 1000 FTU/g respectively. Supplementation of phytase to the control diet increased ($P < 0.05$) bone P concentration by 6.0, 12.3, and 20.0% for 250, 500, and 1,000 ftu supplementation levels, respectively. Bone P had an estimated available P equivalent of 0.041, 0.088, and 0.146% for 250, 500, and 1,000 ftu supplementation levels, respectively. Supplementation of phytase increased serum P by 23.0, 35.6, and 42.7% for 250, 500, and 1,000 ftu phytase levels, respectively ($P < 0.001$). Serum P had an estimated available P equivalent of 0.066, 0.106, and 0.128% for 250, 500, and 1,000 ftu supplementation levels, respectively. Lastly, supplementation with phytase linearly ($P < 0.001$) increased the digestibility of P, improving P digestibility by 23.8, 38.4,

and 60.3% for the 250, 500, and 1,000 ftu compared to the control diet. There was an estimated available P equivalent for apparent fecal digestibility of 0.030, 0.046, and 0.090 for 250, 500, and 1,000 ftu supplementation levels, respectively.

Introduction

The bioavailability of P is low for young pigs fed cereal grains such as corn. It is estimated to be around 15% for corn (NRC, 2012). This is because the majority of the P is bound by an indigestible compound, phytate, which is the major storage form of P in grain. The enzyme phytase has been shown to break the bond formed between phytate and P making the P available for use by the animal (Selle and Ravindran, 2008).

About 10-15% of P in corn and 25% of P in soybean meal is available to pigs. (Cromwell 1995). This leads to a large amount of P being excreted in the feces. This could lead to problems with P in the groundwater and pose an environmental concern (Cromwell 1995). Because phytase allows the P in phytate to be available to the animal, less inorganic P would need to be fed to the animal which leads to less P in the manure of the animal.

Therefore, the objective of this study was to determine the bioavailability of phosphorus when using three levels of phytase in the diet of weanling pigs using a slope ratio assay.

Materials and Methods

Animal use protocols were reviewed and approved by the North Carolina State University Institutional Animal Care and Use Committee.

Pig performance and Sampling

A total of 60 pigs (BW was 8.50 ±0.76 kg) were weaned at approximately 21 d into a temperature controlled raised-deck nursery at the Swine Education Unit, Raleigh, NC, and fed a common starter diet for 14 d. At 35 d of age, pigs were transferred to individual pens. Pigs were weighed and assigned to 1 of 6 dietary treatments resulting in 10 pens per treatment in a randomized complete block design. Each pen had 1 nipple water drinker and a double-spaced feeder. Pigs were allowed ad-libitum feed and water access throughout the experiment. Dietary treatments (Table 1) consisted of 1) a negative control diet which was deficient in P (0.55% and 0.17% Ca and available P respectively), 2) negative control diet plus 0.075% additional P which was added with monosodium phosphate, 3) negative control diet plus 0.15% additional P which was added with monosodium phosphate, 4) negative control diet with 250 FTU of phytase5) negative control diet with 500 FTU of phytase, 6) negative control diet plus 1000 FTU of phytase. The phytase used in this study was an *E. coli* phytase (Lohmann Animal Health, Cuxhaven, Germany). A basal diet was manufactured at the North Carolina State University Feed Mill Educational Unit. Corn was ground with a hammer mill (Model 1522, Roskamp Champion, Waterloo, IA) equipped with a 2.2 mm screen. Dry ingredients were blended in a double ribbon mixer (Model TRDB126-0604, Hayes and Stolz, Ft Worth, TX) and fat was added after dry mixing was complete. Experimental diets were manufactured from the basal diet. The basal diet was divided into 6 equal portions to which ground corn, monosodium phosphate, calcium carbonate, or phytase were added as appropriate for each treatment. Thus, one portion of the basal was mixed with 0.5% finely ground corn and served as the negative control diet. For the other diets,

monosodium phosphate, calcium carbonate, or phytase were added to 0.9 kg finely ground corn and mixed in a stainless steel stand mixer (KitchenAid, St. Joseph, MI). This was then mixed again in a bucket with additional finely ground corn to a total of 0.5% of the final batch volume. Subsequently, premixes were mixed with one of the five remaining portions of each basal in a horizontal double ribbon mixer. The mixer was flushed with corn at 10% of the mixer capacity to minimize cross-contamination between diets. This process ensures that diets were identical in their composition with the exception of Ca, P, and phytase content.

Pigs were weighed individually on day 0, and after 14 d and 28 d. Feed intake and feed efficiency were measured during d 0 to 14, d 15 to 28, and d 0 to 28.On d 26, 27, and 28, fecal samples were obtained to determine apparent total tract digestibility of P using titanium dioxide as an indigestible marker. Fecal samples were collected from each pen by grab sampling of freshly voided feces or from fresh feces present in the pen. Pens in this facility are separated by solid partitions and, therefore, samples in each pen are specific to the pig in that pen. On d 28, pigs were humanely euthanized by captive bolt. Blood samples were obtained in a 10-mLtube without any additives for determination of serum P concentration. The left foreleg was removed and frozen for later analysis.

Fecal samples were dried, ground to pass through a 1 mm screen and thoroughly mixed. Concentration of P in feed and fecal samples was determined following wet ashing with nitric acid using either a hot plate or a microwave digestion system. Concentration of P in the digested sample was determined colorimetrically. Titanium dioxide was determined by digesting samples in concentrated H_2SO_4, followed by the addition of 30% H_2O_2, and measuring absorbance using a spectrophotometer at 410 nm (Myers et al 2004).

Bones were cleaned, removing all tissue from the bones. Air-dried metacarpal bones were oven-dried at 102°C for 24 hours and cooled in a desiccator for DM determination. Bones were then placed into a muffle furnace at 550°C for 6 hours to determine bone ash.

For P determination in bones, approximately 0.5 g of bone ash was digested with 10 mL of nitric acid (50% v:v)and brought up to a 25 mL total volume with distilled water. The colorimetric determination of P was conducted by reacting the ashed samples with ammonium molybdate to form phosphomolybdate. A mixture (Fiske and Subbarow solution) of sodium bisulfate, sodium sulfite, and 1-amino-2-naphthol-4-sulfonic acid was added to reduce the phosphomolybdate to a phosphomolybdenum blue complex. The intensity of the blue color is proportional to the phosphate concentration and was measured at 660 nm. Sample P concentration was determined relative to a standard curve consisting of 0, 2.5, 5.0, 7.5, 10.0, and 12.5 mg/dL of P.

For P determination in blood samples, blood was centrifuged at 500 x g to collect serum. Serum was then deproteinated with trichloroacetic acid, centrifuged to collect the clear supernatant, and P concentration was determined colorimetrically as described above.

Statistical analysis

Regression analysis was performing using the Proc Reg procedure of SAS (SAS Inst. Inc., Cary, NC). The model included phosphorus level and phytase level. Regression analysis was conducted by regressing bone ash (and bone P) of pigs fed the negative control diet and the diets containing the two levels of monosodium phosphate on the amount of dietary P to establish a standard curve. Available P equivalent values were then determined from the standard curve from the measured bone ash (or bone P). Other statistical analysis was

performed using the Proc GLM procedure of SAS. Orthogonal contrast comparisons were made to determine linear and quadratic effects of phytase and phosphorus addition.

Results

Levels of phytase in the diet differed among calculated and analyzed values. Phytase values were higher when analyzed in the premix (6,200 ftu/g measured vs. 4,080 ftu/g reported by the manufacturer) and the diets (Table 1). This was confirmed by two separate laboratories (New Jersey Feed Laboratory, Inc. (NJFL), Ewing, N.J.) and Eurofins Scientific (Des Moines, IA). Analyzed dietary phytase concentrations are reported in Table 2 and compared to the calculated dietary phytase concentrations using the analyzed phytase concentration of the premix (6,200 ftu/g for Eurofins and 5,220 ftu/g for NJFL).

In regards to the diets, control diets had a mean of 81 FTU/g for phase 1 and 2 diets. This is due to the amount of phytase in the ingredients and not supplied by the enzyme. When using the analyzed values from Eurofins and NJFL, the diets measured closely for analyzed and calculated values. As the calculated values for phytase increased in the diets, so did the analyzed values. This suggests that the diets were adequately mixed across all treatments.

When diets increased in levels of available P, bone ash content increased (Table 3). When pigs were fed additional P, bone ash content increased ($P < 0.05$) by 11.9 and 18.4% for diets fed 0.075 and 0.15% additional P, respectively. When phytase was added in the diet at 250, 500, and 1,000 ftu/g bone ash increased 7.2, 9.8, and 15.4% respectively. Bone ash (DM basis) responded linearly (Table 3; Figure 1) to added P concentration in the diet ($P < 0.001$) and regression analysis produced the equation:

Bone ash (DM basis) =

24.282 (±0.36) + 29.883 (±3.81) * available P; R^2 = 0.69. ***Eq. 1***

Similarly, supplementing phytase to the control diet also yielded a linear response in bone ash ($P < 0.001$). By adding 250, 500, and 1000 ftu/g, bone ash increased linearly resulting in the following equation to predict bone ash from supplemental dietary phytase:

Bone ash (DM basis) =

24.501 (±0.43) + 0.0035 (±0.0007) * added phytase; R^2 = 0.37. ***Eq. 2***

Using Eq. 1, available P concentrations released by phytase can be calculated to produce bone ash values for each of the added phytase concentrations (Table 4, Figure 2). Similarly, the effectiveness of phytase in liberating available P can be calculated by solving Eq. 1 and Eq. 2 for equal bone ash percent. This results in an equation that allows the estimation of available P from the amount of added phytase, as follows:

Available P (%) = 0.0117 * added phytase (*100 ftu) + 0.0073 **Eq. 3**

Bone P (DM basis) also increased (Table 3) with increasing concentrations of dietary P and dietary phytase. Compared to pigs fed the control diet, bone P concentration was increased ($P < 0.05$) by 11.6 and 19.7% for pigs fed an additional 0.075 and 0.150% available P, respectively. Supplementation of phytase to the control diet increased ($P < 0.05$) bone P concentration by 6.0, 12.3, and 20.0% for 250, 500, and 1,000 ftu supplementation levels, respectively. Bone P increased linearly (Table 3; Figure 3) as P was added in the diet and regression analysis produced the following equation:

Bone P (DM basis) =

4.334 (±0.061) + 5.729 (±0.649) * available P; R^2 = 0.74. ***Eq. 4***

Increasing phytase in the diet also resulted in a linear increase in bone P concentration ($P < 0.001$) resulting in the following equation:

Bone P (DM basis) =

4.349 (±0.102) + 0.00086 (±0.00018) * added phytase; $R^2 = 0.38$. ***Eq. 5***

Available P concentrations released by phytase were calculated from Eq. 4 by using the bone P concentrations for each of the added dietary phytase concentrations and are shown in Table 4. Similarly, the effectiveness of phytase in liberating available P can be calculated by solving Eq. 4 and Eq. 5 for equal bone P concentration. This results in an equation that allows the estimation of available P from the amount of added phytase, as follows:

Available P (%) = 0.015 * added phytase (*100 ftu) + 0.0026 ***Eq. 6***

Similarly to bone ash % and bone P concentration, serum P also increased with increasing levels of P compared to the control and phytase compared to the control. Increasing available P by 0.075 and 0.150% increased serum P by 30.1 and 47.5%, respectively (Table 3, Figure 4). Supplementation of phytase increased serum P by 23.0, 35.6, and 42.7% for 250, 500, and 1,000 ftu phytase levels, respectively.

Serum P increased linearly (Table 3; Figure 5) to added available P concentration in the diet ($P < 0.001$). Regression analysis, using all individual data points, yielded the following equation:

Serum P (mg/dL) = 7.809 (±0.379) + 24.253 (±3.917) * available P; $R^2 = 0.58$. ***Eq. 7***

Supplementation of phytase to the control diet also resulted in a linear increase in bone P concentration ($P < 0.001$). Regression analysis, using all individual data points, resulted in the following equation to predict bone P from supplemental dietary phytase:

Serum P (mg/dL) =

8.247 (±0.392) + 0.003 (±0.00070) * added phytase; R^2 = 0.34. ***Eq. 8***

Concentrations of available P released by phytase were calculated from Eq. 7 using the serum P concentrations for each of the added phytase concentrations (Table 4, Figure 6). Similarly, the effectiveness of phytase in releasing available P can be calculated by solving Eq. 7 and Eq. 8 for equal serumP concentration. This results in an equation that allows the estimation of available P from the amount of added phytase, as follows:

Available P (%) = 0.018 + 0.0124 * added phytase (*100 ftu) ***Eq. 9***

Addition of P from monocalcium phosphate improved the digestibility of P linearly (P = 0.02), although there was no improvement in digestibility of P when added P was increased from 0.075 to 0.150 (Table 3). Supplementation with phytase linearly (P < 0.001) increased the digestibility of P, improving P digestibility by 23.8, 38.4, and 60.3% for the 250, 500, and 1,000 ftu compared to the control diet.

Regression analysis, using all data points, resulted in the following relationship between apparent total tract digestibility of P and supplemental phytase:

Apparent total tract digestibility of P (%) =

38.367 (±1.048) + 0.0213 (±0.0019) * supplemental phytase; R^2=0.78 ***Eq. 10***

Using this regression equation, the estimated digestibility of P for the different diets was 38.4, 43.7, 49.0, and 59.7% for the 0, 250, 500, and 1,000 ftu phytase diets, respectively. When using the digestibility values and the analyzed content (average for phase 1 and 2 diets) of dietary P, the available P content of the diet program can be calculated as 0.167,

0.197, 0.213, and 0.257%, for the 0, 250, 500, and 1,000 ftu of phytase diets, respectively. Thus, the addition of phytase improved digestible (and presumably available) P by 0.030, 0.046, and 0.090% for 250, 500, and 1,000 ftu supplemental phytase, respectively (Table 4).

Discussion

Phytate, which is the salt of phytic acid, is the deposited complex of IP_6 with minerals, potassium, magnesium, and calcium, as it would normally appears in plants (Selle and Ravindran, 2008). Phytate is the major storage form of phosphorus in grain. It represents 50-85% of the total phosphorus in plant seeds (Pallauf and Rimbach, 1997). There are two main ways for the animal to obtain enough P. One is to supplement the animal with dietary P. (Selle and Ravindran 2008). The negative aspect of supplementing the animal with dietary P is there will be an increase in P in the manure which could lead to environmental concerns (Cromwell et al. 1995). Another way of allowing the animal to obtain P is to supplement phytase in the diet, which will hydrolyze the bond between the phytate molecule and P. The animal is then able to absorb P (Selle and Ravindran 2008).

The objective of this study was to determine the bioavailability of phosphorus when using three levels of phytase in the diet of weanling pigs using a slope ratio assay. There are several ways to measure the bioavailability of P when increasing P in the diet or supplementing phytase. One of the most effective ways is measuring bone ash % (Koch and Mahan 1985). As the dietary need for P is met, storage of P occurs in the bones and so a higher bone ash % correlates to higher P. Data for bone ash in the current study clearly demonstrate that phytase was effective in improving bone mineralization as measured by bone ash and that phytase can increase available P as measured in this slope ratio study.

These data are similar to results by Braña et al. (2005) who fed phytase 250, 500, 750, and 1000 FTU. Ausperger et al. (2003) also found an increased tibia ash % when pigs were fed phytase in a phytase titration.

Bone P is another technique to measure bioavailability of P with supplementation of phytase. Similarly to bone ash, as the P needs for the body are met, P is then stored in the bone. Varley et al. 2010 fed 0, 500, and 1,500 ftu/g of a fungal phytase and saw a linear increase in bone P as phytase was added in both nursery and finisher pig diets. In a later study, Varley et al. 2011 also fed 0, 500, and 1,500 ftu/g and noticed an increase in bone P in finishing pigs. Brady et al. 2002 also fed a different fungal phytase and bone P was increased as phytase was added in the diet. In agreement with these studies, results from the current study showed that bone P demonstrated phytase was effective in increasing P availability as evidenced by increased bone P concentrations to levels that were equivalent to bone P concentrations of pigs fed 0.15% supplemental dietary available P.

Serum P is also a good measure of the bioavailability of phosphorus. Koch and Mahan measured serum P and it was decreased in nursery pigs fed diets low in P compared to diets with adequate P. Lei et al. (1993) fed 0, 250, 500, and 750 ftu/g of a fungal phytase and measured normal serum P levels for nursery pigs when they were fed phytase and moderately deficient when they were fed the P-deficient diet. The same was true when Murry et al. (1997) fed a microbial phytase and measured increased levels of serum P when phytase was added in the diet. Similarly with the previous studies, supplementation with dietary phytase to P deficient diets in the current study increased serum P concentrations to levels

similar to those of pigs fed increased amounts of available P, indicating that phytase was able to release bound P and make it available to the pig.

Increasing phytase in the diet of pigs has also been shown to increase P digestibility. Harper et al. 1997 observed an increase in P digestibility by up to 44% when pigs were fed 500 ftu/g of a fungal phytase. Cromwell et al. fed 250, 500, and 1,000 ftu/g and calculated 30, 80 and 90% improvements of P digestibility as phytase was added in the diets. This in agreement with the current study as adding dietary phytase to P deficient diets also increased digestible P concentrations to levels similar to those fed increased amounts of available P, which shows that phytase was successful in releasing the bound P making it available to the pig.

In summary, the phytase used in this study was able to increase bone ash %, bone P concentration, serum P concentration and the digestibility of P linearly. Supplementing nursery pig diets with the current phytase would be able to improve the bioavailability of phosphorus.

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Table 1. Composition of experimental diets

	Phase 1			Phase 2		
Ingredient	**Control**	**+0.075% P**	**+0.150% P**	**Control**	**+0.075% P**	**+0.150% P**
Corn, yellow dent	61.49	61.00	60.52	65.17	64.68	64.19
Soybean meal, 47.5% CP	32.68	32.68	32.68	29.02	29.02	29.02
Soybean oil	3.00	3.00	3.00	3.00	3.00	3.00
L-lysine HCl	0.37	0.37	0.37	0.37	0.37	0.37
DL-methionine	0.13	0.13	0.13	0.11	0.11	0.11
L-threonine	0.10	0.10	0.10	0.10	0.10	0.10
Monosodium phosphate	0.36	0.65	0.94	0.19	0.48	0.77
Limestone	1.18	1.38	1.57	1.05	1.25	1.45
Nursery Vitamin Premix	0.04	0.04	0.04	0.04	0.04	0.04
Nursery Mineral Premix	0.15	0.15	0.15	0.15	0.15	0.15
Salt	0.50	0.50	0.50	0.50	0.50	0.50
Titanium dioxide	-	-	-	0.30	0.30	0.30
Calculated nutrient concentrations						
ME, Mcal/kg	3.30	3.30	3.30	3.30	3.30	3.30
Total lysine, %	1.42	1.42	1.42	1.32	1.32	1.32
CP %	21.17	21.16	21.16	19.74	19.74	19.73
Ca %	0.57	0.65	0.72	0.51	0.59	0.66
Available P	0.17	0.24	0.32	0.12	0.20	0.27
Total P	0.49	0.56	0.63	0.43	0.50	0.57
Digestible P	0.20	0.27	0.33	0.16	0.22	0.29
Standardized ileal digestible amino acids, %						
Lys	1.27	1.27	1.27	1.18	1.18	1.18
Thr	0.75	0.75	0.75	0.70	0.70	0.70
Met	0.41	0.41	0.42	0.38	0.38	0.38
Met+Cys	0.70	0.70	0.70	0.65	0.65	0.65
Trp	0.23	0.23	0.23	0.21	0.21	0.21

Table 2. Comparison of analyzed vs. calculated values of experimental diets

		Phytase supplemented diets, ftu/kg		
Item	**Control**	**250**	**500**	**1,000**
Phase 1				
Calculated (6,200 ftu/g of premix)[1]	0	380	760	1,520
Analyzed	97	470	910	1,800
Calculated (5,220 ftu/g of premix)[2]	0	320	640	1,280
Analyzed	143	432	683	1,730
Phase 2				
Calculated (6,200 ftu/g of premix)[1]	0	380	760	1,520
Analyzed	73	510	880	1,900
Calculated (5,220 ftu/g of premix)[2]	0	320	640	1,280
Analyzed	137	351	772	1,390

[1]Analyzed by Eurofins, Inc., using AOAC 2000.12.
[2]Analyzed by New Jersey Feed Laboratory, Inc., using International Standard ISO 30024 (2009).

Table 3**.** Response of bone ash, bone P, serum P, and apparent total tract digestible P to supplemental P and phytase.

	Added available P			**Added Phytase**			
	0	**0.075**	**0.150**	**250**	**500**	**1000**	**SEM**
Bone ash, % DM[1,2]	24.07[a]	26.94[bc]	28.51[d]	25.81[b]	26.40[bc]	27.77[cd]	0.50
Bone P, % DM[1,2]	4.31[a]	4.81[b]	5.16[c]	4.57[ab]	4.84[bc]	5.17[c]	0.12
Serum P, mg/dL[1,2]	7.65[a]	9.95[bc]	11.28[d]	9.41[b]	10.37[bcd]	10.92[cd]	0.43
Fecal P digestibility, %[3]	36.5[a]	41.9[bc]	40.6[b]	45.2[c]	50.5[d]	58.5[e]	1.3

[1]Linear effect of added available P ($P < 0.001$).
[2]Linear effect of added phytase ($P < 0.001$).
[3]Linear effect of added phytase ($P = 0.02$).
[abcd] Means within a row without a common superscript are different ($P < 0.05$).

Table 4**.** Estimated available P equivalent of supplemental phytase.

	Supplemental phytase, ftu/kg		
Item	**250**	**500**	**1,000**
Estimated available P equivalent (%)			
Based on bone ash (DM basis)	0.051	0.071	0.117
Based on bone P concentration	0.041	0.088	0.146
Based on serum P concentration	0.066	0.106	0.128
Based on apparent fecal P digestibility	0.030	0.046	0.090

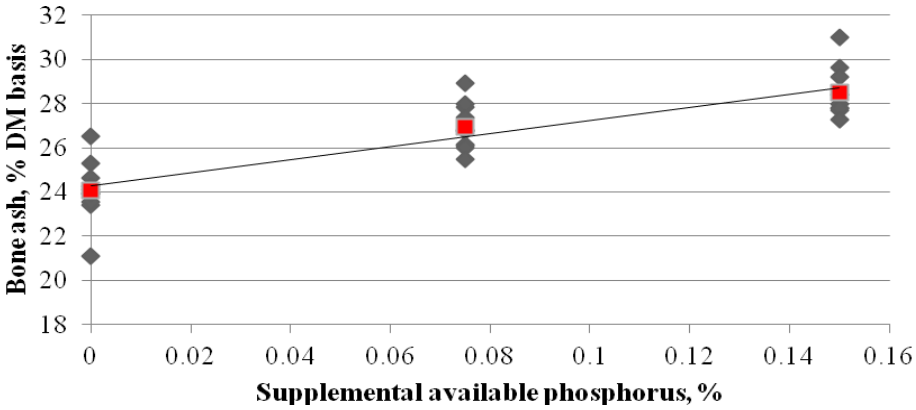

Figure 1**.** Regression analysis of bone ash as a function of supplemental available P.

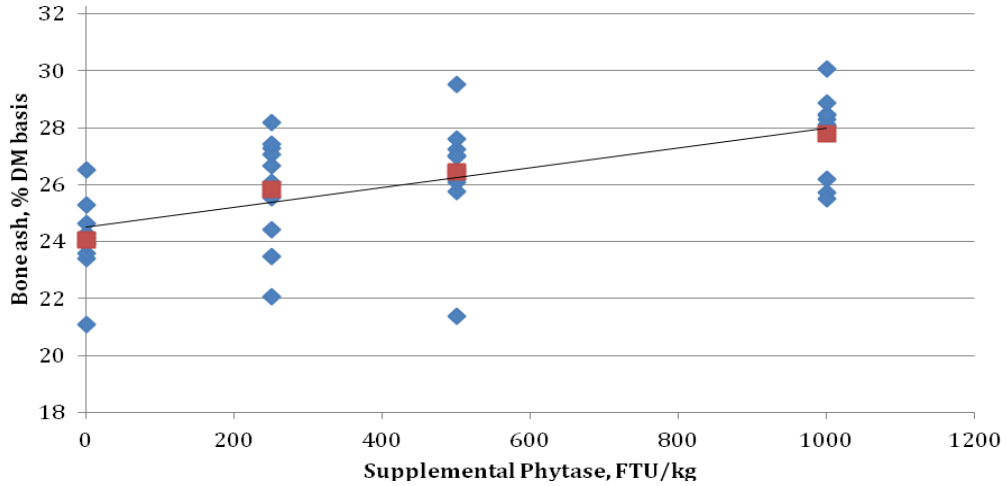

Figure 2**.** Regression analysis of bone ash as a function of supplemental phytase

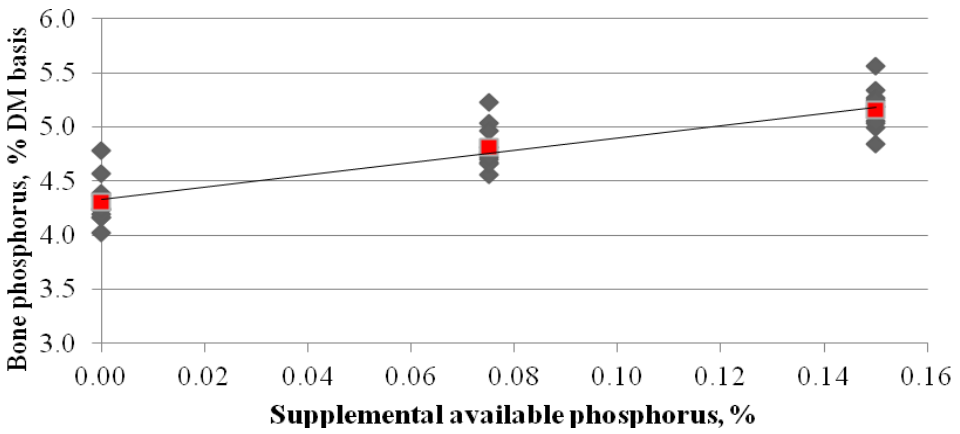

Figure 3**.** Regression analysis of bone P as a function of supplemental available P.

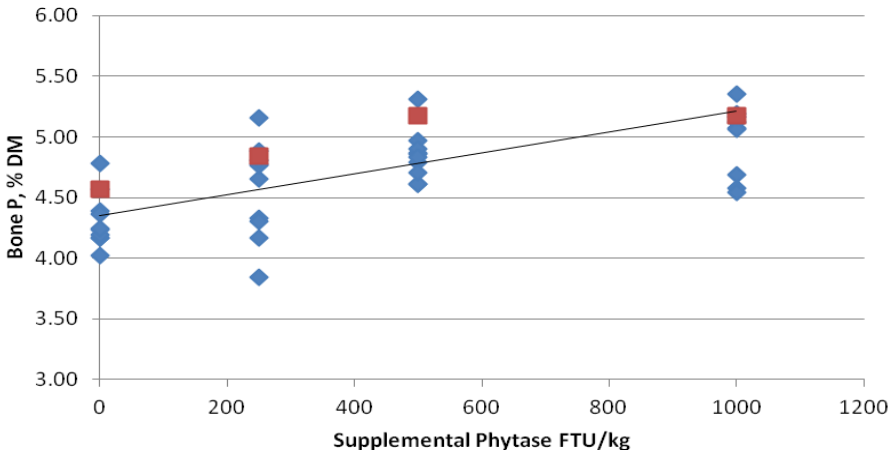

Figure 4**.** Regression analysis of bone P as a function of supplemental phytase.

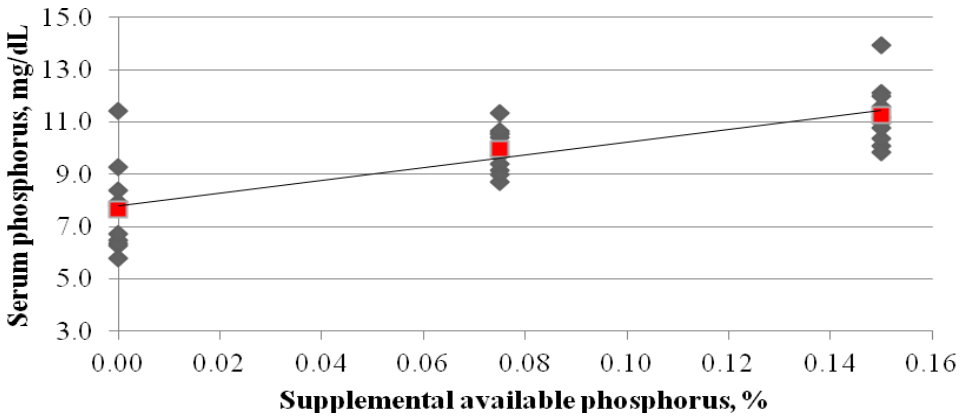

Figure 5**.** Regression analysis of serum P as a function of supplemental available P.

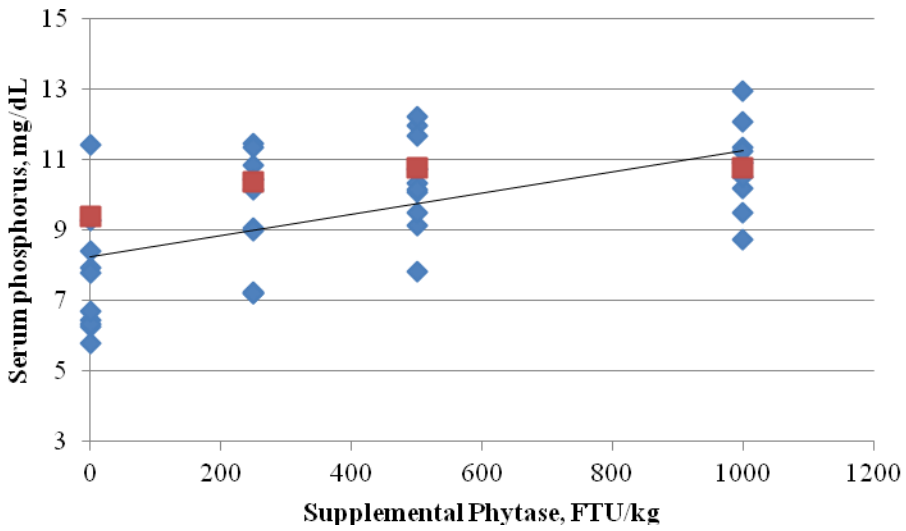

Figure 6**.** Regression analysis of serum P as a function of supplemental phytase.

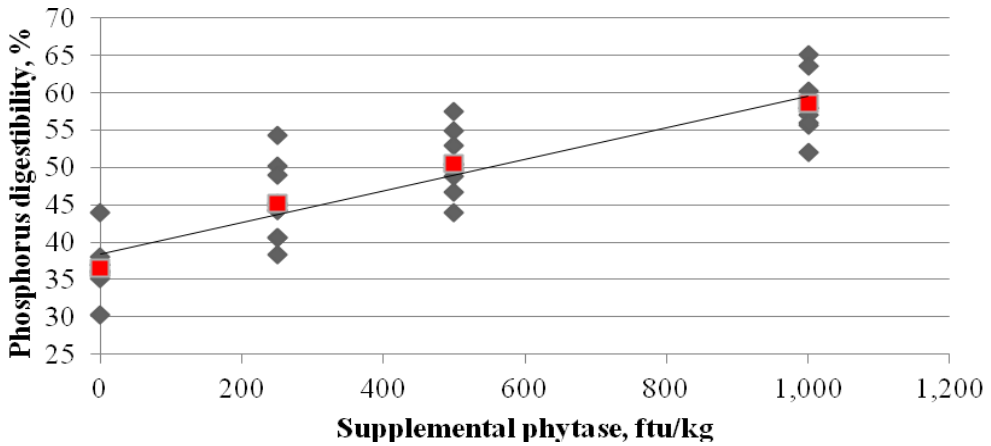

Figure 7**.** Regression analysis of fecal P digestibility as a function of supplemental phytase.