# GUT SECRETIONS AND NUTRIENT ABSORPTION RESPONSES TO DIETARY PHYTIC ACID AND PHYTASE IN PIGLETS

By

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**A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirement of the degree of Doctor of Philosophy**

## ABSTRACT

Phytic acid (**PA**) reduces nutrient digestibility in pigs and poultry, and has been shown to increase endogenous nutrient losses (**ENL**) in poultry. However, there is lack of information on the effect of PA on ENL in pigs, and mechanisms by which PA increases ENL. Three experiments were conducted to determine the effects of PA on ENL in pigs and to establish mechanisms by which PA increases the ENL. The first experiment investigated the effect of PA on ileal digestibility and ileal endogenous nutrient flows. Phytic acid decreased the apparent ileal sodium digestibility to a negative value (-18%). The second experiment investigated the effect of PA on gut enzyme activities, histomorphology and sodium-dependent glucose transporter 1 (**SGLT1**) gene expression. Phytic acid did not affect the gut villous height, villous height to crypt depth ratio, and jejunal SGLT1 gene expression, but decreased gastric pepsin activity and tended to decrease jejunal Na-K-ATPase activity. In the third experiment, the effect of PA on piglet performance and ion uptake in jejunum mounted in Ussing chamber, and jejunal SGLT1 protein level was evaluated. Phytic acid did not affect jejunal SGLT1 protein expression, but lowered piglet performance and jejunal active ion uptake. In conclusion, results from this study show that PA can reduce the apparent ileal digestibility of sodium to a negative value, indicating that PA can increase ileal endogenous sodium loss. The results also show that PA can reduce the pepsin activity and ion uptake in the gut. The reduced pepsin activity implies increased secretion of the enzyme plus hydrochloric acid and hence increased secretion of sodium bicarbonate that neutralizes the acid. The reduced ion uptake by PA implies reduced nutrient absorption.

Because sodium is absorbed partly by co-transportation with other nutrients, the reduced ion uptake by PA implies reduced sodium absorption. Thus, it appears that PA increases ileal endogenous sodium flow partly through reduced pepsin activity and ion uptake in the small intestine. Overall, the results show that phytase (a PA-hydrolysing enzyme), which is added in pig diets to improve phosphorus availability, does not only improve phosphorus availability, but alleviates ant-nutritional effects of PA as well.

## DEDICATION

This thesis is dedicated to my wife Agnes Wanga; my parents Agnes and Andrew Oyengo; and to my siblings Opiyo, Olumi, Cholwa, Were and Onyango.

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## FOREWORD

This thesis was written in a manuscript format and it is composed of five manuscripts. The five manuscripts were prepared from data obtained from three studies that were conducted to achieve the objectives of the thesis research. The first manuscript was prepared using results from the first study. The second, third and fourth manuscripts were prepared using results from the second study, whereas the fifth manuscript was prepared based on the results for the third study. The first and the second manuscripts have been published in British Journal of Nutrition and Livestock Science, respectively, whereas the fourth manuscript has been submitted to Journal of Animal Science. The third and fifth manuscripts are under preparation. Authors to the published or submitted manuscripts are as follows: first manuscript - T. A. Woyengo, A. J. Cowieson, O. Adeola and C. M. Nyachoti; second manuscript - T. A. Woyengo, O. Adeola, C. C. Udenigwe and C. M. Nyachoti; and fourth manuscript - T. A. Woyengo, J. C. Rodriguez-Lecompte, O. Adeola and C. M. Nyachoti. T. A. Woyengo, C. M. Nyachoti and J. C. Rodriguez-Lecompte are from Department of Animal Science, University of Manitoba. C. C. Udenigwe is from Department of Human Nutritional Sciences, University of Manitoba. O. Adeola is from Department of Animal Sciences, Purdue University, West Lafayette, IN, USA, whereas A. J. Cowieson is from Danisco (UK) Limited, Marlborough, UK. In the thesis, the manuscripts were written according to the guidelines for the Journal of Animal Science manuscript preparation.

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| | |
|---|---|
| $C_T$ | Cycle number at an amplification threshold |
| ENL | Endogenous nutrient losses |
| PA | Phytic acid |
| SGLT1 | Sodium-dependent glucose transporter 1 |
| $\Delta C_T$ | Normalized $C_T$ value |
| $\Delta\Delta C_T$ | Comparative $C_T$ value |

# CHAPTER ONE

## GENERAL INTRODUCTION

Vegetable feedstuffs constitute a high proportion of pig and poultry feeds because they are cheaper than the feedstuffs of animal origin. However, approximately two-thirds of phosphorus in vegetable feedstuffs is poorly digested by pigs and poultry because it is bound to phytic acid, which is poorly hydrolysed by pigs and poultry, as they do not produce sufficient amounts of phytase (Mroz et al., 1994; Rutherfurd et al., 2004). Because of the low capacity of pigs and poultry to digest phytic acid-bound phosphorus, inorganic sources of phytic acid-bound phosphorus, which are expensive, are added in feeds to meet P requirements of the pigs and poultry, leading to increased cost of feeding (Selle and Ravindran, 2007, 2008). Furthermore, the phytic acid-bound phosphorus that is not absorbed in gut of pigs and poultry is discharged into environment, which can lead to environmental pollution (Lenis and Jongbloed, 1999; Selle and Ravindran, 2007).

In addition to the poor digestibility of phytic acid-bound phosphorus, phytic acid can also reduce the utilization of other nutrients in pig and poultry diets. This is because phytic acid is negatively charged at acidic, neutral and basic pH (Maenz, 2001), indicating that it has the capacity to bind positively charged molecules in the diet and endogenous gastrointestinal tract secretions such as digestive enzymes and mucins at all pH conditions found in the gastrointestinal tract.

Phytic acid has indeed been reported to limit the digestibility of minerals and amino acids in boilers (Ravindran et al., 2000, 2006; Cowieson et al., 2006), which implies reduced efficiency of nutrient utilization and increased environmental discharge of nutrients as a result of excessive excretion of unabsorbed nutrients due to phytic acid.

Phytic acid has also been shown to increase endogenous losses of minerals and amino acids in broilers (Cowieson et al., 2004; Cowieson and Ravindran, 2007). Because the increased endogenous nutrient losses in the gastrointestinal tract are associated with an increased maintenance requirement for the lost nutrients and of the energy spent on their secretion (Nyachoti et al., 1997a), an increase in endogenous loss of nutrients due to phytic acid implies that there are other adverse effects of phytic acid on the efficiency of nutrient utilization in addition to reducing nutrient digestibility. However, there is lack of information on the effects of phytic acid on the endogenous loss of nutrients in pigs, and on the mechanisms by which phytic acid increases gastrointestinal tract endogenous nutrient losses and the impact of these endogenous nutrient losses on digestive physiology of pigs. Understanding the mechanisms by which phytic acid increases gastrointestinal tract endogenous nutrient losses and the impact of these endogenous nutrient losses on digestive physiology is of critical importance in gaining insight into mechanisms by which phytic acid reduces nutrient utilization in pigs as well as the effectiveness of possible interventions.

Phytic acid may bind to digestive enzymes and dietary protein in the stomach, leading to reduced enzyme activity and hence increased enzyme and acid secretions via negative feedback mechanisms. The resulting acidic digesta may then need to be neutralized by bicarbonates in the small intestine, leading to increased secretion of nutrients, especially minerals. Also, the increased acid secretion may result in increased mucin secretion to protect the gut wall from acid digestion leading to increased amino acid losses.

In addition to binding digestive enzymes in the stomach, phytic acid may also bind to digestive enzymes in the small intestine, leading to increased secretion of digestive enzymes and hence amino acids and their co-factors through negative feedback mechanisms. Furthermore, phytic acid may reduce the re-absorption of the nutrients that are endogenously secreted into the gastrointestinal tract by binding to the endogenously secreted nutrients, thereby increasing the endogenous nutrient losses. Sodium is absorbed in the small intestine partly by co-transport with other nutrients and by solvent drag due to increased solute (nutrient) absorption, and its absorption increases with increase in the absorption of other nutrients (Fordtran, 1975). Thus, phytic acid may also reduce the re-absorption of endogenously secreted sodium by reducing the absorption of other nutrients such as glucose leading to increased endogenous losses of sodium.

Sodium deficiency in chickens has been reported to reduce the activity of Na-K-ATPase in the small intestine and to increase the expression of genes for the same enzyme, which is involved in the absorption of several nutrients including glucose and amino acids (Gal-Garber et al., 2003). Therefore, the increased secretion of sodium may alter the synthesis and activity of Na-K-ATPase, which is involved in nutrient absorption. Also, the increased secretion and reduced (re)absorption of endogenous nutrients may result in reduced synthesis of proteins that are involved in the absorption of macronutrients such as glucose transporter proteins, whose synthesis is positively associated with the availability of nutrients for absorption (Dyer et al., 1997).

Because the villous height reduces with decrease in the availability of nutrients in enterocytes (Pluske et al., 1996), the increased secretion and reduced (re)absorption of endogenous nutrients by phytic acid may result in reduced villous height, leading to a

decrease in surface area for nutrient absorption and hence further reduction in nutrient absorption. Also, because mucins are involved in the lubrication and protection of the gastrointestinal tract epithelium from pathogens, toxins, and acid and enzymatic hydrolyses (Montagne et al., 2004), a decrease in mucin coverage of the epithelium due to phytic acid may result in increased contact between luminal contents and epithelium, increasing the susceptibility of the animals to villous height infections.

Therefore, it was hypothesized that phytic acid increases the endogenous nutrient losses in pigs by reducing the activity of endogenous enzymes and by reducing the (re)absorption of the endogenously secreted nutrients in the gastrointestinal tract, and that the increased endogenous nutrient losses result in reduced capacity of the small intestine to absorb nutrients. The main objective of this research was to determine the effect of dietary phytic acid on the endogenous nutrient losses in pigs and to establish the mechanisms by which phytic acid increases the endogenous nutrient losses in pigs.

# CHAPTER TWO

# LITERATURE REVIEW

## *2.1 Phytic Acid*

Phytic acid, a *myo*-inositol hexakisphosphate (Figure 2.1; Cowieson et al., 2004), is the major storage form of phosphorus in plant tissues (Maenz, 2001). It has 12 protons and of these 6 dissociate at acidic pH, 3 at neutral pH, and the remaining 3 at basic pH, meaning that phytic acid is negatively charged at a wide range of pH conditions (Maenz, 2001). In nature (in plant tissues) phytic acid occurs as phytate (i.e., a mixed salt of cations, mainly potassium and magnesium, and to a lesser extent calcium, iron and zinc), and it is located within protein bodies (Ockenden et al., 2004; Joyce et al., 2005; Lin et al., 2005). Based on the phytate phosphorus content in feed ingredients that are commonly used in the formulation of pig and poultry feeds (Table 2.1), phytic acid concentration in the feed ingredients range from 0.57 to 2.80% with values being higher for cereal milling by-products and oil seed meals followed by grain legumes, and then cereal grains. And based on these phytic acid concentrations in the feed ingredients that are commonly used in formulation of pig and poultry feeds, phytic acid concentration in a typical swine or poultry diet range from 1.0 to 1.8%.

Phytic acid is poorly hydrolyzed by endogenous enzymes of pigs and poultry. For example, Rutherfurd et al. (2004) and Cowieson et al. (2006) found digestibility of phytic acid-bound phosphorus in broilers to be as low as 10%, whereas Jongbloed et al. (1992) and Mroz et al. (1994) reported ileal phytic acid digestibility values of 9.6 and 30% respectively, in growing pigs. Because of the low capacities of pigs and poultry to digest

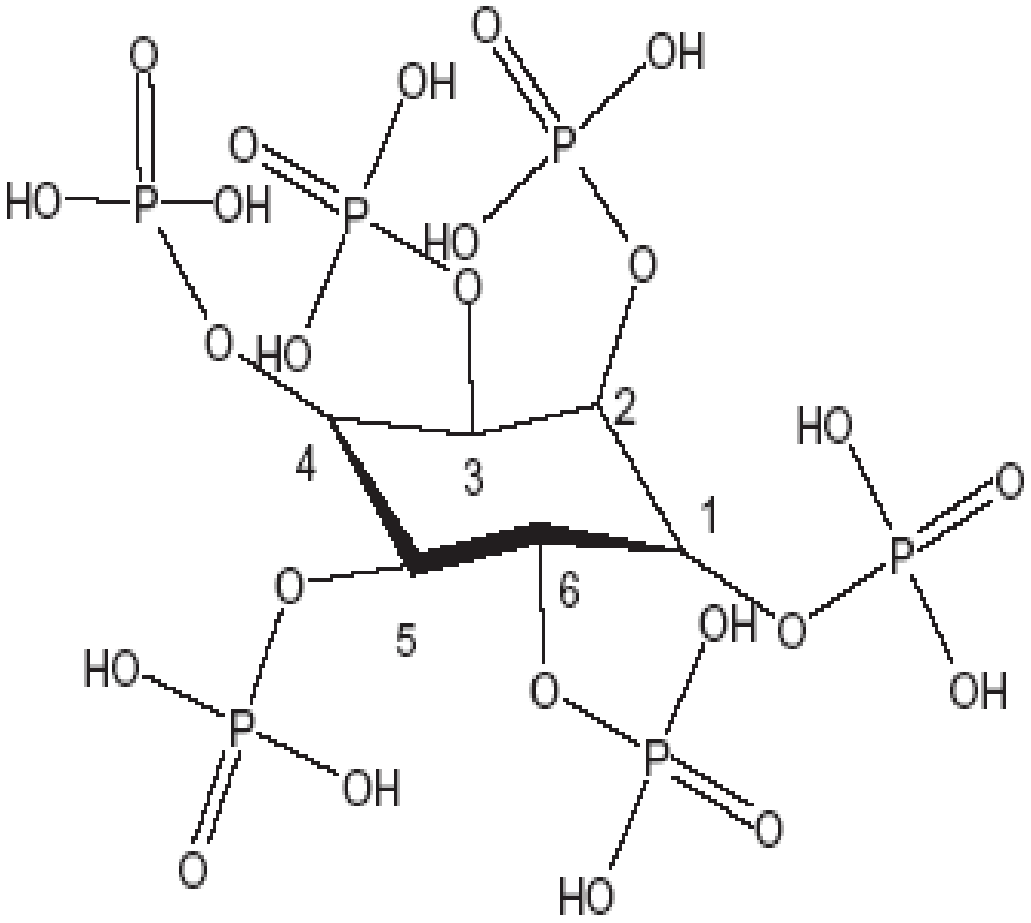


**Figure 2.1.** Structure of phytic acid

**Table 2.1.** Mean concentrations of phytic acid and phytase activities in common feed ingredients

| Ingredient | Phytic acid[1] (%) | Phytase activity (FTU/kg) | Reference |
|---|---|---|---|
| Wheat | 1.03 | 2886 | Steiner et al., 2006 |
| Barley | 0.92 | 2323 | Steiner et al., 2006 |
| Triticale | 0.99 | 2799 | Steiner et al., 2006 |
| Rye | 0.85 | 6016 | Steiner et al., 2006 |
| Corn | 0.73 | <50 | Selle et al., 2003a |
| Sorghum | 0.85 | <50 | Selle et al., 2003a |
| Wheat bran | 2.80 | 9945 | Steiner et al., 2006 |
| Rye bran | 1.74 | 9241 | Steiner et al., 2006 |
| SBM | 1.60 | <50 | Selle et al., 2003a |
| Canola meal | 2.38 | <50 | Selle et al., 2003a |
| Peas | 0.59 | 58 | Selle et al., 2003a |
| Lupins | 0.57 | <50 | Selle et al., 2003a |

[1]Calculated based on the phytate P content in the feed ingredients.

phytic acid-bound phosphorus, inorganic sources of phosphorus, which are expensive, are added in feeds to meet phosphorus requirements of the poultry, leading to increased cost of feeding (Selle and Ravindran, 2007, 2008). Also, phytic acid has capacity to bind other nutrients, thereby reducing their availability for utilization by pigs and poultry (Selle and Ravindran, 2007, 2008). Swine and poultry production in Canada and in other parts of the world is constrained partly by high cost of feeding, which accounts for approximately 70% of the total cost of their (swine and poultry) production. Therefore, the presence of phytic acid in diets for pigs and poultry results in increased cost of production.

In addition to increased cost of production, the unabsorbed phytic acid-bound phosphorus is excreted via feces (manure), which can then be applied to crop fields to supply phosphorus and other nutrients required for crop growth and development. However, application of manure on crop fields can lead to phosphorus accumulation on the fields especially where the availability of manure is high as is the case in areas with intensive livestock production, and where the manure is applied to crop fields based on crop nitrogen needs (as is it is done in most cases) because the nitrogen:phosphorus ratio in livestock manure is lower than that required by the crops (Knowlton et al., 2004; Toth et al., 2006). The excess phosphorus can then runoff into water bodies, resulting in eutrophication (Guyton, 2002; Kleinman et al., 2002). Livestock manure has recently been identified as one of the major sources of phosphorus that pollutes surface water bodies (Knowlton et al., 2004).

### *2.2 Effect of Phytic Acid on Animal Performance*

The presence of phytic acid in diets for non-ruminants has been shown to result in reduced performance and bone mineralization. Liu et al. (2008b, 2009) reported a

decrease in body weight gain of broilers from hatch to 21 days of age by at least 2.6% due to an increase in dietary level of phytic acid from 0.78 to 1.57%. Liu et al. (2008a) also reported a 4.9% reduction in body weight gain of broilers from hatch to 28 days of age due to an increase in dietary level of phytic acid from 0.78 to 1.57%. Onyango and Adeola (2009) investigated the effect of feeding broilers a diet that contained 2% sodium phytate from 8 to 22 days of age on growth performance and bone mineralization. They observed reduced body weight gain, feed intake and tibia ash of broilers by 28, 21 and 2.7%, respectively, due to sodium phytate. Cabahug et al. (1999) also observed reduced body weight gain (from day 7 to day 25 of age) and toe ash (at 25 days of age) of broilers by 7 and 8.4%, respectively, due to an increase in dietary concentration of phytic acid from 1.04 to 1.57%. For Leghorn chicks, Shan and Davis (1994) observed a 44% reduction in body weight gain (from day 28 to day 46 of age) due to an increase in dietary concentration of phytic acid from 0 to 2 %.

The effect of phytic acid on the performance of rats has also been evaluated. Morris and Ellis (1980) and Zhou et al. (1992) observed reduced body weight gain of weanling rats by at least 30%, due to an increase in dietary concentration of phytic acid from 0 to 2.9%, and from 0 to 2.14%, respectively. Earlier on, Likuski and Forbes (1965) fed rats diets with either 0 or 2% phytic acid from 20 to 38 days of age and observed a 46% reduction in body weight gain of rats due to phytic acid. Also, Davies and Nightingale (1975) and Davies and Olpin (1979) reported a decrease in body weight gain of rats due to the addition of 0.74 and 1% of phytic acid to their diets, respectively. More recently, Dilworth et al. (2005) reported a 20% decrease in live body weight of rats due to feeding rats a diet containing 2.9% of phytic acid for a period of 25 days. Gaetke et al.

(2010) also reported a 22% decrease in live body weight of rats due to feeding rats a diet containing 2.3% of phytic acid for a period of 25 days. However, Knuckles et al. (1989) did not observe any effect of increasing the level of phytic acid in diets for rats from 0 to 2.9% on growth performance after 28 days of feeding, and the lack of effect of phytic acid on the performance of rats in this study was not clear. From these studies, it is apparent that phytic acid reduces the growth performance of poultry and rodents. However there is lack of information on the effect of phytic acid on pig performance.

### *2.3 Mechanism of Action of Phytic Acid in Reducing Animal Performance*

***2.3.1 Effect of Phytic Acid on Apparent Mineral Digestibility***. Several studies have been conducted to determine the effect of phytic acid on mineral digestibility in pigs, poultry and rodents. Kemme et al. (1999) reported a 4% reduction in apparent total tract ash digestibility in growing pigs due to an increase in dietary phytic acid concentration from 0.94 to 1.43%. Bohlke et al. (2005) also reported reduced apparent ileal and total tract digestibilities of calcium in growing pigs from 70 to 47.4% and from 69.1 to 49.6%, respectively, due to replacement of low phytate corn with regular corn in diets for the pigs.

In poultry, Ravindran et al. (2006) reported that an increase in the level of phytic acid in broiler diets from 1.04 to 1.36% resulted in reduced apparent ileal digestibility of calcium and iron by 5 and 7%, respectively. Plumstead et al. (2008) also observed reduced apparent ileal calcium digestibility in broilers due to an increase in dietary phytic acid concentration from 0.35 to 1.0%.

In rodents, Urbano et al. (1999) observed a 27% decrease in apparent calcium digestibility in rats due to an increase in dietary phytic acid concentration from 0.48 to

0.65%. Similarly, Yu Wang et al. (2010) reported that the apparent total tract digestibilities of magnesium, iron and zinc in mice were reduced from 39.2 to 32.3%, 29.3 to 20.3% and 21.2 to 15.8%, respectively, due to an increase in dietary phytic acid content from 0 to 1%.

These studies show that phytic acid reduces apparent mineral digestibility. The reduced apparent mineral digestibility by phytic acid could be due to reduced availability of dietary minerals for absorption or increased endogenous secretion of the minerals in the gastrointestinal tract lumen or both. The effects of phytic acid on mineral absorption and secretions are discussed below.

***2.3.2 Effect of Phytic Acid on True Absorption of Minerals***. The effect of phytic acid on the absorption of minerals has been investigated in humans and rats. In humans, Bronner et al. (1954) reported a 55% decrease in $^{45}Ca$ absorption in adolescent boys fed farina meal due to an increase in phytic acid content in their diet from 0 to 0.5%. Similarly, Kim et al. (2007) reported reduced zinc absorption in young women (43 vs. 22%) and in elderly women (34 vs. 20%) due to an increase in dietary phytate intake from 690 to 1623 mg/day and from 760 to 1713 mg/day, respectively. Hunt and Beiseigel (2009) also reported a 25% or 1 mg/day decrease in absolute zinc absorption in women due to an increase in their dietary intake of phytic acid from 440 and 1800 mg/day.

In rats, Lönnerdal et al. (1989) observed an increase in amounts of dietary $^{45}Ca$ in the cecal contents of the rats from 0.45 to 17.5% due to the dietary phytic acid, indicating reduced absorption of calcium in rats due to phytic acid. Kim et al. (1993) also reported a 33% decrease in dietary $^{59}Fe^{3+}$ absorption in rats fed a cereal meal due to addition of 0.13% phytic acid to the meal. With regard to zinc absorption, Rubio et al. (1994)

reported a 18% reduction in absorption of $^{65}$Zn in rats fed a phytic acid-free diet after supplementing the diet with phytic acid at 0.3%. Similarly, Lönnerdal et al. (1989) reported a 84% reduction in dietary $^{65}$Zn uptake by rats' liver due to including phytic acid in the solution, indicating that phytic acid reduced absorption of zinc in rats. In an *in situ* study with rats, Davies and Nightingale (1975) reported a decrease in $^{65}$Zn absorption in ligated loops of rat duodenum (containing 1 ml of a saline solution with 5 µg of $^{65}$Zn) by 96% due to addition of 5.07 µg of phytic acid into the solution. There is, however, lack of information on the effect of phytic acid on true absorption of minerals in pigs and poultry.

In summary, phytic acid can reduce the absorption of dietary minerals leading to reduced mineral availability to the animals, which could partly explain the reduced performance of non-ruminants due to phytic acid. The mechanisms by which phytic acid can reduce the availability of minerals for absorption have been suggested. Phytic acid can reduce the absorption of minerals because it is poorly hydrolyzed by endogenous enzymes of animals (Mroz et al., 1994; Rutherfurd et al., 2004; Cowieson et al., 2006), and in its natural state in plant tissues, it is complexed with minerals in globoids within protein bodies (Ockenden et al., 2004; Joyce et al., 2005; Lin et al,. 2005), implying that it can reduce mineral availability for absorption by masking them. Furthermore, because of its negative charges at acidic, neutral and basic pH conditions, phytic acid can complex cations at all pH conditions in the gastrointestinal tract, thereby reducing the bioavailability of the cations, especially divalent cations, which form insoluble complexes with phytic acid at the pH normally found in the small intestinal (Champagne et al., 1990; Maenz et al., 1999). For instance, Lyon (1984) reported that the addition of

phytic acid to a mineral solution at neutral pH resulted in precipitation of 99.5, 75, 83 and 62% of zinc, iron, calcium and magnesium in the solution, respectively, whereas Davies and Olpin (1979) reported that the addition of phytic acid to a mineral solution at neutral pH resulted in precipitation of 98, 91, and 80% of zinc, copper and manganese in the solution, respectively. However, sodium forms weaker bonds with phytic acid than divalent cations (Erdman, 1979), and hence its solubility is not affected by phytic acid (Scheuermann et al., 1988). Therefore, it appears that the availability of monovalent cations such as sodium for absorption is not affected by phytic acid. However, there is lack of information on the effect of phytic acid on true absorption of monovalent cations in the gastrointestinal tract of non-ruminants.

***2.3.3 Effect of Phytic Acid on Endogenous Losses of Minerals***. The effect of phytic acid on endogenous losses of minerals has been investigated. Cowieson et al. (2004) observed a 68, 32, 300 and 47% increase in endogenous excretion of calcium, iron, sodium and sulfur, respectively, in broilers that had been precision fed a glucose solution over a 48-hour period due to addition of 1 g of phytic acid to the glucose solution. Cowieson et al. (2006) precision fed broilers a solution containing 5 g of casein with or without 1 g of phytic acid and also reported increased excretion of calcium, magnesium, manganese, and sodium by 187, 39, 87, and 174%, respectively, over a 48-hour period, due to phytic acid. However, Liu and Ru (2010) did not observe any effect of increasing the dietary level of phytic acid from 0.71 to 1.42% on the ileal endogenous flows of manganese, zinc, magnesium, potassium and calcium in broilers. Instead, they reported 15 and 18% decreases in ileal endogenous flows of iron and copper,

respectively, and the reason why results from their study were different from those studies of Cowieson et al. (2004, 2006) is not clear.

In rats, dietary phytic acid was shown to reduce the biological half-life for $^{65}Zn$ in the body (Davies and Nightingale, 1975), indicating that phytic acid increased the endogenous losses of zinc in the rats. However, there is lack of information on the effect of phytic acid on endogenous losses of minerals in pigs. Pigs and poultry differ in the anatomy of their digestive tracts. Also, pigs and poultry differ in the physiology of their digestive tracts. For instance, the digesta for pigs is more watery and less viscous, and its passage rate in the gastrointestinal tract is faster than that for poultry (Bedford and Schuzle, 1998). These differences may result in differences between the species with regard to endogenous secretion and (re)absorption of nutrients. Therefore, effects on phytic acid on the endogenous losses of minerals and other nutrients in pigs and poultry might differ.

From these studies, it is apparent that phytic acid increases the endogenous losses of minerals. Four mechanisms by which phytic acid can increase the endogenous secretion of minerals are proposed. First, amino groups found on side chains of basic amino acids and at amino terminal ends of proteins possess a net positive charge at a pH below the isoeletric point (Prattley et al., 1982; Hídvégi and Lásztity, 2002). Hence, phytic acid may interact with dietary protein and endogenous protein (pepsin, and pepsinogen and its activating peptide) in the stomach, where the pH is acidic (Cowieson et al., 2006), thereby reducing the activity of pepsin. Kies et al. (2006) has indeed observed complete dissolution of casein (*in vitro*) at pH 2 in the absence of phytic acid, and almost complete precipitation (99%) in the presence of 0.01% phytic acid. Also,

Knuckles et al. (1989) reported a 9 to 14% reduction in pepsin digestion of casein and bovine serum albumin *in vitro* due to addition of phytic acid to the incubation media, whereas Vaintraub and Bulmaga (1991) reported a 60 to 92% reduction in pepsin digestion of casein, haemoglobin, bovine serum albumin and soybean protein *in vitro* due to addition of phytic acid to the incubation media. Furthermore, Liu et al. (2009) reported a 6.3% reduction in activity of pepsin in the proventriculus of broiler chickens due to dietary phytic acid.

The presence of undigested feed or inactivation of digestive enzymes in the gastrointestinal tract results in increased secretion of the latter, whereas the absence of undigested feed or presence of unbound active digestive enzymes in the gastrointestinal tract results in reduced secretion of the digestive enzymes through negative feedback mechanisms (Hara et al., 2000; Morisset, 2008). Binding of tannic acid to pepsin in the stomach of rats was shown to result in increased pepsin and hydrochloric acid secretions (Mitjavila et al., 1973). Therefore, the binding of phytic acid to pepsinogen and its activating peptide in the stomach can result in an increase in the secretion of the pepsinogen and hydrochloric acid via negative feedback mechanisms. The resulting acidic digesta may then need to be neutralized in the small intestine by mineral bicarbonates secreted by the intestine (Allen et al., 1993) and pancreas (Zebrowska et al., 1983) to protect small intestinal mucosa from acid digestion and to optimize the activities of pancreatic and intestinal digestive enzymes, resulting in increased endogenous secretion and hence losses of the minerals. Pancreatic juice is richer in sodium than other minerals (Zebrowska et al., 1983). Therefore, it appears that sodium is the mineral whose

endogenous secretion is most dramatically affected by increased secretion the pepsinogen and hydrochloric acid due to phytic acid.

Second, several digestive enzymes in the gastrointestinal tract including α-amylase, alkaline phosphatase, aminopeptidases and carboxypeptidases are metalloenzymes (Malmstrom and Neilands, 1964; Wouters and Husain, 2001). For instance α-amylase is a metalloenzyme that requires calcium as a co-factor (Argent et al., 1973), whereas alkaline phosphatase, aminopeptidases and carboxypeptidases are metalloenzymes that require zinc as a co-factor (Malmstrom and Neilands, 1964; Wouters and Husain, 2001). These metal ions are tightly bound to the enzymes to the extent that they are an integral part of the structure of the enzyme (Malmstrom and Neilands, 1964), meaning that an increase in secretion of a metalloenzyme will automatically lead to increased secretion of its co-factor. An increase in secretion of pancreatic α-amylase in the pancreatic juice has indeed been shown to result in an increased secretion of calcium (Argent et al., 1973). The presence of undigested feed in the small intestine has been reported to stimulate the pancreas to secrete digestive enzymes through negative feedback mechanisms (Hara et al., 2000; Morisset, 2008). Therefore, phytic acid may bind to enzyme co-factors (which are positively charged) in the gastrointestinal tract to form insoluble phytic acid-mineral complexes resulting in reduced activity of the enzymes. Also, protein is negatively charged at neutral pH (Maenz, 2001), and thus in the small intestine, where the pH is neutral, phytic acid may bind dietary protein or endogenous (digestive enzymes) protein or both through multivalent cations to form insoluble phytic acid-mineral-protein complexes (Maenz, 2001), leading to increased secretion of the digestive enzymes. This ability of phytic acid

to bind protein via cations was clearly demonstrated by Prattley et al. (1982), who incubated serum albumin with phytic acid either in the presence or absence of calcium and observed formation of phytic acid-protein complexes only in the presence of calcium. Also, Deshpande and Cheryan (1984) observed reduced α-amylase activity *in vitro* due to phytic acid, whereas Liu et al. (2008b) reported a 8.3% reduction in α-amylase activity in the duodenum of broilers due to dietary phytic acid. Therefore, phytic acid may bind to enzyme co-factors, enzymatic protein, dietary protein, or all of them, thereby increasing the secretion of the co-factors through negative feedback mechanisms. Of all the endogenously secreted minerals, it is calcium and zinc that are most affected by this process. This is because they are the co-factors of metalloenzymes found in the gastrointestinal tract (Malmstrom and Neilands, 1964).

Third, phytic acid can bind to endogenously secreted minerals thereby preventing their re-absorption. Multivalent cations form insoluble complexes with phytic acid (Maenz, 2001), meaning that phytic acid can reduce their re-absorption. However, monovalent cations form weaker bonds with phytic acid than their multivalent counterparts (Erdman, 1979; Scheuermann et al., 1988), and hence the re-absorption of the endogenously secreted monovalent cations is less likely to be affected by binding to phytic acid than multivalent cations.

Fourth, the active transport of nutrients (solutes) into enterocytes generates an osmotic flow of water into the enterocytes which, in turn, results in an increase in absorption of minerals by solvent drag (Fordtran et al., 1968). Sodium is additionally absorbed from the small intestine by co-transportation with other nutrients including glucose and galactose (Fordtran et al., 1968), and its absorption was shown to increase

with an increase in glucose absorption (Fordtran, 1975; Schiller et al., 1997). Phytic acid has been shown to reduce true nutrient digestibility or absorption (see the previous section on the "*Effect of Phytic Acid on True Absorption of Minerals*", and the sections on "*Effect of Phytic Acid on True Digestibility or Absorption of Amino Acids"* and "*Effect of Phytic Acid on Energy Digestibility*" below). Therefore, phytic acid may reduce the re-absorption of all the endogenously secreted minerals by reducing the absorption of nutrients such as sugars and amino acids in the gastrointestinal tract.

In summary, the increased endogenous loss of minerals due to phytic acid could be a result of their increased secretion in the small intestine or their reduced re-absorption from the small intestine or both. It appears that the increased endogenous loss of sodium due to phytic acid is mainly due to increased secretion as a result of reduced pepsin activity and hence increased secretion of pepsin and hydrochloric acid in the stomach and of mineral bicarbonates to neutralize the acid; reduced re-absorption due to reduced absorption of other nutrients, whose absorption results in increased absorption of sodium; or both. However, there is a need to test these hypotheses.

The increased endogenous losses of minerals due to phytic acid may partly explain the reduced performance of animals by phytic acid. This is because an increase in endogenous losses of nutrients can result in increased dietary demand of the lost nutrients by the animal and of the energy required to secrete them (Nyachoti et al., 1997a). Also, sodium deficiency in chickens has been reported to reduce the activity of Na-K-ATPase in the gastrointestinal tract and to increase the expression of genes for the same enzyme which is involved in the absorption of several nutrients including glucose and amino acids (Gal-Garber et al., 2003). Therefore, the increased secretion of sodium may alter

the synthesis and activity of ATPases, which are involved in nutrient absorption. Liu et al. (2008b) have shown reduced activity of Na-K-ATPase in broilers due to ingestion of phytic acid. Additionally, villous height is positively correlated with absorptive capacity of small intestine (Montagne et al., 2003). An infusion of sodium into the small intestine of rats has been shown to increase villous height (Clarke, 1977). Therefore, the increased secretion and reduced (re)absorption of sodium due to dietary phytic acid can result in reduced villous height and hence nutrient absorption. However, there is lack of information on the effect of phytic acid on gastrointestinal tract Na-K-ATPase activity and histomorphology of pigs. Therefore, there is a need to determine the effect of phytic acid on gastrointestinal tract Na-K-ATPase activity and histomorphology of pigs.

#### *2.3.4 Effect of Phytic Acid on Apparent Nitrogen and Amino Acids Digestibilities.*

The digestibility of nitrogen and amino acids has been found to be negatively affected by phytic acid. Ravindran et al. (2006) reported a decrease in the apparent ileal digestibility of aspartic acid, threonine, glutamic acid, glycine, alanine, valine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine in broilers fed a corn-soybean meal-based diet from 80.0 to 76.3%, 77.3 to 73.3%, 86.5 to 84.3%, 77.4 to 73.4%, 81.3 to 78.9%, 80.5 to 76.9%, 83.4 to 81.2%, 81.7 to 78.7%, 83.2 to 80.4%, 79.0 to 74.2%, 87.3 to 84.5%, and 88.9 to 86.6%, respectively, due to an increase in dietary phytic acid concentration from 1.04 to 1.36%. Similarly, Ravindran et al. (2000) reported a 3% decrease in the apparent ileal digestibility of nitrogen in broilers due to an increase in dietary phytic acid concentration from 1.04 to 1.57%. They also reported reduced apparent ileal digestibility of all the essential amino acids due to the increase in dietary phytic acid concentration.

In growing pigs fed a corn-soybean meal-based diet, Bohlke et al. (2005) observed reduced apparent ileal digestibility of arginine, lysine, phenylalanine, threonine, valine, aspartic acid and glycine from 79.6 to 75.1%, 65.5 to 57.4%, 86.8 to 77.8%, 61.8 to 57.0%, 67.4 to 61.6%, 69.2 to 65.8%, and 44.7 to 34.9%, respectively, due to replacement of low phytate corn with regular corn in diets for the pigs. Liao et al. (2005) also reported a 4.0% (mean) reduction in the apparent ileal digestibility of all amino acids (except glycine) in weanling pigs due to an increase in concentration of phytic acid in corn-soybean meal-based diet from 0.78 to 1.70%. In their study, the apparent ileal nitrogen digestibility was also reduced by 4.6% due to the increase in dietary phytic acid concentration. However, Knuckles et al. (1989) did not observe any effect of increasing the level of phytic acid in casein-corn starch-based diets for rats from 0 to 2% on apparent ileal protein digestibility. Other studies also did not report increased apparent ileal amino acid digestibility in pigs due to phytase (which hydrolyses phytic acid) despite increased phytic acid hydrolysis as evidenced by improved phosphorus digestibility (Johnston et al., 2004; Woyengo et al., 2008). It is not clear why phytic acid has a variable effect on amino acid digestibility in pigs.

Like minerals, the reduced apparent amino acid digestibility due to phytic acid could be due to reduced availability of dietary amino acids for absorption or increased endogenous losses of amino acids or both. These factors are discussed below.

***2.3.5 Effect of Phytic Acid on True Digestibility or Absorption of Amino Acids.*** Phytic acid supplementation was shown to decrease true ileal digestibility of amino acids by averages of 8.9 and 11.9% due to the addition of 0.5 and 1 g of phytic acid to 5 g of casein that had been precision fed to growing broilers, respectively (Cowieson et al.,

2006). Cowieson et al. (2003) also precision fed growing broilers with 5 g of casein and observed a decrease in true metabolizable nitrogen by 120.4 and 153.7% due to the addition of 0.5 and 1 g of phytic acid to 5 g of casein, respectively. Onyango et al. (2008) perfused the jejunum of broilers with a solution containing labelled amino acids to determine the effect of phytic acid on absorption of amino acids. They reported a linear decrease in absorption of a labelled leucine due to an increase in magnesium-potassium phytate concentration in the perfusate from 0 to 500 mM. In their study, the phytate also tended to linearly reduce the absorption of lysine and glutamic acid. However, there is lack of information on the effect of phytic acid on true ileal digestibility or absorption of amino acids in pigs, and hence there is a need to fill this knowledge gap.

Two mechanisms have been proposed to explain the detrimental effects of phytic acid on true amino acids digestibility or absorption. First, phytic acid in its natural state is complexed with amino acids in protein bodies (Ockenden et al., 2004; Joyce et al., 2005; Lin et al., 2005) and thus it can minimize the availability of these nutrients for digestion and absorption by masking them (Lenis and Jongbloed, 1999). Second, phytic acid can bind to dietary protein and proteolytic enzyme proteins in the stomach and in the small intestine (see previous section on the "*Effect of Phytic Acid on Endogenous Losses of Minerals*"), thereby reducing the availability of protein for digestion in the stomach and the small intestine. In addition to reducing pepsin activity (see previous section on the "*Effect of Phytic Acid on Endogenous Losses of Minerals*") phytic acid was also shown to increase inhibition of activity of trypsin (from 2.7 to 42.5%) *in vitro* at pH 7.5 in an incubation medium that contained casein as substrate due to an increase in level of phytic acid concentration in the medium from 10 to 90 mM (Singh and Krikorian, 1982).

Similarly, Vaintraub and Bulmaga (1991) reported reduced hydrolysis of casein by trypsin *in vitro* due to phytic acid. In an *in vivo* study, Liu et al. (2009) reported a 24.2% reduction in trypsin activity in the jejunum of chickens due to an increase in dietary phytic acid level from 0.78 to 1.56%. Thus, phytic acid can reduce performance partly by reducing the digestibility of dietary amino acids.

***2.3.6 Effect of Phytic Acid on Endogenous Losses of Amino Acids***. Studies on the effect of phytic acid on endogenous losses of amino acids have generally shown a positive relationship between dietary phytic acid concentration and endogenous losses of amino acids. Liu and Ru (2010) observed that an increase in dietary level of phytic acid from 0.71 to 1.42% resulted in an increase in the ileal endogenous losses of valine (11%), methionine (14%), leucine (7%), arginine (18%), aspartic acid (15%), serine (16%), glumatic acid (15%), proline (19%), glycine (18%), alanine (14%) and tyrosine (18%) in broilers. Cowieson et al. (2008) similarly reported that an increase in the dietary concentration of phytic acid from 0 to 1.45% resulted in an increase in the ileal endogenous losses of nitrogen (64%), threonine (57%), valine (35%), isoleucine (48%), leucine (98%), phenylalanine (145%), histidine (112%), lysine (38%), arginine (57%), methionine (56%), aspartic acid (72%), serine (79%), glutamic acid (72%), proline (17%), glycine (64%), alanine (52%), tyrosine (120%) and cysteine (32%) in broilers. Cowieson and Ravindran (2007) also reported increased endogenous losses of amino acids by an average of 87% due to an increase in phytic acid content of broiler diets from 0.85 to 1.45%.

The effect of phytic acid on endogenous losses of amino acids in poultry has also been investigated by Cowieson et al. (2004) and Onyango et al. (2009) using a precision

feeding technique. Cowieson et al. (2004) precision fed 6 week-old broilers on a glucose solution and observed an increase in endogenous excretion of nitrogen by 20% over a 48-hour period due to addition of 1 g of phytic acid to the glucose solution. In their study, the addition of 1 g of phytic acid to the glucose solution also increased the excretions of aspartic acid (31%), serine (12%), glutamic acid (28%), alanine (34%), histidine (57%), threonine (25%), arginine (22%), valine (30%), phenylalanine (32%), isoleucine (34%) leucine (32%) and lysine (22%). Similarly, Onyango et al. (2009) reported increased excretions of endogenous arginine, threonine, glycine, proline and serine in 10-week-old broilers fed a dextrose solution for a period of 54 hour by 30, 28, 22, 29 and 30%, respectively due to addition of phytic acid in the solution at rate of 2 g/bird/day. However, there is a lack of information on the effect of phytic acid on endogenous losses of amino acids in pigs. Therefore, there is a need to determine the effects of phytic acid on endogenous losses of amino acids in pigs.

Endogenous nitrogen and amino acids in the gastrointestinal tract originate from digestive enzymes, mucoproteins and sloughed cells of gastrointestinal tract epithelium (Nyachoti et al., 1997a). The amounts of endogenous amino acids that appear at the terminal ileum are a proportion of what is secreted but not re-absorbed (Nyachoti et al., 1997a). Thus, the increased endogenous flow of amino acids at the terminal ileum might be a result of their increased secretion or reduced re-absorption or both. Phytic acid may increase the endogenous secretion of amino acids by binding to dietary protein and endogenous digestive enzymes in the gastrointestinal tract, thereby increasing the secretion of the enzymes through negative feedback mechanisms (see previous section on the "*Effect of Phytic Acid on Endogenous Losses of Minerals*"). The increased secretion

of digestive enzymes may in turn result in increased secretion of mucins, leading to further increase in endogenous losses of amino acids. This is because mucins are partly composed of protein (Forstner and Forstner, 1994), and an increase in the secretion of gastric pepsin has been shown to result in increased secretion of mucins to protect the gastrointestinal tract mucosa from digestion by the enzyme and by hydrochloric acid, whose secretions increase with increases in pepsin secretion (Munster et al., 1987). Cowieson et al. (2004) have observed increased secretion of sialic acid, which is a major component of mucin, in broilers due to dietary phytic acid. Onyango et al. (2009) have also observed increased secretion of sialic and mucins in broilers due to dietary phytic acid indicating that the secretion of mucins is indeed increased by phytic acid.

In addition to increasing the secretion of digestive enzymes and mucin, phytic acid may increase the endogenous losses of amino acids by reducing the re-absorption of the endogenously secreted amino acids in the small intestine. This is because phytic acid has been shown to reduce true amino acids digestibility or absorption (see previous section on the "***Effect of Phytic Acid on True Digestibility or Absorption of Amino Acids***"), implying that it can also reduce the digestibility of the endogenously secreted proteins and hence the re-absorption of the endogenous amino acids.

Therefore, it appears that phytic acid increases the endogenous amino acids losses by increasing the secretion of digestive enzymes and mucins, and by reducing the re-absorption of the endogenously secreted amino acids in the small intestine. Because the increased endogenous nitrogen and amino acid losses in the gastrointestinal tract are associated with increased maintenance requirement of the lost nitrogen and amino acids and of the energy spent on their secretion (Nyachoti et al., 1997a), an increase in

endogenous losses of nitrogen and amino acids due to phytic acid imply that the presence of the phytic acid in diets for non-ruminants results in increased maintenance requirements of energy and amino acids, thereby reducing the availability of these nutrients for tissue deposition. Therefore, the reduced animal performance due to dietary phytic acid could partly be explained by the increased endogenous losses of nitrogen and amino acids. Also, villi have been reported to be shorter in piglets fed restricted milk diet than in piglets fed the milk diet ad-libitum (Pluske et al., 1996; van Beers-Schreurs et al., 1998). Therefore, the increased secretion and reduced (re)absorption of amino acids due to dietary phytic acid can result in reduced villous height and hence nutrient absorption. Because mucins are involved in the lubrication and protection of the gastrointestinal tract epithelium from pathogens, toxins and acid and enzymatic hydrolyses (Montagne et al., 2004), a decrease in mucin coverage of the epithelium due to phytic acid may result in increased contact between luminal contents and epithelium, increasing the susceptibility of the animals to gut infections.

***2.3.7 Effect of Phytic Acid on Energy Digestibility.*** An increase in phytic acid concentration in corn-soybean meal-based diets for broilers from 1.0 to 1.36% from the addition of rice bran was reported to result in a decrease in the apparent metabolizable energy content of the diet by 2.1% (3353 vs 3281 kcal/kg; Ravindran et al., 2006). Liao et al. (2005) also reported reduced apparent ileal and total tract digestibilities of energy in weanling pigs by 7.5 and 6.1%, respectively due to an increase in dietary phytic acid concentration from 0.78 to 1.70%.

Phytic acid can cause a reduction in energy digestibility by reducing the digestibility of energy generating molecules such as carbohydrates, lipids and protein.

For example, Thompson et al. (1987) reported that dephytinization of navy beans resulted in a 25% increase in starch digestibility *in vitro*. They also observed decreased carbohydrate malabsorption (estimated by breath hydrogen) and improved glycemic index in human subjects by 81 and 126%, respectively, due to consumption of bread made from dephytinized navy beans. In mice, Lee et al. (2006) reported reduced fasting and non-fasting blood glucose levels in male diabetic KK mice fed a casein-corn starch-based diet by 21 and 15%, respectively, due to an increase in the dietary level of phytic acid from 0 to 1.0%, indicating reduced glucose absorption as a result of the dietary phytic acid. In broilers, an increase in dietary phytic acid level from 0.79 to 1.57% was shown to result in a decrease in blood serum glucose concentration by 6% (Liu et al., 2008b), indicating reduced glucose absorption due to the phytic acid. Onyango et al. (2008) also observed a tendency for phytic acid to linearly reduce the absorption of glucose in jejunum of broilers after perfusion of the jejunum with a solution that contained a labelled glucose and phytic acid at increasing concentrations (0, 50, 250 or 500 mM).

Deshpande and Cheryan (1984) observed reduced activity of α-amylase *in vitro* due to phytic acid, whereas Liu et al. (2008b) reported reduced activities of α-amylase, sucrase and maltase in the duodenum of broilers by 8.3, 11.4 and 6.0%, respectively. Thus, it appears that phytic acid reduces the absorption of carbohydrates likely by reducing the activity of digestive carbohydrases. The reduced glucose absorption by phytic acid could partly explain the reduced energy digestibility by phytic acid. However, research is required to probe the effect of phytic acid on digestibility or absorption of carbohydrates in pigs.

Glucose is absorbed in the small intestine by sodium-dependent glucose transporter 1 (**SGLT1)** protein whose expression declines with a decrease in the availability of glucose for absorption (Dyer et al., 1997). Therefore, the reduction of glucose absorption by phytic acid is expected to result in reduced expression of the SGLT1 protein and the capacity of the small intestine to absorb sodium in addition to the glucose. Also, an infusion of glucose into the small intestine of rats has been shown to increase villous height (Clarke, 1977), indicating that reduced availability of glucose for absorption in the intestinal lumen can result in reduced villous height. Therefore, the reduced glucose absorption by phytic acid may result in reduced villous height.

With regard to lipid digestibility, Lee et al. (1997) reported increased fecal cholesterol and bile acid excretion in rats due to dietary phytic acid, indicating that phytic acid reduced the (re)absorption of cholesterol and bile acids. Lee et al. (2005) fed diabetic KK mice with diets containing 0, 1 and 1.5% phytic acid to investigate the effect of phytic acid on the levels of lipids in the serum and liver. They observed lower serum total cholesterol and low-density lipoprotein cholesterol concentrations as well as hepatic total lipid and total cholesterol due to an increase in the level of dietary phytic acid from 0 to 1.5%. Similarly, Lee et al. (2007) reported lower serum low-density lipoprotein cholesterol and hepatic triacylglycerol in aged mice due to an increase in dietary phytic acid from 0 to 1.5%. In their study, the apparent absorption of total lipid and cholesterol was also reduced, whereas fecal cholesterol and lipid contents were increased by the dietary phytic acid. Yuangklang et al. (2005) also reported increased excretion of bile acids in feces and reduced concentration of cholesterol in the liver of rats fed a cholesterol rich diet by 28 and 22%, respectively, due to an increase in dietary phytic acid

concentration from 0 to 0.33%. However, the increased fecal excretion of bile acids did not translate in reduced fat digestibility and serum cholesterol concentration and the reason for this is not clear. It could probably be attributed to the fact that the dietary level of phytic acid (0.33%) was not adequate or enough to impact fat digestibility.

In poultry, Liu et al. (2009) reported reduced serum total cholesterol and hepatic triacylglycerol content due to an increase in the level of phytic acid level in the diets for broilers from 0.7 to 1.4%. Thus, results from these studies indicate that phytic acid reduces the (re)absorption of fat, cholesterol and bile acids, leading to reduced serum hepatic and serum lipid levels.

Phytic acid has been reported to reduce the activity of porcine pancreatic lipase *in vitro* (Knuckles et al., 1988) and pancreatic lipase activity in the small intestine of broilers (Liu et al., 2009), implying that the reduced fat digestibility by phytic acid could partly be a result of reduced activity of lipase. Phytic acid could also reduce fat digestibility and absorption by binding bile acids via divalent cations such as calcium to form insoluble phytic acid-mineral-bile acids complexes, thereby reducing fat digestion and absorption, and bile acids re-absorption. However, this hypothesis needs to be tested.

With regard to protein digestibility, phytic acid has been shown to reduce amino acid digestibility and to increase the endogenous losses of nitrogen and amino acids (see previous sections on the "*Effect of Phytic Acid on True Digestibility or Absorption of Amino Acids*" and on the "*Effect of Phytic Acid on Endogenous Losses of Amino Acids*"), meaning that phytic acid can reduce energy digestibility partly by reducing the digestibility and increasing the endogenous losses of the same. For instance, Cowieson et al. (2008) reported that an increase in the concentration of phytic acid in a diet for

broilers from 0.85 to 1.45% resulted in an endogenous loss of energy in the form of protein by 23 kcal/kg of dry matter intake.

### *2.4 Summary and Perspectives*

Phytic acid can reduce animal performance partly through reduced digestibility and increased endogenous losses of nutrients including amino acids and minerals. The reduction in nutrient digestibility by phytic acid is because of binding of phytic acid to nutrients or digestive enzymes, thereby reducing nutrient digestibility. The increased endogenous losses of nutrients due to phytic acid might be the result of their increased secretion in the gastrointestinal tract or reduced re-absorption of the endogenously secreted nutrients in the gastrointestinal tract or both. However, all studies that have been conducted so far on the effect of phytic acid on endogenous losses of nutrients have been limited to poultry. There is lack of information on the effect of phytic acid on the endogenous losses of nutrients in pigs. Furthermore, the above-mentioned hypothesis about mechanisms by which phytic acid could increase the endogenous losses of nutrients has not been tested. Hence, there is a need to determine the effect of phytic acid on the endogenous losses of nutrients in pigs and to establish the mechanisms by which phytic acid increases the endogenous losses of nutrients. The results from these areas of research will provide data on the effect of phytic acid on endogenous losses of nutrients in pigs and help elucidate novel mechanisms by which phytic acid may increase endogenous losses of nutrients. Understanding the mechanisms by which phytic acid increases gastrointestinal tract endogenous nutrient losses is of critical importance in gaining insight into mechanisms by which phytic acid reduces nutrient utilization in animals as well as the potential success of possible interventions.

# CHAPTER THREE

## HYPOTHESES AND OBJECTIVES

In this thesis, it was hypothesized that phytic acid increases the endogenous losses of amino acids and minerals in pigs by:

1. Reducing the activity of digestive enzymes in the gastrointestinal tract, thereby increasing the secretion of (i) enzymes and hydrochloric acid in the gastrointestinal tract through negative feedback mechanisms, (ii) mucins to protect the gastrointestinal tract wall from acid and enzyme digestions, and (iii) mineral bicarbonates to neutralize the acid.
2. Reducing the re-absorption of the endogenously secreted nutrients.

It was also hypothesized that the increased endogenous losses of nutrients by phytic acid results in reduced villous height and the synthesis of the nutrient transporter proteins.

The overall objective of this study was to determine the effect of phytic acid on the endogenous losses of nutrients in pigs, and to establish the mechanisms by which phytic acid increases the endogenous nutrient losses in pigs.

The specific objectives of this study were:

1. To determine the effect of phytic acid on endogenous nutrient losses in piglets with the view of developing a phytic acid containing diet that promotes endogenous nutrient losses in piglets.

2. To determine the effect of phytic acid on enzyme activities in the gastrointestinal tract of piglets.
3. To determine the effect of phytic acid on the synthesis of nutrient transporter proteins in the gastrointestinal tract and gastrointestinal tract histomorphology of piglets.
4. To determine the effect of phytic acid on nutrient absorption in piglets.

# CHAPTER FOUR

## Ileal digestibility and endogenous flow of minerals and amino acids: Responses to dietary phytic acid in piglets[1]

**4.1 ABSTRACT:** The objective of this study was to investigate the effects of phytic acid on ileal mineral and amino acid digestibilities and ileal endogenous amino acids flow in piglets. Seven ileal-cannulated weanling pigs were fed a casein-corn starch-based diet with phytic acid (as sodium phytate) at 0, 0.5, 1.0 or 2.0% in 4 x 4 Latin square design with three added columns to give seven observations per treatment. The basal diet was formulated to meet National Research Council energy, essential amino acids, vitamin and mineral recommendations for piglets. The respective ileal digestibility and endogenous lysine flow were determined by indicator and homoarginine methods. The apparent ileal digestibility of sodium, potassium and phosphorus was linearly and quadratically reduced ($P < 0.05$) by increased dietary phytic acid concentration, whereas that of calcium and magnesium was only reduced linearly ($P < 0.05$) by the dietary phytic acid. The apparent ileal digestibility values for magnesium and sodium were negative (-3.0 and -18.0%, respectively) when phytic acid was supplemented at 2.0%. The apparent ileal digestibility of isoleucine, leucine and valine responded quadratically to dietary phytic acid concentration, though the differences between the apparent ileal digestibility values of the amino acids due to change in dietary phytic acid concentration were marginal (at most by 1·8 percentage units).

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[1]The material presented in chapter four of this thesis has been published in British Journal of Nutrition: T. A Woyengo, A. J. Cowieson, O. Adeola, and C. M. Nyachoti. 2009. Ileal digestibility and endogenous flow of minerals and amino acids: Responses to dietary phytic acid in piglets. Br. J. Nutr. 102:428-433.

Furthermore, dietary phytic acid did not affect ($P > 0.05$) endogenous amino acid losses. The results suggest that phytic acid has limited effect on the digestibility and endogenous losses of amino acids in piglets, but can reduce apparent ileal digestibility of magnesium and sodium partly by increasing endogenous losses of these minerals as evidenced by their negative apparent ileal digestibility values.

**Key words:** endogenous mineral and amino acid flow, mineral and amino acid digestibility, phytic acid, piglets, terminal ileum

## 4.2 INTRODUCTION

Phytic acid (myo-inositol hexaphosphate) is a constituent of plant seeds, where it serves as a storage form of phosphorus (Maenz, 2001). Plant seeds form the bulk of pig and poultry feeds. Phytic acid-bound phosphorus in plant seeds (except in viscous grains like rye and wheat, which have high endogenous phytase activity) is, however, poorly digested by pigs and poultry because they do not produce sufficient amount of phytase enzyme that hydrolyzes phytic acid (Mroz et al., 1994; Rutherfurd et al., 2004).

Furthermore, each of the 6 phosphate groups on phytic acid has 2 protons, which can dissociate to leave phytic acid with 12 negative charges and hence the potential to bind positively charged molecules such as minerals and basic amino acids (Maenz, 2001). Of the 12 protons on phytic acid, 6 can dissociate at acidic pH, 3 at neutral pH and the remaining 3 at basic pH, suggesting that phytic acid has the capacity to bind positively charged molecules in diet and endogenous gastrointestinal secretions such as digestive enzymes and mucin at all pH conditions found in the gut (Maenz, 2001).

Phytic acid has indeed been reported to limit the digestibility of minerals and amino acids in boilers (Ravindran et al., 2000, 2006; Cowieson et al., 2006), which implies reduced efficiency of nutrient utilisation and increased environmental discharge of nutrients as a result of excessive excretion of unabsorbed nutrients due to phytic acid (Lenis and Jongbloed, 1999). Phytic acid has also been shown to increase endogenous losses of minerals and amino acids in broilers (Cowieson et al., 2004; Cowieson and Ravindran, 2007). Because the increased endogenous nutrient losses in the gut are associated with increased maintenance requirement for the lost nutrients and of the energy spent on their secretion (Nyachoti et al., 1997a), an increase in endogenous losses of nutrients due to phytic acid imply that there are other adverse effects of phytic acid on the efficiency of nutrient utilisation in addition to reducing nutrient digestibility. However, there is a lack of information on the effect of phytic acid on endogenous nutrient losses in pigs. Furthermore, the effect of phytic acid on digestibility of nutrients, especially amino acids, has been variable. For instance, Bohlke et al. (2005) and Liao et al. (2005) reported a reduction in amino acid digestibility in pigs due to phytic acid, whereas Johnston et al. (2004) and Woyengo et al. (2008) did not report improvement in amino acid digestibility due to phytase (which hydrolyses phytic acid) despite increased phytic acid hydrolysis as evidenced by improved phosphorus digestibility in their studies. The objective of the present study was to determine the effect of phytic acid on the ileal mineral and amino acid digestibilities and endogenous flow of amino acid at the terminal ileum of weanling pigs.

## 4.3 MATERIALS AND METHODS

### *4.3.1 Animals*

Seven Genesus (Yorkshire-Landrace female x Duroc male) barrows (mean initial BW 9·50 ± 0.42 kg (mean ± SD) were obtained from the Glenlea Swine Research Unit, University of Manitoba immediately after weaning, and group-housed in pens and monitored for consumption of a commercial starter diet to ensure that piglets were healthy and able to eat and hence ready for surgeries. After 3 days, piglets were transferred to metabolic crates to adapt to the crates for 2 days, and then surgically fitted with a simple T-cannula at the distal ileum as described by Nyachoti et al. (2002). After surgery, piglets were returned to the metabolic crates and allowed a 7 days recovery period before the commencement of the experiment. During the recovery period, they were fed twice daily increasing amounts of the starter diet starting with 50 g of the feed after surgery and then increasing the amount offered by 50 g/day until feed consumption was at 2·6 times maintenance energy requirement (ARC, 1981) based on their body weight. Piglets had unlimited access to water throughout the study. The mean body weight at the start of the experiment was 10·9 ± 0.48 kg (mean ± SD). The use of animals in the present study was reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee, and piglets were handled in accordance with the guidelines described by the Canadian Council on Animal Care (CCAC, 1993).

### *4.3.2 Diets*

During the experimental period, four casein-corn starch based diets formulated to meet NRC (1998) energy, essential amino acids, vitamin and mineral recommendations

for piglets (Table 4.1) were fed. The diets included a control diet either unsupplemented or supplemented with 0.5, 1.0 or 2.0% phytic acid (as sodium phytate; Sigma-Aldrich Corporation, St Louis, MO, USA). Two sets of 4 diets were prepared: one set of diets contained chromic oxide (0.3%) as an indigestible marker and intact casein; whereas the other set contained titanium oxide (0.3%) as an indigestible marker and 50% of the dietary casein was guanidinated to convert lysine to homoarginine for determination of endogenous amino acid flow at the terminal ileum by the homoarginine method (Nyachoti et al., 2002). Casein was guanidinated as described by Nyachoti et al. (1997b, 2002).

### *4.3.3 Experimental Design and Procedure*

The experiment was conducted as a 4 x 4 Latin Square Design combined with a 4 x 3 Youden square design to give seven replicates per diet. Each period lasted for 7 days. Piglets were fed their respective un-guanidinated diets during the first 6 days and guanidinated diets during day 7 of each period. On days 5 and 6, ileal digesta was collected continuously from each pig from 0800 to 2000 hours daily and stored frozen at -22˚C for later determination of apparent ileal nutrient digestibility. On day 7, ileal digesta was collected continuously from 1200 hours (when the chromic oxide (green colour) from the un-guanidinated diets had disappeared from the digesta) to 1000 hours the following day (before the appearance of chromic oxide from un-guanidinated diets fed on the same day at 0800 hours) and similarly stored frozen for later determination of endogenous amino acid flow at the terminal ileum and true ileal digestibility of amino acids. During the experiment, pigs were fed the four experimental diets at 2·6 times maintenance energy requirement (ARC, 1981) based on their body weight at the

**Table 4.1.** Composition of basal diet as fed basis

| Ingredient[1] | Amount, % |
|---|---|
| Cornstarch | 48.6 |
| Lactose | 20.0 |
| Casein | 20.0 |
| Vegetable oil | 3.0 |
| Cellulose | 3.0 |
| Monocalcium phosphate | 1.3 |
| Limestone | 1.3 |
| Salt | 0.5 |
| Potassium carbonate | 0.4 |
| Magnesium oxide | 0.1 |
| Marker[2] | 0.3 |
| Vitamin/mineral premix[3] | 1.5 |
| Analyzed nutrient content[4] | |
| Crude protein, % | 17.7 |
| Calcium, % | 0.94 |
| Total phosphorus, % | 0.50 |
| Sodium | 0.53 |
| True digestible amino acid content, % | |
| Lysine | 1.37 |
| Methionine | 0.77 |
| Threonine | 0.84 |

[1]The basal diet was prepared in 2 sets; in one set, all the casein was intact, whereas in the other set, 50% of casein in diet had been guanidinated. The four diets containing 0, 0.5, 1 and 2% phytic acid were created from the basal diet by supplementing the basal diet with the phytic acid.

[2]Chromic oxide was used as an indigestibility marker in un-guanidinated diet, whereas titanium oxide was used as an indigestibility marker in guanidinated diet.

[3]Vitamin/mineral premix supplied the following per kg of finished diet: retinol, 2479 μg; cholecalciferol, 25 μg; α-tocopherol, 13.4 mg; phylloquinone, 1.1 mg; riboflavin, 5 mg; nicotinamide, 36.8 mg; cyanocobalamin, 25 mg; pyridoxine, 4.4 mg; biotin, 200 mg;

Pteroyl(mono)glutamic acid, 1 mg; choline, 781 mg; copper, 6 mg; iodine, 0.28 mg; iron, 100 mg; manganese, 40; selenium, 0.30 mg; and zinc, 100 mg.

[4]Analysed nutrient content was done on un-guanidinated diet.

beginning of each experimental period. The diets were offered in 2 equal portions at 0800 and 1530 hours on days 1 to 6 and in three equal portions at 0800, 1530 and 2030 hours on day 7.

### *4.3.4 Sample Preparation and Chemical Analyses*

The ileal digesta collected from each pig in each period were pooled for days 5 and 6 (un-guanidinated diet) and for day 7 (guanidinated diet), homogenised in a blender (Waring Commercial, Torrington, CT, USA), sub-sampled and freeze-dried. The dried ileal digesta were finely ground in a grinder (CBG5 Smart Grind; Applica Consumer Products Inc., Shelton, CT, USA), and thoroughly mixed prior to analysis. Diet and digesta dry matter content was determined according to AOAC (1998; Procedure 4.1.06), and nitrogen was determined using a nitrogen analyser (Model NS-2000; LECO Corporation, St. Joseph, MI, USA). Samples for calcium, phosphorus, potassium, sodium and magnesium analyses were ashed for 24 hours and digested according to AOAC (1990) procedures (method 990.08) and read on a Varian Inductively Coupled Plasma Mass Spectrometer (Varian Inc., Palo Alto, CA, USA). Samples for amino acid analysis were prepared by acid hydrolysis according to AOAC (1998; Procedure 4.1.11 alternative 3). Samples for analysis of sulphur-containing amino acids (methionine and cysteine) were subjected to performic acid oxidation prior to acid hydrolysis. Tryptophan was not determined. Samples for chromium analysis were ashed and digested according to procedures described by Williams et al. (1962) and read on a Varian Inductively Coupled Plasma Mass Spectrometer. Titanium was determined according to the method of Myers et al. (2004).

### *4.3.5 Calculations and Statistical Analysis*

The extent of conversion of lysine to homoarginine was calculated as described by Nyachoti et al. (2002). Apparent ileal dry matter, mineral and amino acid digestibilities, and true ileal digestibility of amino acids and endogenous amino acid flow at the terminal ileum were calculated as described by Nyachoti et al. (1997b; see Appendix 1). Data obtained were subjected to ANOVA as a 4 x 4 Latin square design (Cochran and Cox, 1957) combined with a 4 x 3 Youden square design (Cochran and Cox, 1957) with 6 degrees of freedom for piglets, 3 degrees of freedom for periods, 3 degrees of freedom for diets and 15 degrees of freedom for the error term using Mixed procedure (SAS, 2002). Linear and quadratic contrasts for unequally spaced levels (Gill, 1978) were performed to assess the effect of increasing dietary concentration of phytic acid.

## 4.4 RESULTS

The extent of conversion of Lys to homoarginine in guanidinated casein was 96·3%. The effects of dietary treatment on apparent ileal digestibility of dry matter, calcium, phosphorus, potassium, sodium and magnesium are presented in Table 4.2. Phytic acid linearly reduced ($P < 0.05$) the apparent ileal digestibility of dry matter. The apparent ileal digestibility of sodium, potassium and phosphorus were linearly and quadratically reduced ($P < 0.05$) by increased dietary concentration of phytic acid, whereas that of calcium and magnesium was only linearly reduced ($P < 0.05$) by the dietary phytic acid. The quadratic reduction in apparent ileal digestibility of phosphorus was such that the decrease in the digestibility was greater when the level of phytic acid

**Table 4.2.** Effect of phytic acid on apparent ileal dry matter and mineral digestibilities in piglets fed a casein-corn starch-based diet

| Item | Dietary phytic acid, % | | | | SEM | *P* values | |
|---|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 2.0 | | Linear | Quadratic |
| Dry matter | 88.6 | 88.1 | 88.5 | 86.2 | 0.38 | 0.032 | 0.168 |
| Calcium | 78.2 | 71.1 | 53.4 | 36.9 | 1.66 | 0.037 | 0.388 |
| Magnesium | 29.1 | 18.8 | 13.7 | -0.30 | 2.18 | 0.035 | 0.346 |
| Phosphorus | 80.1 | 65.4 | 50.5 | 37.2 | 1.78 | 0.017 | 0.023 |
| Sodium | 49.2 | 42.7 | 46.6 | -18.2 | 7.29 | 0.019 | 0.002 |
| Potassium | 87.6 | 86.6 | 89.0 | 81.1 | 1.31 | 0.034 | 0.044 |

was increased from 0 to 1.0% than when it was increased from 1.0 to 2.0%. On the other hand, the quadratic reduction in apparent ileal digestibility of sodium and potassium was such that the decrease in the digestibility of these minerals was lower when the concentration of phytic acid was increased from 0 to 1.0% than when it was increased from 1.0 to 2.0%. The apparent ileal digestibility values for sodium and potassium were negative when phytic acid was supplemented at 2.0%, with the apparent ileal digestibility value for sodium being the lowest.

Tables 4.3 to 4.5 show the apparent ileal digestibility, endogenous losses and true ileal digestibility of amino acids, respectively. Increasing dietary concentration of phytic acid resulted in a quadratic response ($P < 0.05$) in apparent ileal digestibility of isoleucine, leucine, valine, glutamic acid, proline and serine such that the apparent ileal digestibility of these amino acids increased when the dietary concentration of phytic acid was increased from 0 to 1.0% and then declined when the dietary phytic acid level was further increased to 2.0%, though the differences between the apparent ileal digestibility values of the amino acids at dietary phytic acid supplementation of 0 and 1.0% were marginal (on average by 1.8 percentage points). A similar trend was observed for true ileal digestibility of isoleucine, leucine, glutamic acid and proline when the dietary concentration of phytic acid was increased. However, the apparent ileal digestibility and true ileal digestibility values of other amino acids, and the endogenous amino acid losses, were unaffected ($P > 0.05$) by the dietary concentration of phytic acid.

**Table 4.3**. Effect of phytic acid on apparent ileal digestibilities of nitrogen and amino acids in piglets fed a casein-corn starch-based diet

| Item | Dietary phytic acid, % | | | | SEM | *P* values | |
|---|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 2.0 | | Linear | Quadratic |
| Nitrogen | 90.4 | 90.5 | 92.0 | 90.2 | 0.45 | 0.911 | 0.032 |
| Indispensable amino acids | | | | | | | |
| Arginine | 91.7 | 91.2 | 93.2 | 91.7 | 0.47 | 0.962 | 0.091 |
| Histidine | 94.5 | 94.5 | 95.7 | 94.7 | 0.34 | 0.973 | 0.116 |
| Isoleucine | 93.3 | 93.6 | 95.1 | 93.3 | 0.27 | 0.978 | 0.030 |
| Leucine | 95.5 | 95.2 | 96.6 | 95.3 | 0.28 | 0.917 | 0.048 |
| Lysine | 94.5 | 94.3 | 95.0 | 94.5 | 0.23 | 0.945 | 0.087 |
| Methionine | 95.1 | 94.7 | 95.7 | 94.6 | 0.24 | 0.239 | 0.244 |
| Phenylalanine | 95.6 | 94.7 | 96.4 | 95.2 | 0.29 | 0.953 | 0.328 |
| Threonine | 87.6 | 86.6 | 88.6 | 86.8 | 0.49 | 0.344 | 0.442 |
| Valaline | 92.0 | 91.8 | 93.0 | 91.5 | 0.30 | 0.389 | 0.046 |
| Dispensable amino acids | | | | | | | |
| Alanine | 87.0 | 86.8 | 89.2 | 87.3 | 0.56 | 0.905 | 0.176 |
| Aspartic acid | 89.2 | 88.6 | 90.3 | 88.1 | 0.54 | 0.572 | 0.141 |
| Cysteine | 94.8 | 93.8 | 95.2 | 94.6 | 0.19 | 0.919 | 0.232 |
| Glutamic acid | 94.6 | 94.7 | 95.6 | 93.2 | 0.54 | 0.343 | 0.049 |
| Glycine | 71.4 | 72.0 | 77.5 | 69.7 | 2.34 | 0.274 | 0.182 |
| Proline | 92.5 | 94.4 | 95.5 | 93.5 | 0.96 | 0.930 | 0.043 |
| Serine | 88.7 | 89.7 | 91.3 | 89.6 | 0.65 | 0.939 | 0.027 |
| Tyrosine | 96.2 | 94.7 | 97 | 95.2 | 0.54 | 0.468 | 0.226 |

**Table 4.4.** Effect of phytic acid on endogenous losses of amino acids (mg/kg dry matter) in piglets fed a casein-corn starch-based diet

| Item | Dietary phytic acid, % | | | | | *P* values | |
|---|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 2.0 | SEM | Linear | Quadratic |
| Indispensable amino acids | | | | | | | |
| Arginine | 602 | 714 | 602 | 690 | 58 | 0.801 | 0.303 |
| Histidine | 338 | 360 | 305 | 344 | 15 | 0.801 | 0.303 |
| Isoleucine | 563 | 600 | 509 | 573 | 24 | 0.801 | 0.303 |
| Leucine | 900 | 959 | 814 | 916 | 29 | 0.801 | 0.303 |
| Lysine | 675 | 719 | 611 | 687 | 29 | 0.801 | 0.303 |
| Methionine | 225 | 240 | 204 | 229 | 9.7 | 0.801 | 0.303 |
| Phenylalanine | 675 | 719 | 611 | 687 | 29 | 0.801 | 0.303 |
| Threonine | 1013 | 1079 | 916 | 1030 | 44 | 0.801 | 0.303 |
| Valaline | 788 | 839 | 713 | 802 | 34 | 0.801 | 0.303 |
| Dispensable amino acids | | | | | | | |
| Alanine | 951 | 1013 | 860 | 967 | 41 | 0.801 | 0.303 |
| Aspartic acid | 1587 | 1691 | 1435 | 1612 | 69 | 0.801 | 0.303 |
| Cysteine | 360 | 384 | 326 | 366 | 16 | 0.801 | 0.303 |
| Glutamic acid | 2251 | 2398 | 2036 | 2291 | 98 | 0.801 | 0.303 |
| Glycine | 999 | 1065 | 904 | 1017 | 43 | 0.801 | 0.303 |
| Proline | 736 | 784 | 666 | 749 | 32 | 0.801 | 0.303 |
| Serine | 1035 | 1103 | 936 | 1053 | 45 | 0.801 | 0.303 |
| Tyrosine | 450 | 480 | 407 | 458 | 20 | 0.801 | 0.303 |

**Table 4.5.** Effect of phytic acid level on true ileal amino acids and nitrogen digestibilities in piglets fed a casein-corn starch-based diet

| Item | Dietary phytic acid, % | | | | | *P* values | |
|---|---|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 2.0 | SEM | Linear | Quadratic |
| Nitrogen | 92.2 | 92.2 | 93.5 | 92.0 | 0.37 | 0.913 | 0.034 |
| Indispensable amino acids | | | | | | | |
| Arginine | 103.0 | 103.2 | 103.4 | 103.2 | 0.24 | 0.962 | 0.986 |
| Histidine | 101.5 | 101.9 | 102 | 110.9 | 0.13 | 0.476 | 0.493 |
| Isoleucine | 99.3 | 99.9 | 100.6 | 99.4 | 0.25 | 0.947 | 0.046 |
| Leucine | 100.6 | 100.6 | 101.2 | 100.5 | 0.15 | 0.917 | 0.039 |
| Lysine | 99.3 | 99.4 | 99.4 | 99.4 | 0.07 | 0.540 | 0.338 |
| Methionine | 98.6 | 98.5 | 99 | 98.2 | 0.17 | 0.461 | 0.414 |
| Phenylalanine | 102.5 | 102.4 | 102.7 | 102.3 | 0.15 | 0.985 | 0.923 |
| Threonine | 99.3 | 99.1 | 99.2 | 98.8 | 0.42 | 0.273 | 0.764 |
| Valaline | 98.5 | 98.8 | 98.9 | 98.2 | 0.24 | 0.658 | 0.049 |
| Dispensable amino acids | | | | | | | |
| Alanine | 104.4 | 105.4 | 105.0 | 105.1 | 0.51 | 0.461 | 0.478 |
| Aspartic acid | 101.3 | 101.5 | 101.3 | 100.4 | 0.38 | 0.334 | 0.442 |
| Cysteine | 122.7 | 123.6 | 120.5 | 123.1 | 1.2 | 0.959 | 0.372 |
| Glutamic acid | 99.8 | 100.3 | 100.3 | 98.5 | 0.47 | 0.668 | 0.047 |
| Glycine | 98.3 | 100.6 | 101.8 | 97.1 | 1.77 | 0.204 | 0.122 |
| Proline | 95.8 | 98.0 | 98.5 | 96.9 | 0.94 | 0.963 | 0.041 |
| Serine | 98.6 | 100.3 | 100.3 | 99.7 | 0.57 | 0.976 | 0.033 |
| Tyrosine | 101.6 | 100.5 | 101.9 | 100.5 | 0.46 | 0.451 | 0.868 |

## 4.5 DISCUSSION

Phytic acid has been shown to reduce apparent ileal digestibility of cationic minerals in pigs (Bohlke et al., 2005) and broilers (Ravindran et al., 2006), which is consistent with current observations. However, in the current study, phytic acid supplementation at 2.0% resulted in negative apparent ileal digestibility values of sodium and magnesium, indicating increased endogenous flow of these minerals at the terminal ileum due to phytic acid. Cowieson et al. (2004) also observed increased excretion of endogenous minerals in broilers due to phytic acid. In both the present study and that of Cowieson et al. (2004), sodium was the mineral most affected. Sodium deficiency in chickens has been reported to reduce the activity of intestinal Na-K-ATPase in the gut, which is involved in the absorption of glucose and other nutrients (Gal-Garber et al., 2003). Thus, the increase in endogenous secretion of minerals such as sodium due to phytic acid may have significant nutritional and physiological implications as it could result in alteration of electrolyte balance and hence Na-K-ATPase activity and nutrient absorption in the small intestine. This is supported by results from a broiler study by Liu et al. (2008b) which showed reduced activity of Na-KATPase in broilers due to ingestion of phytic acid. However, in pigs unlike in poultry, the minerals that are endogenously secreted in the small intestine may be re-absorbed in the large intestine. Hence, the alteration in the electrolyte balance due to phytic acid might be local (in the mucosa of the small intestine), but not systemic (in the whole body) as the deficiency of the minerals would be corrected by re-absorption in the large intestine. It will be interesting to see the effects of phytic acid on sodium retention in the body, and on the activity of Na-K-ATPase and nutrient absorption in the small intestine.

The mechanisms by which phytic acid increases the endogenous secretion of minerals in the gastrointestinal tract have not yet been established. However, two mechanisms are proposed. First, phytic acid may bind to enzyme co-factors in the gut, resulting in increased mineral secretion through negative feedback mechanisms. Second, amino groups found on side-chains of basic amino acids and at amino terminal ends of proteins possess a net positive charge at pH below the isoelectric point (Hídvégi and Lásztity, 2002). Hence phytic acid may interact with dietary protein, and endogenous protein (pepsin, and pepsinogen and its activating peptide) in the stomach, where the pH is acidic (Cowieson et al., 2004), thereby reducing the activity of pepsin and hence increasing the secretion of the enzyme and hydrochloric acid via negative feedback mechanisms (Cowieson et al., 2006). The resulting acidic digesta may then need to be neutralised in the small intestine by mineral-bicarbonates secreted by the intestine (Allen et al., 1993) and pancreas (Zebrowska et al., 1983) to protect the small intestinal mucosa from acid digestion and to optimise pancreatic and intestinal digestive enzymes, resulting in increased endogenous secretion and hence losses of the minerals. Because pancreatic juice is richer in sodium than other minerals (Zebrowska et al., 1983) and it is sodium that was most affected by phytic acid, it appears that phytic acid increases the endogenous losses of minerals (especially sodium) mainly by increasing the endogenous secretion of mineral bicarbonates in the small intestine to neutralize the acidic digesta exiting the stomach. This hypothesis, however, needs to be proven.

In the current study, the reduction in apparent ileal digestibility of monovalent cations (sodium and potassium) due to increased dietary concentration of phytic acid was quadratic such that the reduction in apparent ileal digestibility of these cations was lower

when the dietary phytic acid level was increased from 0 to 1.0%, than when it was increased from 1.0 to 2.0%. On the other hand, the reduction in apparent ileal digestibility of divalent cations (calcium and magnesium) due to dietary phytic acid was linear such that the magnitude by which dietary phytic acid reduced the apparent ileal digestibility of these cations did not change when the level of phytic acid was increased from 0 to 1.0% or from 1.0 to 2.0%. Phytic acid is known to form weaker bonds with monovalent cations than with divalent cations (Erdman, 1979). This implies that the reduction in apparent ileal digestibility of cations due to their binding to dietary phytic acid could be more for divalent than monovalent cations. Therefore, the different responses in the apparent ileal digestibility of monovalent cations compared with that of their divalent counterparts (due to increasing level of dietary phytic acid) is most likely due to differences in mechanisms by which phytic acid reduces the apparent ileal digestibility of the two categories of cations (monovalent versus divalent).

Phytic acid supplementation reduced the apparent ileal digestibility of phosphorus in the current study. This is interesting because phosphorus, unlike other minerals whose apparent ileal digestibility was measured in the current study, is negatively charged. The reduced apparent ileal digestibility of phosphorus due to phytic acid could have been due to binding of phytic acid to both endogenous and dietary non- phytic acid phosphorus via divalent cations to form phytic acid-cation-phosphorus complexes. Because the true digestibility of phosphorus in pigs is lower for diets with higher phytic acid concentration than for lower concentration of phytic acid (Dilger and Adeola, 2006), the lower apparent ileal digestibility of phosphorus due to phytic acid could also have been as a result of higher content of phytic acid-bound phosphorus in phytic acid-supplemented diets.

However, it is not clear why the decrease in apparent ileal digestibility of phosphorus, in contrast to that of other minerals measured in the present study, was greater when the level of phytic acid was increased from 0 to 1.0% than when it was increased from 1.0 to 2.0%.

The rate of conversion of lysine into homoarginine in casein (96.3 %) was within the range of values reported by Nyachoti et al. (2002) (89.2%) and Schmitz et al. (1991) (99.6%). The apparent ileal digestibility, endogenous losses and true ileal digestibility of amino acids observed in the current study were generally similar to what Nyachoti et al. (1997b) reported in growing pigs fed casein-corn starch with a casein content (20%) that is similar to what was contained in the basal diet used in the current study. The true ileal digebility of cysteine in all diets was greater than 100%, which indicate that the content of this amino acid in endogenous protein at terminal ileum of piglets is overestimated. Therefore, there is a need to determine whether the composition of the endogenous protein at terminal ileum vary depending on the experimental conditions.

In addition to reducing mineral digestibility, phytic acid is expected to bind to amino acids in the diet and proteolytic enzymes (Cowieson et al., 2004), resulting in reduced true amino acid digestibility. Also by interacting with amino acids in both diet and proteolytic enzymes in the stomach, phytic acid is expected to increase enzyme and hydrochloric acid secretion as previously discussed. Also, phytic acid could bind to enzymes secreted by the pancreas and small intestinal wall, resulting in their compensatory secretion. Because one of the physiological functions of mucin is to protect the gut wall from degradation by acid and proteolytic enzymes (Montagne et al., 2004), the increased proteolytic enzymes and hydrochloric acid secretion is expected to result in

increased mucin secretion to protect the gut wall from the enzyme and acid digestion, resulting in increased endogenous losses of amino acids and nitrogen contained in the digestive enzyme and mucin. Thus, the presence of phytic acid in pig diets is expected to result in reduced true ileal digestibility and increased endogenous losses of amino acids, and hence decreased apparent ileal digestibility of the amino acids. However, in the current study, phytic acid supplementation did not decrease the apparent and true ileal digestibilities of amino acids and promote the endogenous losses of the amino acids. This observation contradicts results of Bohlke et al. (2005) and Liao et al. (2005), who reported decreased apparent ileal digestibility of amino acids due to phytic acid. However, results of the current study are similar to those of studies that did not report reduced amino acid digestibility in pigs due to phytase despite increased phytic acid hydrolysis as evidenced by improved P digestibility (Johnston et al., 2004, Woyengo et al., 2008). It is not clear why phytic acid has variable effects on amino acid digestibility in pigs.

In nature, phytic acid occurs as a mixed salt of cations, mainly potassium and magnesium, and to a lesser extent calcium, iron and zinc in spherical inclusions called globoids within protein bodies (Prattley and Stanely, 1982; Ockenden et al., 2004; Joyce et al., 2005; Lin et al., 2005). In most feed ingredients, phytic acid is concentrated within cells that are rich in fibre (Ockenden et al., 2004; Joyce et al., 2005). Thus, in order to determine the actual effect of phytic acid on nutrient utilisation, a phytic acid-free diet should be fed with and without phytic acid in a fibre-free form because fibre also affects the digestibility and endogenous losses of nutrients. However, it was difficult to get phytic acid in a form that is naturally found in feed ingredients (mixed salt of cations) in

the market. Most of the phytic acid products that were available in the market at the time when the study was conducted are salts of one cation and hence the reason why sodium phytate was used in the current study. Nonetheless, in pigs, the mode of action of intrinsic phytic acid is expected to be the same as that used in the present study due to the following two reasons. First, phytic acid and the cationic minerals disassociate at acidic pH found in the stomach because they are both soluble at this pH (Maenz, 2001). Therefore, the form in which phytic acid exerts its effects in the stomach is independent of the original form in which it is supplied in the diet. Second, at small intestinal pH, phytic acid reacts with free cationic minerals to form phytate (Maenz, 2001). The major cationic mineral in practical diets is calcium. Therefore, phytic acid is likely to complex more calcium than other cations regardless of its original form in the diet. However, it should be noted that the pH in the crop of poultry is not acidic enough to cause disassociation between phytic acid and cationic minerals, and therefore, phytic acid may exert its effects in the crop in a form that is similar to the form that it is supplied in the diet, resulting in an overall influence of the dietary form of phytic acid on response to its ingestion.

In conclusion, our results suggest that dietary phytic acid has limited effect on the digestibility and endogenous losses of amino acids, but can reduce apparent ileal mineral digestibility in piglets partly due to their increased endogenous secretion at the terminal ileum as evidenced by negative apparent ileal digestibility values for some minerals. The increase in endogenous flow of minerals such as sodium may be particularly important as an increased presence of these minerals in the gut will effectively alter electrolyte balance. The altered electrolyte balance may result in reduced activity of Na-K-ATPase

and hence the capacity of the enterocytes to transport glucose and other nutrients. The present data are thus suggestive of important physiological responses in piglets to the ingestion of phytic acid.

# CHAPTER FIVE

## Gastro-intestinal digesta pH, pepsin activity and soluble mineral concentration responses to supplemental phytic acid and phytase in piglets[1]

**5.1 ABSTRACT**: The objective of this study was to investigate the effects of phytic acid and phytase on gastro-intestinal digesta pH, soluble mineral concentration and pepsin activity in piglets. Twenty four piglets (initial body weight = 7.60 ± 0.73 kg; mean ± SD) were randomly assigned to 3 experimental diets to give 8 piglets per diet. The diets consisted of a casein-corn starch-based diet with 0, 2% phytic acid (as sodium phytate), or 2% phytic acid plus phytase at 500 FTU/kg. The basal diet was formulated to meet National Research Council's recommendations of energy, essential amino acids, minerals and vitamins for piglets. After consuming the experimental diets for 10 days, the piglets were killed and digesta sampled from the stomach and jejunum for pH, pepsin activity, and soluble mineral determination. Phytic acid decreased ($P < 0.01$) jejunal digesta pH from 7.13 to 6.61. Phytic acid also decreased ($P < 0.05$) stomach digesta pepsin activity by 46%; jejunal $Ca^{2+}$ and $Mg^{2+}$ concentrations by 60 and 85%, respectively; but increased ($P < 0.0001$) jejunal Na+ concentration by 57%. Phytase did not influence ($P > 0.05$) any of the response criteria measured in this study. It is concluded that supplemented phytic acid reduces pepsin activity in the stomach, pH in jejunum, and concentrations of soluble

[1]The material presented in chapter five of this thesis has been published in Livestock Science: T. A Woyengo, C. C. Udenigwe, O. Adeola, and C. M. Nyachoti. 2010. Gastro-intestinal digesta pH, pepsin activity and soluble mineral concentration responses to supplemental phytic acid and phytase in piglets. Livest. Sci. 134:91-93.

calcium and magnesium in the jejunum; and increases sodium secretion in the jejunum of piglets. Thus, it appears that phytic acid reduces nutrient utilization in pigs partly through reduced stomach pepsin activity and altered mineral solubility in the small intestine.

**Key words:** gastrointestinal pH, mineral concentration, pepsin activity, phytase, phytic acid, piglet

## 5.2 INTRODUCTION

Phytase supplementation improves mineral utilization in pigs and poultry due to hydrolysis of its substrate, phytic acid, which is naturally present in plant feedstuffs (Adeola et al., 2004; Rutherfurd et al., 2004). Phytic acid is negatively charged and hence it can reduce the availability of positively charged minerals by binding to them (Bedford, 2000). Phytic acid has also been shown to increase endogenous mineral losses in poultry (Cowieson et al., 2004), and to reduce the apparent digestibility of Na and Mg in piglets to negative values (Woyengo et al., 2009), indicating increased endogenous losses of these minerals in pigs due to phytic acid. However, there is no information on the mechanisms by which phytic acid increases the endogenous mineral losses. Binding of tannic acid to pepsin in the stomach of rats was shown to result in increased pepsin and hydrochloric acid secretions (Mitjavila et al., 1973). Thus, phytic acid may bind to positively charged amino acids on the pepsinogen molecule in the stomach, leading to reduced pepsin activity and hence increased pepsin and hydrochloric acid secretions via negative feedback mechanisms. The resulting acidic digesta may then require neutralization by mineral-bicarbonates in small intestine, resulting in increased endogenous mineral losses. The objective of this study was to determine the effect of

phytic acid and phytase on gastric digesta pepsin activity, and pH and total and soluble mineral concentrations in the stomach and jejunal digesta of piglets.

## 5.3 MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee, and pigs were handled in accordance with guidelines described by the Canadian Council on Animal Care (CCAC, 1993). Twenty-four Genesus (Yorkshire-Landrace X Duroc) piglets (balanced for sex) with an initial body weight of 7.60 ± 0.73 kg (mean ± SD) were obtained immediately after weaning, and group-housed in pens and fed a commercial starter diet. After 3 days, piglets were housed individually in pens (1.5 x 1.2 m) with smooth sides and plastic covered expanded metal flooring in a temperature-controlled room (30 ± 2ºC). They were then randomly assigned to 3 experimental diets to give 8 piglets per diet. The diets were a casein-corn starch-based diet that was supplemented with 0, 2% phytic acid (as sodium phytate; Sigma-Aldrich Corporation, St Louis, MO, USA), or 2% phytic acid plus an *Escherichia coli*-derived phytase at 500 FTU/kg. The basal diet was formulated to meet NRC (1998) energy, amino acid, mineral and vitamin recommendations for piglets (Table 4.1). After consuming the experimental diets ad-libitum for 10 days, the piglets were anesthetized by an intramuscular injection of ketamine:xylazine (20:2 mg/kg; Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada), and killed by an intravenous injection of sodium pentobarbital (50 mg/kg of BW; Bimeda-MTC Animal Health Inc.). The abdominal cavity was exposed by midline laparotomy, and the contents of the stomach and jejunum (from 80 cm below the pylorus to 80 cm above the ileal-cecal junction) were obtained and thoroughly mixed.

The stomach contents were immediately divided into 2 equal portions (one for determining pepsin activity and the other for determining pH and mineral concentration), and together with jejunal contents, they were frozen in liquid nitrogen and stored at -80ºC for determination of pepsin activity, pH and total and soluble calcium, magnesium, sodium and potassium contents.

Stomach digesta samples for pepsin activity analysis were thawed, and an aliquot was taken and centrifuged for 15 min at 14 000 x *g* at 4ºC. The supernatants were then collected and the pepsin activity was determined as described by Rick and Fritsch (1974). Samples for pH and mineral concentration analyses were thawed, pH immediately determined and sampled for total and soluble mineral concentrations determination. Aliquots for determining total mineral content were freeze-dried, finely ground, ashed for 12 hour, digested according to AOAC (1990) procedures (method 990.08), and read on a Varian Inductively Coupled Plasma Mass Spectrometer (Varian Inc, Palo Alto, CA, USA). Aliquots for determining soluble mineral contents were centrifuged for 10 min at 2 440 x *g* at 4ºC, and supernatants were obtained and read as described for total mineral content determination. Data were subjected to analysis of variance using the GLM procedure (SAS, 2002). The effects of phytic acid and phytase were determined using specific contrasts (Steel et al., 1997).

## 5.4 RESULTS AND DISCUSSION

The analyzed phytase activities in the control, control plus phytic acid, and control plus phytic acid and phytase diets were 56, 78 and 554 FTU/kg, respectively (Appendix 2). The effects of phytic acid and phytase on pepsin activity, pH as well as soluble mineral concentration in digesta are presented in Table 5.1. Phytic acid decreased

**Table 5.1.** Effect of phytic acid and phytase on gut digesta pepsin activity, pH and soluble mineral content

| | Diet[1] | | | | *P* values | |
|---|---|---|---|---|---|---|
| Item | Control | PA | Phytase | SEM | Control vs PA | PA vs phytase |
| Pepsin activity[2], PU/ml | 265.5 | 142.5 | 161.9 | 35.5 | 0.028 | 0.713 |
| Stomach digesta pH | 4.60 | 4.84 | 4.48 | 0.277 | 0.554 | 0.379 |
| Jejunal digesta pH | 7.13 | 6.61 | 6.91 | 0.122 | 0.009 | 0.113 |
| Jejunal mineral content, ppm | | | | | | |
| $Ca^{2+}$ | 270.2 | 108.5 | 128.2 | 36.8 | 0.007 | 0.719 |
| $K^{+}$ | 653.4 | 691.2 | 740.7 | 75.8 | 0.737 | 0.661 |
| $Mg^{2+}$ | 468.5 | 69.1 | 113.4 | 98.7 | 0.012 | 0.762 |
| $Na^{+}$ | 2,670.2 | 4,191.9 | 3,987.8 | 163.9 | <0.001 | 0.405 |

[1]PA = control plus 2% phytic acid, Phytase = control plus 2% phytic acid plus phytase.

[2]Determined in stomach digesta.

($P < 0.05$) stomach digesta pepsin activity, which could be due to binding of the phytic acid to pepsinogen. Pig pepsinogen, but not pepsin contains basic amino acids (Stepanov et al., 1973), which are positively charged at the acidic pH found in the stomach, and hence they can form electrostatic bonds with phytic acid, which is negatively charged at the same pH (Prattley et al., 1982). By binding to the basic amino acids of pepsinogen, phytic acid may interfere with its conversion to pepsin, leading to reduced activity of the latter.

The stomach and jejunal pH values were similar to values reported by Moore and Tyler (1955) in piglets. Phytic acid did not affect ($P > 0.05$) stomach pH, but decreased ($P < 0.01$) jejunal digesta pH. The reduced pepsin activity due to phytic acid was expected to result in increased secretion of the enzyme and hydrochloric acid, resulting in reduced pH of the digesta in the stomach and the upper part of small intestine (duodenum and jejunum). It is not clear why phytic acid did not affect the stomach digesta pH.

Phytic acid did not affect ($P > 0.05$) soluble sodium, calcium, magnesium and potassium concentrations in the stomach digesta (Appendix 3), but decreased $Ca^{2+}$ and $Mg^{2+}$ concentrations and increased $Na^{+}$ concentration in the jejunal digesta ($P < 0.05$). The phytic acid-related reduction in $Ca^{2+}$ and $Mg^{2+}$ concentrations in the jejunal digesta could be due to lower solubility of these cations that were bound to phytic acid because the total content of these minerals in jejunal digesta were not reduced by phytic acid (data not presented). The increased $Na^{+}$ content could be due to its reduced absorption or increased endogenous secretion or both because its total concentration was also increased by phytic acid (Table 6.2). However, the increased $Na^{+}$ content in jejunal digesta could mainly have been due to its increased endogenous secretion because we have observed

negative apparent ileal digestibility of sodium due to phytic acid (Woyengo et al., 2009), indicating its increased endogenous loss due to the same (phytic acid). Phytase did not influence ($P > 0.05$) any of the response criteria measured in this study, which could be attributed to the fact that the basal diet was formulated to be adequate in calcium and available phosphorus, which can reduce the efficacy of phytase. Phytic acid reacts with divalent cations at intestinal pH to form insoluble phytic acid-mineral complexes that cannot be hydrolyzed by phytase (Maenz, 2001). Thus, high (adequate) dietary concentration of calcium can reduce the efficacy of phytase on phytic acid hydrolysis. Also, the catalytic activity of phytase is inhibited by its end product, inorganic phosphorus (Greiner et al., 1993), indicating that high dietary concentration of inorganic (available) P can result in decreased hydrolysis of phytic acid by phytase.

In conclusion, supplemented phytic acid in the form of sodium phytate reduced pepsin activity in the stomach digesta, pH and soluble calcium and magnesium contents in the jejunal digesta; and increased soluble sodium content in the jejunal digesta of piglets. The reduced soluble calcium and magnesium contents, and increased soluble sodium content implies reduced calcium and magnesium availability for absorption, and increased endogenous secretion of sodium, respectively. Thus, it appears that supplemented phytic acid reduces nutrient utilization in pigs partly through reduced pepsin activity in the stomach and altered mineral solubility in the small intestine.

# CHAPTER SIX

## Effect of dietary phytic acid and phytase on small intestinal ATPase activity and digesta mineral content of piglets

**6.1 ABSTRACT:** The effects of phytic acid and phytase on jejunal Na-K-ATPase activity and jejunal mineral concentrations of piglets was investigated. Twenty-four piglets (average body weight = 7.60 kg) were fed one of 3 diets (8 piglets per diet) for 10 days. The diets were a casein-corn starch-based diet with 0, 2% phytic acid, or 2% phytic acid plus phytase at 500 FTU/kg. Phytic acid, but not phytase, increased ($P$ = 0.002) calcium concentration in jejunal digesta, and tended to reduce total ATPase ($P$ = 0.082) and Na-K-ATPase ($P$ = 0.075) activities in the jejunum. There was a tendency for phytic acid to increase ($P$ = 0.058) and for phytase to reduce ($P$ = 0.062) potassium content in jejunal digesta. In conclusion, phytic acid can reduce Na-K-ATPase activity and utilization of calcium in the jejunum of pigs.

**Key words:** ATPase activity, phytase, phytic acid, piglet

## 6.2 INTRODUCTION

Phytic acid reduces mineral availability in pigs by binding to the minerals (Selle and Ravindran, 2008). Phytic acid has also been shown to reduce the apparent ileal sodium digestibility in piglets to a negative value (Woyengo et al., 2009), indicating increased endogenous loss of this mineral at the terminal ileum due to phytic acid. However, there is lack of information on the mechanisms by which phytic acid increases the endogenous Na loss and the implications of this loss on digestive physiology of pigs.

Phytic acid may interact with basic amino acids of both dietary and endogenous (digestive enzymes) sources in the stomach, where basic amino acids are positively charged, resulting in reduced activity of pepsin and hence increased secretion of the enzyme and hydrochloric acid via negative feedback mechanisms (Cowieson et al., 2004). The resulting acidic digesta may require neutralisation by sodium bicarbonate, which is secreted into the small intestine, resulting in increased endogenous sodium secretion. We have observed reduced pepsin activity in the stomach of pigs due to dietary phytic acid (Woyengo et al., 2010), implying that the increased endogenous flow of the sodium could indeed be partly due to increased sodium bicarbonate secretion as a result of increased hydrochloric acid secretion in the stomach. Also, sodium is absorbed in the small intestine partly by co-transport with other nutrients and by solvent drag due to increased solute (nutrient) absorption, and its absorption increases with increases in the absorption of other nutrients (Fordtran, 1975). Phytic acid is known to reduce nutrient digestibility, implying that it may increase the endogenous sodium secretion partly by reducing the re-absorption of the endogenously secreted sodium.

Sodium deficiency in chickens has been reported to reduce the synthesis and activity of gastro-intestinal Na-K-ATPase, which is involved in the absorption of several nutrients including glucose and amino acids (Gal-Garber et al., 2003). Thus, the increased endogenous sodium flow at the terminal ileum due to phytic acid may result in altered synthesis and activity of Na-K-ATPase in the small intestine because of the reduced re-absorption of endogenous sodium and lowered availability of sodium in the enterocytes. The objective of this study was to determine the effect of phytic acid and phytase on Na-K-ATPase activity and digesta mineral concentration in the jejunum of piglets.

## 6.3 MATERIALS AND METHODS

Details on the experimental animals and diets are presented in Chapter Five. Briefly, 3 diets were offered ad-libitum to 24 piglets for 10 days in a completely randomised design with 8 piglets per diet. The diets were a casein-corn starch-based diet that was supplemented with 0, 2% phytic acid (as sodium phytate; Sigma-Aldrich Corporation, St Louis, MO, USA), or 2% phytic acid plus an *Escherichia coli*-derived phytase at 500 FTU/kg, and they were fed in mash form. At the end of the experiment, the piglets were killed, and the contents of the jejunum (from 80 cm below the pylorus to 80 cm above the ileal-cecal junction) were obtained and thoroughly mixed, frozen in liquid nitrogen and stored at -80ºC. Jejunal tissue samples (160 cm below pylorus) were also obtained, were frozen in liquid nitrogen and stored at -80ºC for latter determination of total content of minerals, and of ATPase activity, respectively.

Jejunal digesta samples for measuring total mineral contents were freeze-dried, finely ground, and analyzed for calcium, magnesium, sodium and potassium contents as described by Woyengo et al. (2009). Jejunal tissue samples were thawed, flushed using ice-cold physiological saline and opened longitudinally. The mucosal layer was then scraped gently with a microscope slide and sub-samples for determining Na-K-ATPase activity and protein content were taken. The total and ouabain insensitive ATPase activities were determined as described by Del Catstillo and Robinson (1985), whereas the protein content was determined according to a modified Lowry method (Markwell et al., 1978) using bovine serum albumin (Sigma-Aldrich Corporation, St Louis, MO, USA) as a standard. The Na-K-ATPase activity (micro-moles of phosphate liberated per

milligram protein per minute) was calculated as the difference between total activity and ouabain insensitive activity.

Data were subjected to analysis of variance as a completely randomized design using the GLM procedure of SAS (SAS, 2002). The effects of phytic acid and phytase were determined using specific contrasts (Steel et al., 1997).

## 6.4 RESULTS AND DISCUSSION

Phytic acid supplementation tended to reduce total ATPase ($P$ = 0.082) and Na-K-ATPase ($P$ = 0.075) activities in the jejunum (Table 6.1). This tendency for phytic acid to reduce the activity of Na-K-ATPase probably arose from increased endogenous secretion and reduced (re)absorption of sodium in the jejunum because of the following reasons. First, sodium deficiency in chickens has been reported to reduce the activity of intestinal Na-K-ATPase (Gal-Garber et al., 2003), meaning that increased secretion and reduced (re)absorption of sodium may alter the synthesis and activity of Na-K-ATPase. Second, we have observed phytic acid-induced negative apparent ileal digestibility of sodium (Woyengo et al., 2009), indicating increased endogenous secretion and reduced (re)absorption of sodium in the small intestine.

Phytic acid supplementation increased ($P$ = 0.002) the total calcium concentration in the jejunal digesta (Table 6.2), which could be due to its reduced solubility (and hence availability for absorption) as a result of binding to phytic acid. We have previously reported reduced soluble calcium concentration in jejunal digesta due to phytic acid (Woyengo et al., 2010). Phytic acid has previously been shown to reduce the digestibility of calcium in pigs (Woyengo et al., 2009), which could be explained by the reduced solubility of the calcium in the small intestine. The increased total content of calcium, but

**Table 6.1.** Effect of phytic acid and phytase on jejunal mucosal ATPase activity (μmol/mg)

| | Diet[1] | | | | *P* values | |
|---|---|---|---|---|---|---|
| Activity | Control | PA | Phytase | SEM | Control Vs. PA | PA vs. phytase |
| Total ATPase | 10.91 | 6.68 | 7.75 | 1.52 | 0.082 | 0.644 |
| Oubain insensitive ATPase | 5.29 | 3.39 | 4.10 | 1.30 | 0.340 | 0.718 |
| Na-K-ATPase | 5.62 | 3.29 | 3.65 | 0.82 | 0.075 | 0.772 |

[1]Control = a casein-cornstarch-based diet without phytic acid and phytase, PA = control plus 2% phytic acid, Phytase = control plus 2% phytic acid plus phytase at 500 FTU/kg.

**Table 6.2.** Effect of phytic acid and phytase on total mineral content in jejunal digesta (g/kg dry matter)

| | Diet[1] | | | | *P* values | |
|---|---|---|---|---|---|---|
| Item | Control | PA | Phytase | SEM | Control vs. PA | PA vs. phytase |
| Calcium | 17.5 | 37.3 | 36.5 | 3.1 | 0.002 | 0.864 |
| Potassium | 4.0 | 5.9 | 4.1 | 0.6 | 0.058 | 0.062 |
| Magnesium | 8.1 | 5.6 | 5.3 | 1.2 | 0.210 | 0.876 |
| Sodium | 30.4 | 65.4 | 36.3 | 12.6 | 0.096 | 0.144 |

[1]Control = a casein-cornstarch-based diet without phytic acid and phytase, PA = control plus 2% phytic acid, Phytase = control plus 2% phytic acid plus phytase at 500 FTU $kg^{-1}$.

not magnesium, sodium and potassium in the jejunal digesta due to phytic acid could be due to more binding of calcium than the other minerals to phytic acid. This is because calcium has been reported to have a higher affinity to bind phytic acid than magnesium (Lyon, 1984); whereas monovalent cations (sodium and potassium) form weaker bonds with phytic acid, meaning that their availability for absorption is least affected by phytic acid (Scheuermann et al., 1988).

Phytic acid supplementation tended to increase total sodium ($P = 0.096$) and K ($P = 0.058$) concentrations in jejunal digesta (Table 9). The increase in sodium content in the digesta due to phytic acid could be partly due to increased endogenous secretion. This is because we (Woyengo et al., 2010) observed reduced stomach pepsin activity due to phytic acid, which can result in increased acidity of the digesta that reach the small intestine, which may then need to be neutralized by sodium bicarbonate that is secreted into the small intestine, leading to increased endogenous sodium secretion. Also, it could partly be due to reduced absorption of both dietary and endogenous sodium as a result of reduced absorption of other nutrients by phytic acid because of the following three reasons. First, sodium is absorbed in the small intestine partly by co-transport with other nutrients and by solvent drag due to increased nutrient absorption, and its absorption increases with increases in the absorption of other nutrients (Fordtran, 1975). Second, phytic acid can reduce the digestibility and hence absorption of nutrients including carbohydrates, amino acids and divalent cations by binding to the nutrients or digestive enzymes or both (Selle and Ravindran, 2008). Third, sodium forms a weaker bond with phytic acid and hence its availability for absorption is unaffected by phytic acid. The reason for increased potassium content in the jejunal digesta is not clear.

Phytase did not influence any of the response criteria measured in this study except for jejunal potassium content, which tended to decrease ($P$ = 0.062) with phytase supplementation. This is contrary to expectations. It had been assumed that phytase would alleviate the negative effects caused by phytic acid. This lack of effect of phytase is likely due to the fact that the basal diet was formulated to be adequate in calcium and available phosphorus, which can reduce phytase efficacy.

In conclusion, supplemental phytic acid can reduce Na-K-ATPase activity in the jejunum; and increase calcium and sodium contents in the jejunal digesta of piglets. The reduced Na-K-ATPase activity implies reduced absorption of sodium and other nutrients. Thus, it appears that phytic acid increases the endogenous flow of sodium at the terminal ileum of pigs partly through reduced sodium absorption in the small intestine.

# CHAPTER SEVEN

## Histomorphology and small intestinal sodium-dependent glucose transporter 1 gene expression in piglets fed phytic acid and phytase-supplemented diets

**7.1 ABSTRACT:** The objective of this study was to investigate the effects of dietary phytic acid and phytase supplementation on histomorphology of the small intestine and sodium-dependent glucose transporter 1 (**SGLT1**) gene expression in piglets. Twenty-four piglets with an average initial body weight of 7.60 ± 0.73 kg (mean ± SD) were randomly assigned to 3 experimental diets to give 8 piglets per diet. The diets were a casein-corn starch-based diet that was supplemented with 0, 2% phytic acid (as sodium phytate), or 2% phytic acid plus an *Escherichia coli*-derived phytase at 500 FTU/kg. The basal diet was formulated to meet National Research Council's recommendations of energy, essential amino acids, minerals and vitamins for piglets. After 10 days of feeding, the piglets were killed to determine small intestine histomorphology and small intestinal SGLT1 gene expression. Phytic acid supplementation did not affect villous height and villous height to crypt depth ratio, but decreased ($P < 0.05$) crypt depth in the jejunum. Phytase supplementation did not affect villous height, crypt depth and villous height to crypt depth ratio. There was no effect of phytic acid or phytase supplementation on SGLT1 gene expression in the duodenum, jejunum and ileum. In conclusion, phytic acid reduced the crypt depth in the jejunum, but had no effect on villous height, crypt depth, villous height to crypt depth ratio and SGLT1 gene expression. Therefore, it appears that phytic acid does not reduce nutrient utilization in pigs through reduced villous height or expression of the SGLT1.

**Key words:** histomorphology, phytase, phytic acid, piglets, SGLT1expression

## 7.2 INTRODUCTION

Phytic acid reduces mineral digestibility in pigs (Kemme et al., 1999; Bohlke et al., 2005) and has recently been shown to lead to negative apparent ileal digestibility values for sodium in piglets (Woyengo et al., 2009), indicating increased sodium endogenous flow at the terminal ileum due to phytic acid. Although it has been reported that phytic acid binds divalent cations thus reducing their availability for absorption (Maenz, 2001), there is lack of information on the mechanisms by which phytic acid increases the endogenous sodium flow. Sodium, a monovalent cation, forms weaker bonds with phytic acid than divalent cations, and thus its availability for absorption is unaffected by phytic acid (Scheuermann et al., 1988).

Sodium is (re)absorbed from the small intestine partly by co-transportation with glucose (Fordtran, 1975), and its absorption was shown to increase with an increase in glucose absorption (Schiller et al., 1997). Because phytic acid has been shown to reduce ileal energy digestibility (Liao et al., 2005) and starch, which yields glucose is a major energy source in practical swine diets, the increased endogenous flow of sodium at the terminal ileum may be partly due to reduced absorption of glucose by phytic acid. Glucose is transported across the brush border membrane of enterocytes by sodium-dependent glucose co-transporter 1 (**SGLT1**) protein, whose expression reduces with decrease in availability of glucose for absorption (Dyer et al., 1997). Thus, the effect of phytic acid on SGLT1 synthesis is indicative of its (phytic acid) effect on glucose absorption and hence sodium absorption. Also, because the villous height reduces with a decrease in the availability of nutrients for absorption (Pluske et al., 1996), the effect

of phytic acid on histomorphology of the small intestine may be reflective of its effect on nutrient absorption. However, the effect of phytic acid on intestinal histomorphology and SGLT1 expression in pigs has not been determined. The objective of this study was to determine the effect of phytic acid and phytase on the histomorphology of the small intestine and SGLT1 gene expression in piglets.

## 7.3 MATERIALS AND METHODS

### *7.3.1 Experimental Protocol*

Details on the diet formulations, animals, management, and treatment allocation are presented in the companion paper (see Chapter Five). Twenty-four piglets were fed 3 experimental diets (8 piglets per diet) ad-libitum. The diets were a casein-cornstarch-based diet that was supplemented with 0, 2% phytic acid (as sodium phytate; Sigma-Aldrich Corporation, St Louis, MO, USA), or 2% phytic acid plus an *Escherichia coli*-derived phytase at 500 FTU/kg. The basal diet was formulated to meet NRC (1998) recommendations of energy, essential amino acids, minerals and vitamins for piglets (Table 4.2). After 10 days of feeding, the piglets were anesthetized and the abdominal cavity was exposed by midline laparotomy as previously described (see Chapter Five). Two 2-cm sections (samples) of duodenum (10 cm below the pylorus), jejunum (160 cm below pylorus) and ileum (10 cm above the ileo-cecal junction) were obtained for determination of histomorphology and SGLT1 gene expression. Samples for histomorphology analysis were fixed by immersion in 10% neutral buffered formalin in vials at room temperature. The samples for SGLT1 gene expression analysis were

immediately rinsed using phosphate buffered saline, frozen in liquid N and stored at -80ºC until the analysis.

### *7.3.2 Histomorphology Analysis*

After 48 hours in 10% neutral buffered solution, samples were transferred into vials containing 50% ethanol for 15 min, transferred to vials containing 70% ethanol, and then sent for processing (making histology slides) in a commercial laboratory (Veterinary Diagnostic Services Manitoba Agriculture, Food and Rural Initiatives, Winnipeg, MB). Villous height from the tip of the villi to the villous-crypt junction and crypt depth from the villous-crypt junction to the base were measured at x10 magnification using Axiostar Plus Microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Cannon camera, and NIH image J Soft Ware (US NIH, Bethesda, MD) in 10 well-oriented villi and crypt columns. The villous height to crypt depth ratio was calculated.

### *7.3.3 Messenger RNA Quantification*

Duodenal, jejunal, and ileal samples were removed from -80°C storage and put on ice until when they completely thawed. A total of 70 to 80 mg of tissue was added to 1 mL of ice-cold Trizol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) and homogenized with a homogenizer for 1 minute. The homogenate was transferred to a 1.5-mL tube, and 200 µL of chloroform was added to the Trizol mixture. After centrifugation at 13,400 × *g* for 10 min at 4°C, the aqueous phase was carefully transferred to a new 1.5-mL tube, and the RNA was precipitated with 500 µL of isopropanol and washed with 75% ethanol. The RNA pellet was re-suspended in 50 to

100 μL of DNase-RNase-free water (Invitrogen Canada Inc., Burlington, Ontario, Canada), and total concentration was measured at 260 nm on a DU800 Spectrophotometer (Beckman Coulter Canada Inc., Mississauga, Ontario, Canada). The absorbance ratio at wavelength 260:280 nm was ≥ 2.0.

The cDNA was derived from the total RNA by reverse transcription (**RT**) according to the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). From the obtained cDNA mixture, SGLT1 mRNA expression was determined by real-time PCR using Fast SYBR® Green Master Mix kit (Applied Biosystems, Foster City, CA) and β-actin as an endogenous control. The gene specific primers are listed in Table 7.1. cDNA was amplified using the StepOne Real Time PCR System (Applied Biosystems, Foster City, CA), with results given as the cycle number (**$C_T$**) at which a cDNA transcript reached a selected amplification threshold. All PCR were performed in triplicate, and under the following conditions: 95°C for 10 min and 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. The specificity of PCR products were confirmed by melting curve analysis, which was done under the following conditions: 95°C for 15 seconds, 60°C for 1 minute, and then an increase from 60°C to 95°C at an interval of 0.3°C. Data were expressed by normalizing the expression (in $C_T$) of SGLT1 against the expression of the β-actin (internal control; Schmittgen and Livak, 2008) using the following equation:

Normalized CT value (**$\Delta C_T$**) = $C_T$ for SGLT1 - $C_T$ for β-actin

**Table 7.1.** Primer sequences for the PCR amplification of specific genes

| Gene | Sequence |
|---|---|
| β-actin | |
| Forward primer | CTCGATCATGAAGTGCGACGT |
| Reverse primer | GTGATCTCCTTCTGCATCCTGTC |
| Sodium-dependent glucose transporter | |
| Forward primer | GGCTGGACGAAGTATGGTGT |
| Reverse primer | ACAACCACCCAAATCAGAGC |

The SGLT1 gene expression for the diet containing phytic acid alone was related to that for the control diet by the comparative $C_T$ ($\mathbf{\Delta\Delta C_T}$) method (Schmittgen and Livak, 2008) using the following equation:

$\Delta\Delta C_T$ = $\Delta C_T$ for phytic acid containing diet - $\Delta C_T$ for the control diet.

The SGLT 1 gene expression for the diet containing phytic acid plus phytase was related to that for the diet containing phytic acid alone also by the same method as follows:

$\Delta\Delta C_T$ = $\Delta C_T$ for the phytase and phytic acid containing diet - $\Delta C_T$ for the phytic acid containing diet.

Fold change in expression of SGLT1 gene due to phytic acid or phytase supplementation was calculated using the following equation as described by Schmittgen and Livak (2008):

Fold change due to supplementation = $2^{-\Delta\Delta CT}$.

If the $2^{-\Delta\Delta CT}$ value was greater than 1, then the SGLT1 gene expression was increased by the supplementation (Schmittgen and Livak, 2008). If the $2^{-\Delta\Delta CT}$ value was less than one, then the SGLT1 gene expression was decreased by the supplementation, and fold change reduction in expression was calculated as the negative inverse of the $2^{-\Delta\Delta CT}$ value (Schmittgen and Livak, 2008).

### *7.3.4 Statistical Analysis*

The histomorphology data and $\Delta C_T$ values were subjected to Analysis of Variance as a completely randomized design (Steel et al., 1997) using the GLM

procedure (SAS, 2002). The effects of phytic acid and phytase were determined using specific contrasts (Steel et al., 1997).

## RESULTS AND DISCUSSION

Phytic acid supplementation reduced ($P$ = 0.03) crypt depth in the jejunum, but not duodenum and ileum (Table 7.2). Villous cells originate from stem cells, which are located within the crypt (Shen, 2009). As the villous cells move up the crypt-villous axis, they differentiate into goblet cells and absorptive enterocytes; and they are expelled from the villi when they reach the villous tips (Shen, 2009). Crypt depth is positively correlated with the rate of its cell proliferation (Hedemann et al., 2003). The presence of nutrients in the lumen of the small intestine stimulates cell proliferation in the crypt (Goodlad and Wright, 1984), and phytic acid has been shown to reduce nutrient digestibility (Selle and Ravindran, 2008), indicating that phytic acid reduces nutrient availability in the lumen. Therefore, the reduced crypt depth in the jejunum by phytic acid supplementation might have been as a result of reduced proliferation of cells in the crypts due to reduced nutrient availability in the jejunal lumen. The lack of effect of phytic acid on the crypt depth in the duodenum and ileum is not clear. It could have been due to the fact that they are not the major sites of absorption of macro-nutrients (monosaccharides, amino acids, peptides and fatty acids; Shen, 2009), whose availability can be reduced by phytic acid (Singh and Krikorian, 1982; Liu et al., 2008a, 2009).

Phytic acid supplementation did not affect villous height and the villous height to crypt depth ratio (Table 7.2).Villous height is positively correlated with the number of

**Table 7.2.** Effect of dietary treatment on small intestinal histomorphology

| | Diet[1] | | | | Contrasts | |
|---|---|---|---|---|---|---|
| Item[1] | Control | PA | Phytase | SEM | Control vs. PA | PA vs. phytase |
| Villous height, μm | | | | | | |
| Duodenum | 1,005 | 809 | 780 | 107 | 0.23 | 0.86 |
| Jejunum | 528 | 602 | 689 | 59 | 0.40 | 0.32 |
| Ileum | 511 | 508 | 485 | 64 | 0.98 | 0.81 |
| Crypt depth, μm | | | | | | |
| Duodenum | 745 | 716 | 692 | 71 | 0.79 | 0.82 |
| Jejunum | 403 | 320 | 308 | 24 | 0.03 | 0.73 |
| Ileum | 397 | 402 | 367 | 37 | 0.94 | 0.53 |
| Villous height to crypt depth ratio | | | | | | |
| Duodenum | 1.38 | 1.09 | 1.19 | 0.15 | 0.19 | 0.65 |
| Jejunum | 1.43 | 1.89 | 2.27 | 0.21 | 0.16 | 0.23 |
| Ileum | 1.30 | 1.32 | 1.33 | 0.14 | 0.94 | 0.97 |

[1]PA = control plus phytic acid, Phytase = control plus phytic acid plus phytase.

cells within the villi, which in turn, is a function of cell proliferation and migration rates along the crypt-villous axis, and cell loss rate at the tip of the villi (Ferraris, 2000). Villous cell number has been shown to be lower in starved than in fed mice (Goodlad and Wright, 1984), whereas the villi have been reported to be shorter in piglets fed a restricted amounts of milk diet than in piglets fed a milk diet ad-libitum (Pluske et al., 1996; van Beers-Schreurs et al., 1998). Also, an infusion of nutrients into the small intestine of rats has been shown to increase villous height (Clarke, 1977), indicating that reduced availability of nutrients in the intestinal lumen can result in reduced villous cell number and hence villous height. Thus, we hypothesized that phytic acid supplementation will result in reduced villous height because the phytic acid has been shown to reduce nutrient availability. However, this was not the case. This lack of effect of phytic acid on villous height could have been due to slower migration and maturation rates of cells along the villi and cell loss rate at the tip of the villi. However, the mechanisms by which phytic acid could cause these changes are not clear.

Phytase supplementation did not affect villous height (Table 7.2), which may have been due to lack of effect of phytic acid on the same response criterion. It could also be attributed to the fact that the control diet to which phytic acid and phytase were added was formulated to be adequate in calcium and available phosphorus, which can reduce the efficacy of phytase. Pirgozliev et al. (2007) also observed no effect of phytase on villous height in chickens. Phytase supplementation did not affect crypt depth in all sections of the small intestine despite the reduced crypt depth in jejunum by phytic acid, however, the reason for this is not clear. It could have been due to low efficacy of phytase due to the higher calcium and available phosphorus contents in the

basal diet. The duodenal, jejunal and ileal SGLT1 $\Delta C_T$ values were not affected by phytic acid supplementation (Table 7.3), which was contrary to the results of Liu et al. (2008b) who observed reduced SGLT1 expression in the duodenum of chickens due to phytic acid. Phytic acid is negatively charged at the pH found in the stomach and small intestine, meaning that it may bind to positively charged nutrients in the gastrointestinal tract, thereby reducing their availability for absorption (Selle and Ravindran, 2008). Also, phytic acid can bind to endogenous digestive enzymes, thereby reducing their ability to digest nutrients (Cowieson et al., 2006; Selle et al., 2006). Phytic acid has indeed been shown to reduce gastric pepsin activity in piglets (Woyengo et al., 2010), trypsin activity *in vitro* (Singh and Krikorian, 1982) and amylase activity in chickens (Liu et al., 2008b), and to reduce the digestibility of various nutrients in pigs including energy (Liao et al., 2005). The synthesis of SGLT1 in the small intestine is positively correlated with glucose availability in the intestinal lumen (Ferraris and Diamond, 1993; Dyer et al., 1997; Ferraris, 2001). Therefore, dietary phytic acid would be expected to reduce SGLT1 expression due to reduced availability of glucose for absorption by the same (phytic acid). However, this was not the case in the current study, implying that phytic acid does not affect glucose absorption in pigs. Therefore, there is a need to establish whether or not phytic acid reduces glucose absorption in pigs. Phytase supplementation did not affect SGLT1 gene expression in the small intestine of pigs, which could have been due to low efficacy of phytase as discussed above.

In conclusion, phytic acid reduced the crypt depth in the jejunum, but had no effect on villous height, villous height to crypt depth ratio and SGLT1 gene

**Table 7.3.** Effects of phytic acid and phytase on sodium-dependent glucose transporter 1 (SGLT1) gene expression in duodenum, jejunum and ileum of piglets

| GIT[1] section | Item[2] | Diet[3] | | | SEM | Contrasts | |
|---|---|---|---|---|---|---|---|
| | | Control | PA | Phytase | | Control vs. PA | PA vs. phytase |
| Duodenum | $\Delta C_T$ | 5.46 | 5.61 | 6.60 | 0.70 | 0.888 | 0.353 |
| | $\Delta\Delta C_T$ | | 0.15 | 1.00 | | | |
| | Fold change | | -1.11 | -2.00 | | | |
| Jejunum | $\Delta C_T$ | 7.29 | 9.72 | 8.32 | 1.13 | 0.179 | 0.413 |
| | $\Delta\Delta C_T$ | | 2.43 | -1.40 | | | |
| | Fold change | | -5.39 | 2.65 | | | |
| Ileum | $\Delta C_T$ | 5.49 | 6.73 | 8.72 | 1.31 | 0.507 | 0.349 |
| | $\Delta\Delta C_T$ | | 1.23 | 1.99 | | | |
| | Fold change | | -2.35 | -3.98 | | | |

[1]GIT = gastrointestinal tract.

[2]$\Delta C_T$ = normalized CT value, which was calculated as $C_T$ for SGLT1 minus $C_T$ for β-actin; $\Delta\Delta C_T$ = comparative $C_T$ value, which was calculated as $\Delta C_T$ for PA containing diet minus $\Delta C_T$ for the control diet (for PA diet) and $\Delta C_T$ for the phytase and PA containing diet minus $\Delta C_T$ for the PA containing diet (for Phytase diet); Fold change = fold change in expression of SGLT1 gene due to PA or phytase supplementation.

[3]PA = control plus phytic acid, Phytase = control plus phytic acid plus phytase.

expression. Because phytic acid increases ileal endogenous sodium flow, and SGLT1 is involved in sodium absorption, the lack of effect of phytic acid on SGLT1 expression by phytic acid implies that the latter does not increase the endogenous flow of sodium at the terminal ileum of pigs through reduced expression of the SGLT1. It also implies that phytic acid does not reduce nutrient utilization in pigs through reduced villous height and expression of the SGLT1.

# CHAPTER EIGHT

## Effect of dietary phytic acid on performance and nutrient uptake in the small intestine of piglets

**8.1 ABSTRACT:** An experiment was conducted with piglets to determine the effect of dietary phytic acid supplementation on performance, electrophysiological properties of jejunum mounted in Ussing chambers, sodium-dependent glucose transporter 1 (**SGLT1**) protein expression in jejunum, and blood plasma glucose and sodium levels. Sixteen piglets with an average initial body weight of 7.40 ± 0.36 kg (mean ± SD) were randomly assigned to 2 experimental diets to give 8 piglets per diet. The diets were a casein-corn starch-based diet that was either unsupplemented or supplemented with 2% phytic acid (as sodium phytate). The basal diet was formulated to meet National Research Council's recommendation of energy, amino acids, minerals and vitamins for piglets. The experiment lasted for 21 days at the end of which body weight gain and feed consumption were determined, and blood samples were collected for determination of plasma glucose and sodium concentrations. The piglets were then killed to determine jejunal electrophysiological properties (transmural potential difference and short-circuit current) and SGLT1 protein expression. Phytic acid supplementation reduced average daily gain ($P$ = 0.002), average daily feed intake ($P$ = 0.017) and gain to feed ratio ($P$ = 0.001) from 316.1 to 198.2 g, 437.4 to 360.3 g, and 0.721 to 0.539 g/g, respectively. Phytic acid supplementation also tended to reduce ($P$ = 0.088) potential difference (-3.80 vs. -2.23 mV), and reduced ($P$ = 0.023) short-circuit current from 8.07 to 0.1 $\mu$A/cm$^2$. However, phytic acid supplementation did not affect SGLT1 protein, blood

plasma glucose and sodium concentrations. In conclusion, dietary phytic acid reduced growth performance, transmural short-circuit current in the jejunum of piglets. The reduced transmural short-circuit current in the jejunum by phytic acid implies reduced active nutrient transport in the jejunum by the same. Therefore, it appears that dietary phytic acid reduces growth performance of pigs partly through reduced capacity of the small intestine to absorb nutrients.

**Key words:** growth performance, phytic acid, pigs, SGLT1 protein expression, transmural electrophysiological properties

## 8.2 INTRODUCTION

Phytic acid has been shown to increase endogenous sodium loss in broiler chickens (Cowieson et al., 2004), and to reduce the apparent ileal sodium digestibility in piglets to a negative value (Woyengo et al., 2009), indicating increased ileal endogenous sodium flow in pigs due to phytic acid. However, there is lack of information on the mechanisms by which phytic acid increases the endogenous flow of sodium at the terminal ileum. Phytic acid may bind dietary and endogenous (digestive enzymes) protein in the stomach, resulting in reduced activity of pepsin and hence increased secretion of the enzyme and hydrochloric acid via negative feedback mechanisms (Cowieson et al., 2006). The resulting acidic digesta may require neutralization by sodium bicarbonate secreted by the pancreas into the small intestine, resulting in increased endogenous sodium secretion. In a recent study, we observed reduced pepsin activity in the stomach of pigs due to dietary phytic acid (Woyengo et al., 2010), implying that the increased endogenous sodium secretion may partly be due

to increased secretion of sodium bicarbonate as a result of increased pepsin and hydrochloric acid secretion in the stomach.

The amount of the endogenous secreted nutrient that appears at the terminal ileum represents a proportion of that which is not re-absorbed in the small intestine. Sodium is (re)absorbed from the small intestine partly by co-transportation with glucose (Fordtran, 1975). Consequently, glucose uptake leads to increased sodium absorption (Schiller et al., 1997). Phytic acid has been shown to reduce α-amylase and maltase activities in the duodenum of broilers (Liu et al., 2008b), implying that phytic acid reduces glucose absorption due to the reduced abundance of this monosaccharide. It was thus, hypothesized that the increased ileal endogenous sodium flow by phytic acid is partly due to reduced availability of glucose for absorption. The objective of this study was to determine the effect of dietary phytic acid piglet performance, electrophysiological properties, jejunal sodium-dependent glucose transporter 1 (**SGLT1**) protein expression and blood plasma indices.

## 8.3 MATERIALS AND METHODS

### *8.3.1 Experimental Animals and Housing*

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee, and pigs were handled in accordance with guidelines described by the Canadian Council on Animal Care (CCAC, 1993). Sixteen Genesus (Yorkshire-Landrace X Duroc) piglets (balanced for sex) with an initial BW of 7.40 ± 0.36 kg (mean ± SD) were obtained immediately after weaning, and group-housed in pens (8 pigs/diet) and monitored for consumption

of a commercial starter diet to ensure that they were healthy piglets that were able to eat. After 3 days, piglets were housed individually in pens (1.5 x 1.2 m) with smooth sides and plastic covered expanded metal flooring in a temperature-controlled room (30 ± 2ºC) and fed the experimental diets.

### *8.3.2 Experimental Diets and Procedure*

The two diets fed were a casein-corn starch-based diet without phytic acid (control) and the control plus 2.0% phytic acid (as sodium phytate, Sigma-Aldrich Corporation, St Louis, MO). Phytic acid was supplemented at 2.0% because we had previously observed increased endogenous loss of of sodium and magnesium due to phytic acid, when the latter was supplemented at 2.0% (Woyengo et al., 2009). The basal diet was formulated to meet NRC (1998) energy, amino acids, minerals and vitamins recommendations for piglets (Table 4.2). The diets were fed ad-libitum to the piglets for 21 days in a completely randomized design with 8 piglets per diet. At the end of the experiment, body weight gain and feed consumption were determined. In addition, blood samples (10 mL) were collected from each pig via jugular vein puncture into vacutainer tubes coated with lithium heparin (Becton Dickinson & Co, Franklin Lakes, NJ). The samples were immediately centrifuged at 2,000 x *g* for 10 min at 4°C to recover plasma, which was immediately stored at -20°C until used for glucose, and sodium analyses.

After the blood collection, the piglets were anesthetized by an intramuscular injection of ketamine:xylazine (20:2 mg/kg; Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada), and euthanized by an intravenous injection of sodium pentobarbital (50 mg/kg of body weight; Bimeda-MTC Animal Health Inc., Cambridge,

Ontario, Canada). The abdomen and the thorax were cut open by a midline incision. Two 10-cm pieces (samples) of the jejunum (160 cm below the pylorus) were obtained from piglets by cutting off the mesentery at the line of its attachment to the intestine for determination of SGLT1 protein level and electrophysiological properties. Samples to be used for SGLT1 protein expression were immediately frozen in liquid nitrogen and stored at -80ºC until required for analysis. The samples for determination of electrophysiological properties were rinsed with ice-cold Ringer buffer with the following composition (mmol/L): NaCl, 115; $NaHCO_3$, 25; $K_2HPO_4$, 2.4; $CaCl_2$, 1.2; $MgCl_2$, 1.2; $KH_2PO_4$, 0.4; D-glucose, 10. The pH of the buffer had been adjusted to 7.4. After rinsing, they were transported in the ice-cold Ringer buffer to the laboratory, where they were opened along the mesenteric border and the mucosa inspected for digesta particles. If digesta particles were present, they were gently washed off with the Ringer buffer at 4˚C. Till experimentation, the samples of jejunum were kept in the ice-cold Ringer buffer which was continuously gassed with a mixture of 95% $O_2$ and 5% $CO_2$, until required for measurements.

### *8.3.3 Determination of Electrophysiological Properties*

The electrophysiological properties (transmural potential difference and short-circuit current) were determined using a modified Ussing chambers (VCC-MC6; Physiologic Instruments Inc., San Diego, CA) containing pairs of current (Ag wire) and voltage (Ag/AgCl pellet) electrodes housed in 3% agar bridges and filled with 3 *M* KCl. Four milliliters of the Ringer buffer solution was added to mucosal chambers, and four milliliters of Ringer buffer solution enriched with with 10 mmol/L D-mannitol instead of D-glucose, was added to serosal chambers. Both the mucosal and serosal chambers

were continuously gassed with a mixture of 95% $O_2$ and 5% $CO_2$. The temeprature of the chambers was maintained at 37°C. The possible potential difference existing between the mucosal and serosal chambers was offset before tissue mounting. After gently stripping off serosal and longitudinal muscle layers using micro-forceps, the tissues were mounted in the Ussing chambers employing a tissue holder with an aperture of 1 $cm^2$. The tissues were left to equilibrate for 10 minutes followed by the recording of the potential difference and short-circuit current 15 minutes and 30 minutes after mounting, respectively, for determination of the effect of dietary treatment on the total potential difference and short-circuit current. Thereafter, the Ringer buffer solution on the mucosal side was replaced with a similar Ringer buffer solution that contained 0.5 mmol/L phloretin, a SGLT1 inhibitor (Sigma-Aldrich Corporation, St Louis, MO) and potential difference and short-circuit current were recorded at 45 and 60 minutes after the mounting. By this, potential difference and short-circuit current were determined that were due to the ion absorption by SGLT1 protein. The potential difference and short-circuit current generated due to ion absorption by SGLT1 protein were calculated as the difference between the potential difference and short-circuit current values that were recorded 15 and 30 minutes after mounting, and the values that were recorded at 45 and 60 minutes after mounting.

### *8.3.4 Determination of Sodium-Dependent Glucose Transporter 1 Protein Level*

Sodium-dependent glucose transporter 1 protein level in the jejunum was determined by Western Immunoblotting Analysis. In brief, approximately 1 g of mucosa from pig jejunum was removed and transferred to cold lysis buffer containing 20 *M* Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2.5

mmol/L Sodium Pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Sodium Orthovanadate, 1% Triton X-100, 2.1 µmol/L Leupeptin and 1 mmol/L Phenylmethylsulfonyl Fluoride. The mucosa was homogenized and sonicated before centrifugation for 5 min at 3,000 x *g*. Supernatant was collected for determining protein concentration using the Bradford assay. Proteins (100 µg) were separated by electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel. Partitioned proteins were transferred to a nitrocellulose membrane. The membrane was probed with rabbit anti-SGLT1 polyclonal antibody (Millipore, Billerica, MA) at 1:2000 dilutions. Horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Inc., Danvers, MA) at 1:1000 dilutions was used as the secondary antibody. The corresponding protein bands were visualized using enhanced chemiluminescence reagents and analyzed with a gel documentation system (Bio-Rad Gel Doc1000, Hercules, CA).

### *8.3.5 Blood Analysis*

Blood plasma was assayed for glucose and sodium using a Nova Stat Profile M Blood Gas and Electrolyte Analyzer (Nova Biomedical Corporation, Waltham, MA).

### *8.3.6 Statistical Analysis*

The data were subjected to analysis of variance as a completely randomized design (Steel et al., 1997) using the GLM procedure (SAS, 2002). Treatment means (control vs. control plus phytic acid) were compared using the t-test procedure (Steel et al., 1997).

## 8.4 RESULTS

Phytic acid supplementation reduced average daily gain ($P$ = 0.002), average daily feed intake ($P$ = 0.017) and gain to feed ratio ($P$ = 0.001) of the pigs (Table 8.1). Data on the effect of dietary phytic acid on the electrophysiological properties of piglets' Jejunum when mounted in Ussing chambers is presented in Table 8.2. Phytic acid supplementation tended to reduce ($P$ = 0.088) total potential difference, and reduced ($P$ = 0.023) total short-circuit current. However, phytic acid supplementation did not reduce SGLT1 sensitive potential difference and short-circuit current ($P$ > 0.10).

Phytic acid supplementation did not affect the SGLT1 protein expression ($P$ = 0.243) (Figure 8.1). Also, phytic acid supplementation did not affect the blood plasma glucose and sodium concentrations (Table 8.3).

## 8.5 DISCUSSION

Phytic acid supplementation reduced pig performance, which was likely a result of reduced digestibility and increased endogenous losses of nutrients due to dietary phytic acid. Phytic acid has been shown to reduce apparent digestibility of minerals (Woyengo et al., 2009) and energy (Liao et al., 2005) in pigs, implying that it reduces the availability of nutrients for utilization by the animals. Phytic acid has also been shown to reduce the apparent ileal sodium and magnesium digestibilities in piglets to negative values (Woyengo et al., 2009), indicating increased endogenous flow of these minerals at the terminal ileum of pigs. An increase in endogenous nutrient losses in the

**Table 8.1.** Growth performance of piglets fed casein-corn starch-based diet without or with phytic acid for 21 days

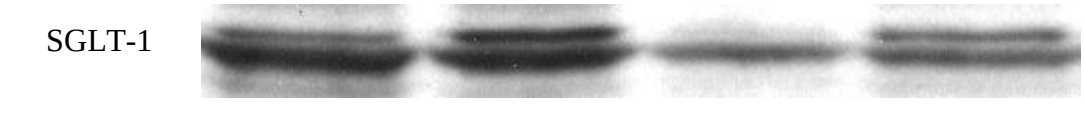
| Item | Diet[1] | | SEM | *P*-value |
|---|---|---|---|---|
| | Control | Phytic acid | | |
| Average daily feed intake, g | 437 | 360 | 20 | 0.017 |
| Average daily gain, g | 316 | 198 | 21 | 0.002 |
| Gain to feed ratio, g/g | 0.72 | 0.54 | 0.03 | 0.001 |

[1]Control = a casein-corn starch-based diet without phytic acid, Phytic acid = Control diet plus 2% phytic acid.

**Table 8.2.** Effect of dietary phytic acid on electrophysiological properties of piglets jejunum mounted in Ussing chambers

| Item | Diet[1] | | SEM | *P*-value |
|---|---|---|---|---|
| | Control | Phytic acid | | |
| Total potential difference, mV | -3.80 | -2.23 | 0.60 | 0.088 |
| SGLT-1 dependent potential difference, mV | -0.73 | -0.54 | 0.17 | 0.440 |
| Total short-circuit current, $\mu$A/cm$^2$ | 8.07 | 0.10 | 0.87 | 0.023 |
| SGLT-1 dependent short-circuit current, $\mu$A/cm$^2$ | 6.50 | 0.04 | 3.41 | 0.275 |

[1]Control = a casein-corn starch-based diet without phytic acid, Phytic acid = Control diet plus 2% phytic acid.



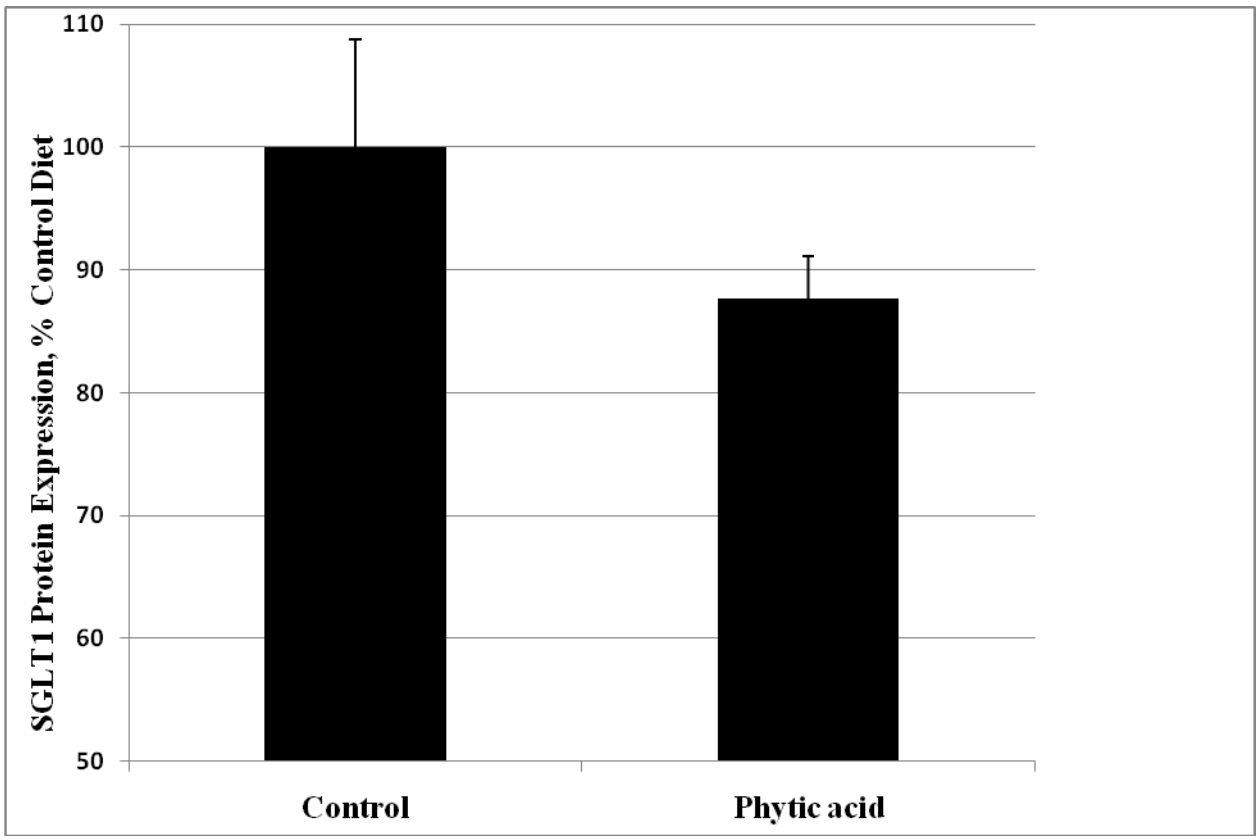




**Figure 8.1.** Effect of dietary phytic acid on sodium-dependent glucose transporter 1 (SGLT1) protein expression in the jejunum of piglets

Control = casein-corn starch based diet without phytic acid; Phytic acid = casein-corn starch based diet plus 2% phytic acid*; P* = 0.243

**Table 8.3.** Effect of dietary phytic acid on piglets plasma sodium and glucose levels

| Item | Diet[1] | | SEM | *P*-value |
|---|---|---|---|---|
| | Control | Phytic acid[1] | | |
| Glucose, mg/dL | 132 | 131 | 14 | 0.968 |
| $Na^+$, mmol/L | 131 | 132 | 2 | 0.606 |

[1]Control = a casein-corn starch-based diet without phytic acid, Phytic acid = Control diet plus 2% phytic acid.

gut is associated with increased maintenance requirements for the lost nutrients and of the energy spent on their secretion (Nyachoti et al., 1997a). Therefore, an increase in endogenous loss of nutrients due to phytic acid implies that the presence of the phytic acid in diets for the pigs results in increased maintenance requirements of energy and other nutrients, thereby reducing the availability of energy and nutrients for tissue deposition.

It is a well established fact that dietary phytase supplementation improves phosphorus availability in pigs due to phytic acid hydrolysis (Selle and Ravindran, 2008). Because of this improved availability of phosphorus due to phytase, the available phosphorus in swine diets has been reduced without any significant effect on performance (Harper et al., 1997; Matsui et al., 2000; Stahl et al. 2000). However, in the current study, the basal diet to which phytic acid was added was formulated to be adequate in available phosphorus. Therefore, the reduced performance of piglets due to phytic acid implies that dietary phytase supplementation can improve pig performance not only by improving phosphorus availability, but by alleviation of the other anti-nutritional effects of phytic acid as well.

The potential difference and short-circuit current were lower in the jejunum of piglets that had been fed phytic acid-supplemented diet when compared to piglets fed the control diet. In Ussing chambers, the potential difference reflects the transmural potential difference that is generated by ion movement across the epithelium, whereas short-circuit current reflects the net transmural ion movement (Ussing, 1994; Wright and Loo, 2000). In the current study, the Ringer buffer solution that was used to bath the mucosal side was the same as the one used on the serosal side except that glucose

was added in the mucosal solution, whereas mannitol was added at the same molarity to the serosal solution to maintain osmotic balance across the tissue. Therefore, potential difference and short-circuit current reflected active transport of ions from the mucosal side to the serosal side, and hence phytic acid reduced the active absorption of ions in the jejunum.

Sodium absorption is the major generator of the potential difference and short-circuit current across the epithelium (Ussing and Zerahn, 1951; Ussing, 1994; Grubb, 1991). The Na-K-ATPase, which is located on the basolateral membrane of the enterocytes actively pumps sodium ions from the same cells to interstitial fluid, creating an electrochemical gradient that serves as the driving force for movement of sodium from the lumen to enterocytes. Because of this movement of sodium ions from the intestinal lumen into the enterocytes and then to interstitial fluid, transepithelial potential difference and short-circuit current are created. Sodium is absorbed partly by co-transportation with other nutrients such as glucose (Fordtran, 1975). Therefore, an increase in absorption of nutrients co-transported with sodium results in an increase in the absorption of the latter, leading to changes in transepithelial potential difference and (Wright and Loo, 2000). Also, the transport of nutrients (solutes) such as glucose and minerals into enterocytes generates an osmotic flow of water into the enterocytes which, in turn, can results in an increase in absorption of sodium by solvent drag (Fordtran et al., 1968), leading to further changes in transepithelial potential difference and short-circuit current when the sodium ions are actively pumped from the enterocytes.

Phytic acid has been reported to reduce *in vitro* starch digestibility and carbohydrate absorption (estimated by breath hydrogen) and glycemic index in humans (Thompson et al., 1987), and to reduce piglets' ileal digestibility of energy (Liao et al., 2005) whose major source in practical swine diets is starch. Phytic acid has also been reported to decrease the activity of α-amylase *in vitro* (Deshpande and Cheryan, 1984) and to reduce activities of α-amylase, sucrase and maltase in the duodenum of broilers (Liu et al., 2008b), implying that phytic acid lowers carbohydrate digestibility and hence availability of glucose for absorption.

Glucose and galactose are absorbed in the small intestine by SGLT1 protein, whose expression reduces with a decrease in the availability of glucose for absorption (Dyer et al., 1997). Therefore, the reduction of glucose absorption by phytic acid is expected to result in a reduced expression of the SGLT1 protein, and hence the capacity of the small intestine to absorb glucose and sodium. However, in the current study, phytic acid did not significantly reduce the SGLT1 sensitive potential difference and short-circuit current. Also, phytic acid supplementation did not significantly reduce SGLT1 protein expression in jejunum of piglets. Therefore, it appears that the reduced ion uptake in jejunum of piglets fed the phytic acid-supplemented diet was also due to other processes.

In addition to sodium, other ions in the Ringer buffer solutions, such as calcium and magnesium are actively transported from the mucosal to serosal side of the jejunum also generating a positive short-circuit current. These ions are actively transported in the small intestine by proteins whose activity increases with a decrease in their availability in diets in an effort to maintain adequate levels of calcium and magnesium

in the body (Hoenderop and Bindels, 2005; Khanal and Nemere, 2008). However, phytic acid binds to magnesium and calcium and thereby reduces the availability of these ions, meaning that the expression of proteins that are involved in the active transport of these ions may be enhanced in animals fed a phytic acid-containing diet. Therefore, it is difficult to explain other mechanisms (apart from reduced SGLT1 protein expression) by which active ion uptake in the jejunum (mounted in Ussing chambers) of piglets fed the phytic acid-supplemented diet could be reduced. However, as previously discussed, sodium is absorbed not only by co-transportation with other nutrients, but by solvent drag as well (Fordtran et al., 1968). The reduced ion absorption in jejunum from piglets fed the phytic acid-supplemented diet implies that the concentration of solutes in the cytoplasm of enterocytes was lower for jejunum from piglets fed the phytic acid-supplemented diet, meaning that the absorption of sodium by solvent drag in jejunum from piglets fed the phytic acid-supplemented diet was also lower. Therefore, the sodium absorption may have been lower in the jejunum from piglets fed the phytic acid-supplemented diet regardless of whether reduction was due to a decrease in SGLT1 protein expression or not. The ileal endogenous flow of a nutrient is dependent on its secretion and re-absorption. Therefore, the phytic acid-induced increase in the ileal endogenous sodium flow at the terminal ileum that we have previously observed (Woyengo et al., 2009) could partly be due to reduced absorption of sodium.

Because phytic acid was shown to increase the endogenous flow of sodium at the terminal ileum of piglets (Woyengo et al., 2009), piglets fed the phytic acid-supplemented diet in the current study were expected to have lower blood plasma

sodium concentration. However, this was not the case as there was no effect of phytic acid on the plasma sodium concentrations. This lack of effect of phytic acid on the plasma sodium concentration could be attributed to an increase in (re)absorption of sodium in the large intestine. It should, however, be noted that the basal-lateral membrane of enterocytes is impermeable to sodium ions, and therefore, the enterocytes rely on sodium ions coming from the intestinal lumen for their functioning (Ussing, 1994). Hence, the increased ileal endogenous flow of sodium due to dietary phytic acid that we have previously observed can still result in reduced availability of sodium to enterocytes, thereby leading to sodium deficiency in the same cells.

In the current study, phytic acid supplementation did not significantly reduce blood plasma glucose concentration. This could have been due to a non-significant reduction in glucose absorption by phytic acid as evidenced by a non-significant reduction in SGLT1sensitive active ion uptake in the jejunum, and a non-significant reduction in jejunal SGLT1 protein expression.

In conclusion, dietary phytic acid can reduce growth performance and active ion transport in jejunum of piglets. The reduced active ion transport by phytic acid implies that the latter reduces the capacity of the small intestine to absorb nutrients. Because sodium is absorbed partly by co-transportation with other nutrients and by solvent drag and phytic acid has been reported to increase the endogenous flow of sodium at terminal ileum of pigs, the reduced capacity of the small intestine (by phytic acid) to absorb nutrients implies that phytic acid increases the ileal endogenous sodium flow partly by reducing the re-absorption of endogenously secreted sodium. The results also

show that dietary phytic acid reduces growth performance of pigs partly through reduced capacity of the small intestine to absorb nutrients.

# CHAPTER NINE

## GENERAL DISCUSSION

The general objective of this thesis was to determine the mechanisms by which phytic acid increases the endogenous nutrient losses in pigs and the impact of these losses on the digestive physiology of pigs. Phytic acid has been shown to reduce nutrient digestibility in pigs and poultry (Selle and Ravidran, 2007, 2008) and to increase endogenous nutrient losses in poultry (Cowieson et al., 2004, 2006; Liu and Ru, 2010), leading to reduced efficiency of nutrient utilization. This is because a decrease in nutrient digestibility results in reduced availability of dietary nutrients for utilization by the animal, whereas an increase in endogenous nutrient losses results in increased maintenance requirements of the lost nutrients and of the energy that is spent during their synthesis and secretion.

Phytic acid has been reported to reduce nutrient digestibility in pigs and poultry by binding to the nutrients and to digestive enzymes (Selle and Ravidran, 2007, 2008). However, there is limited information on the mechanisms by which phytic acid increases the endogenous losses of nutrients and the impact of these losses on the digestive physiology of pigs.

Phytic acid may bind amino acids in both the diet and digestive enzymes, thereby increasing the secretion of the enzymes and hydrochloric acid through negative feedback mechanisms (Cowieson et al., 2004) and hence increased secretion of nitrogen, amino acids and co-factors (minerals). This is because binding of tannic acid to pepsin in the stomach has been shown to result in increased pepsin and hydrochloric acid secretions (Mitjavila et al., 1973), whereas the presence of undigested feed in the gastrointestinal

tract has been reported to stimulate the pancreas to secrete enzymes with the view of digesting the feed (Hara et al., 2000; Morisset, 2008). Therefore, the reduced pepsin activity may result in increased pepsinogen and hydrochloric acid secretions through negative feedback mechanisms.

The increased secretion of digestive enzymes and hydrochloric acid is in turn expected to result in increased secretion of mucins, leading to further increases in endogenous nitrogen and amino acids secretions. The increased hydrochloric acid secretion can also result in increased secretion of mineral-bicarbonates to neutralize the acid, leading to increased secretion of the minerals in the gastrointestinal tract. Phytic acid may also increase the endogenous losses of nutrients by reducing the re-absorption of the endogenously secreted nutrients by the same mechanisms that it reduces the absorption of nutrients of dietary origin.

In the current thesis research project, phytic acid decreased piglet performance, and the apparent ileal digestibility of sodium and magnesium to negative values, indicating increased endogenous flow of these minerals at the terminal ileum of piglets due to phytic acid. Phytic acid also reduced stomach digesta pepsin activity, jejunal digesta pH, active uptake of ions in jejunum mounted in Ussing chambers, and tended to reduce Na-K-ATPase activity in the jejunum.

The reduced pig performance by dietary phytic acid was likely a result of reduced digestibility and increased endogenous losses of nutrients due to dietary phytic acid. Supplemental phytase is added to pig diets to improve performance because it improves phosphorus availability due to phytic acid hydrolysis (Harper et al., 1997; Matsui et al., 2000; Stahl et al. 2000). However, in the current study, the basal diet to which phytic

acid was added was formulated to be adequate in available phosphorus. Therefore, reduced performance of piglets due to phytic acid implies that dietary phytase supplementation can be added in pig diets to improve pig performance not only by improving phosphorus availability, but by alleviation of the other anti-nutritional effects of phytic acid as well.

The increased endogenous losses of sodium by phytic acid could have been due to its increased secretion and reduced re-absorption in the small intestine because of the following reasons. First, phytic acid reduced the stomach digesta pepsin activity, implying that it also increased the secretion of hydrochloric acid, leading to secretion of mineral-bicarbonates which are rich in sodium. Second, phytic acid reduced active uptake of ions in the jejunum mounted in Ussing chambers, implying that it can reduce the absorption of sodium by solvent drag. Third, phytic acid reduced ion uptake in jejunum mounted in Ussing chambers, implying that it can limit sodium absorption by solvent drag. Other studies with poultry have also shown increased endogenous losses of sodium due to dietary phytic acid (Cowieson et al., 2004, 2006).

The primary source of mineral-bicarbonates in the small intestine is the pancreas, (McGuigan, 1978). However, there is lack of information on the effect of dietary phytic acid on pancreatic secretions in pigs. Therefore, there is a need to do further research on the effect of phytic acid on mineral bicarbonate secretion by the pancreas to establish whether the increased ileal endogenous flow of sodium is partly due to increased pancreatic mineral-bicarbonate secretion as a result of increased digesta acidity. In order to quantify pancreatic secretions, pancreatic duct need to be chronically catheterized for collection of pancreatic juice. We have developed a protocol for chronic catheterization

of pancreatic duct of piglets (see Appendix 4) in an effort to develop a piglet model that can be used to study the effect of dietary treatment on pancreatic secretions. So far we have been able to locate the pancreatic duct. However, it has been difficult to insert the catheter it in the duct because the latter is too tender; the duct ruptures during the insertion. Therefore, there is a need to develop a gentle method of inserting the catheter in the duct.

The increase in endogenous loss of magnesium by phytic acid could have been due to the binding of the latter to the endogenously secreted magnesium. This is because phytic acid forms insoluble complexes with divalent cations such as magnesium, leading to their reduced absorption (Maenz et al., 1999). Cowieson et al. (2006) have also shown increased endogenous losses of magnesium in poultry due to dietary phytic acid. It is, however, interesting to note that phytic acid did not reduce the apparent ileal digestibility of calcium to a negative value as it did for magnesium. Phytic acid has a higher affinity for calcium than for magnesium (Lyon, 1984; Maenz et al., 1999), and hence it was expected to have more adverse effects on the apparent digestibility of calcium than on apparent digestibility of magnesium. Indeed, phytic acid has been shown to have more adverse effects on endogenous loss of calcium than on that of magnesium in poultry (Cowieson et al., 2006). It should, however, be noted that in the poultry study of Cowieson et al. (2006), the diets were formulated to be deficient in minerals, whereas in the current research project the diets were formulated to be adequate in minerals. The dietary requirement of calcium by pigs is higher than that of magnesium (NRC, 1998), and hence its inclusion in diets used in the current study was higher than that for magnesium. Therefore, the higher dietary concentration of calcium than that of

magnesium may have masked the actual effects of phytic acid on the apparent calcium digestibility.

The reduced pepsin activity in the stomach digesta due to phytic acid could as a result of binding of phytic acid to the basic amino acids in pepsinogen. Pig pepsinogen, but not pepsin, contains the positively-charged basic amino acids lysine, arginine and histidine, which can form electrostatic bonds with phytic acid at the acidic pH found in the stomach (Stepanov et al., 1973; Prattley et al., 1982). By binding to the basic amino acids, phytic acid may interfere with the activation of pepsinogen to pepsin, leading to reduced activity. Others have also reported reduced pepsin activity *in vitro* (Knuckles et al., 1989; Vaintraub and Bulmaga, 1991) and in the proventriculus of broiler chickens (Liu et al., 2009) due to phytic acid.

The decreased jejunal digesta pH by phytic acid could have been due to increased hydrochloric acid content in jejunal digesta as a result of reduced stomach pepsin activity and hence increased secretion of pepsin and hydrochloric acid in the stomach. However, in the current study, phytic acid supplementation did not affect stomach pH, which was surprising. The reduced pepsin activity due to phytic acid was expected to result in reduced pH of the digesta in the stomach. Therefore, it is not clear why phytic acid did not affect the stomach digesta pH. This could have been due to method errors as pH was determined in samples that had been frozen and thawed. Also, the saliva and duodenum contents may have contaminated the stomach digesta during collection, masking the effects of phytic acid on pH as there was an effect of phytic acid on jejunal digesta pH. This is because pH has been reported to be higher in the stomach digesta of killed piglets than in the stomach digesta of the same piglets just before the killing (3.6 vs. 1.6), and the

difference has been attributed to contamination of stomach digesta with saliva and duodenal digesta, both of which have higher pH during killing (Maner et al., 1962). Thus, the stomach digesta pH values observed in the current study may not reflect the true pH values in live piglets. However, this needs to be established by determining the effect of dietary phytic acid on pH of the stomach digesta *in situ*.

Sodium is absorbed partly by co-transportation with glucose (Fordtran et al., 1968), and hence glucose uptake leads to increased sodium absorption (Schiller et al., 1997). Glucose is absorbed in the small intestine by SGLT1 protein (Dyer et al., 1997). The SGLT1 activity in the small intestine has been reported to decrease with a decrease in the availability of glucose for absorption (Dyer et al., 1997), whereas the SGLT1 protein level is positively correlated with SGLT1 activity (Dyer et al., 1997; Harmon and McLeod, 2001). Phytic acid has been shown to reduce the activity of maltase and α-amylase in the small intestine of broilers (Liu et al., 2008b), implying that phytic acid reduces glucose absorption due to the reduced availability of this monosaccharide. Therefore, it had been hypothesized that dietary phytic acid will reduce sodium in jejunum of piglets by reducing the capacity of the jejunum to absorb glucose. However, phytic acid did not significantly reduce the SGLT1 sensitive ion uptake and SGLT1 protein expression in jejunum of piglets. Therefore, in the current study, the reduction in total active ion transport in the jejunum by phytic acid could have been achieved by other mechanisms. However, these mechanisms are unknown. There is a need for further research to determine the effect of dietary phytic acid on the total and SGLT1 sensitive active transport of labelled sodium in Ussing chambers to establish if phytic acid reduces the capacity of the small intestine to absorb sodium by other means. There is

also a need for further research to determine the effect of dietary phytic acid on the synthesis of intestinal mucosal proteins that are involved in absorption of other nutrients in addition to glucose.

The tendency for the activity of Na-K-ATPase by phytic acid probably arose from increased endogenous secretion of sodium and reduced absorption of nutrients including glucose and minerals in the jejunum because of the following two reasons. First, sodium deficiency in chickens has been reported to reduce the activity of gastro-intestinal Na-K-ATPase (Gal-Garber et al., 2003), indicating that increased secretion and reduced re-absorption of endogenous sodium may alter the synthesis and activity of Na-K-ATPase. Second, phytic acid reduced the active uptake of ions and SGLT1 protein level in jejunum, meaning that it reduces glucose and mineral absorptions. The activity of Na-K-ATPase has been reported to reduce with decreased availability of nutrients for absorption (Lucas-Teixerira et al., 2000).

However, in the current thesis research, phytic acid did not affect the ileal endogenous losses of nitrogen and amino acids in piglets, which was surprising. It had been hypothesized that phytic acid will increase the endogenous losses of nitrogen and amino acids by binding to digestive enzymes, thereby increasing the endogenous secretion of enzymes and mucins. In the current study, phytic acid reduced the activity of pepsin in the stomach, but this did not translate to increased endogenous losses of nitrogen and amino acids.

Phytic acid has had variable effects on apparent ileal amino acids digestibilities in pigs. For instance, Bohlke et al. (2005) and Liao et al. (2005) reported decreased apparent ileal digestibility of amino acids due to phytic acid, whereas Johnston et al. (2004) and

Woyengo et al. (2008) did not report improved amino acids digestibility in pigs due to phytase (which hydrolyzes phytic acid) despite increased phytic acid hydrolysis as evidenced by improved phosphorus digestibility. It is not clear why phytic acid did not affect endogenous amino acids losses in the current study and why it has had variable effects on apparent ileal amino acids digestibilities in pigs. This could probably be due to the fact that phytic acid increases the endogenous secretion of amino acids (as evidenced by reduced pepsin activity), but it does not affect the digestion and absorption of amino acids in pigs, leading to efficient re-absorption of the endogenously secreted amino acids. However, it should be noted that energy is spent during the synthesis, secretion and re-absorption of endogenous protein. This implies that the reduced pepsin activity may result in increased maintainance requirement of energy without an effect on amino acid digestibility. There is a need to determine the effect of phytic acid on the secretion and activity of digestive enzymes other than pepsin, secretion of mucins and energy expenditure in the gastrointestinal tract of pigs.

Phytic acid supplementation reduced crypt depth in the jejunum, which could have been due to reduced proliferation of cells in the crypts due to reduced availability of nutrients for absorption in the jejunal lumen by phytic acid as evidenced by reduced ion uptake and a trend in reduction in SGLT1 protein level by the phytic acid. The presence of the available nutrients in the lumen of the small intestine stimulate cell proliferation in the crypt (Goodlad and Wright, 1984). However, phytic acid supplementation did not affect villous height and the villous height to crypt depth ratio despite the increased endogenous losses of sodium and magnesium, and the reduced ion uptake in the small intestine by the phytic acid. The reason for the lack of effect of

phytic acid on these histomorphological measurements is not clear. However, these results indicate that phytic acid can increase the endogenous losses of nutrients and reduce nutrient absorption in the small intestine of pigs without an effect on villous height and the villous height to crypt depth ratio.

# CHAPTER TEN

# CONCLUSIONS AND FURTHER RESEARCH

## CONCLUSIONS

Phytic acid supplementation may not affect ileal endogenous amino acids losses in pigs, but can reduce the piglet performance, and apparent ileal digestibility of sodium and magnesium to negative values, indicating that phytic acid can increase the endogenous flows of sodium and magnesium at the terminal ileum of pigs. Phytic acid can also reduce the pepsin activity and active ion transport in the gastrointestinal tract of pigs. The reduced pepsin activity by phytic acid implies increased pepsin and hydrochloric acid secretions in the gastrointestinal tract and hence increased secretion of mineral bicarbonates to neutralize the acid. The reduced active ion uptake by phytic acid implies reduced absorption of sodium and other nutrients. Thus, it appears that phytic acid does not affect the ileal endogenous amino acids losses in pigs, but increases ileal endogenous sodium flow partly through reduced pepsin activity and its (sodium) (re)absorption in the small intestine. It also appears that the increased ileal endogenous sodium and magnesium flows at the terminal ileum and the reduced nutrient absorption due to phytic acid does not affect the villous height and the villous height to crypt depth ratio. Overall, the results show that phytase (a phytic acid -hydrolysing enzyme), which is added in pig diets to improve phosphorus availability, does not only improve phosphorus availability, but alleviates the ant-nutritional effects of phytic acid as well.

In this thesis, it had been hypothesized that phytic acid increases the endogenous losses of amino acids and minerals in pigs by reducing the activity of digestive enzymes

in the gastrointestinal tract and re-absorption of the endogenously secreted nutrients, and that the increased endogenous losses of nutrients by phytic acid results in reduced villous height and the synthesis of the nutrient transporter proteins. The hypothesis has partially been supported by results from the thesis because phytic acid increased the endogenous loss of sodium and magnesium, and reduced gastric pepsin activity and jejunal ion uptake in piglets.

## FURTHER RESEARCH

Further research is suggested to:

1. Determine the effect of dietary phytic acid on the pH of the pigs' stomach digesta *in situ* to confirm whether the lack of effect of dietary phytic acid on stomach digesta pH in this thesis was due to determination of the pH after euthanization of the piglets.
2. Effect of phytic acid on pancreatic mineral and digestive enzymes secretions in the gastrointestinal tract of pigs to confirm whether the increased ileal endogenous flow of sodium is due to increased pancreatic mineral-bicarbonate secretion.
3. Effect of phytic acid on activities of digestive enzymes other than pepsin in because they have not been determined.
4. Effect of phytic acid on the true absorption of nutrients including monosaccharides, fatty acids, glycerol, cholesterols and minerals in pigs because they have not been determined.
5. Effect of phytic acid on synthesis of intestinal mucosal proteins that are involved in the absorption of nutrients other than glucose in pigs to establish

whether the reduced ion uptake by phytic acid that was observed in this thesis was due to the reduced synthesis of intestinal mucosal proteins other than glucose transporter protein.

6. Effect of phytic acid on biliary secretions and absorption of bile acids in the gastrointestinal tract of pigs because phytic acid has a potential of increasing secretion of the bile acids by binding to them via cations in the small intestine, thereby reducing the absorption of fat and fat soluble nutrients.
7. Effect of phytic acid on energy expenditure in the gut relative to the whole body to establish whether the reduced pepsin activity and the increased ileal endogenous flow of sodium and magnesium that were observed in this thesis can result in increased energy expenditure in the gut.

# CHAPTER ELEVEN

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# APPENDICES

**Appendix 1.** Equations that were used for determination of digestibility and endogenous flow of nutrients at terminal ileum of piglets

(i) Apparent ileal nutrient digestibility (%)

= 100 - (100 * ([nutrient]digesta * [Cr]diet)/([nutrient]diet * [Cr]digesta))

where [nutrient]diet and [nutrient]digesta are the concentrations (mg/kg dry matter) of nitrogen or amino acid in the diet and digesta, respectively; and [Cr]diet and [Cr]digesta are the concentrations (mg/kg dry matter) of chromic oxide in the diet and digesta, respectively.

(ii) Total flow (mg/kg dry matter intake) of nutrient

= [nutrient]digesta * ([Marker]diet/[Marker]digesta)

where nutrient flow is the flow of nitrogen, amino acids, or homoarginine at the terminal ileum and [Marker]diet and [Marker]digesta are the concentrations of the appropriate indigestible marker (chromic oxide for days 5 and 6 and titanium oxide for day 7 observations) in the diet and digesta, respectively.

(iii) True lysine digestibility

= ([homoarginine]diet - homoarginine flow) * 100/[ homoarginine]diet

where [homoarginine]diet and homoarginine flow are the homoarginine concentration in the diet (mg/kg dry matter intake) and the flow of homoarginine at the terminal ileum, respectively.

(iv) Endogenous lysine

= lysine flow - ([lysine]diet * (1 - true lysine digestibility * 0.01))

where [lysine]diet is the concentration of lysine in the diet. The endogenous flow of amino acids other than lysine was calculated from the observed flow of endogenous

lysine and the amounts of other amino acids relative to lysine as reported by Boisen and Moughan (1996), except for proline and glycine, for which ratios from de Lange et al. (1989) were used.

(v) True digestibilities of amino acids other than lysine

= ([amino acid]diet - (amino acid flow - endogenous amino acid flow) ) *100/[ amino acid]diet

**Appendix 2.** Analyzed phytase activities for diets used in the second study

| Item | Control | Control + 2% phytic acid | Control + 2% phytic acid and phytase |
|---|---|---|---|
| Phytase activity, FTU/kg | 56 | 78 | 554 |

**Appendix 3.** Effect of phytic acid and phytase on concentration of minerals in stomach digesta of piglets used in the second study

| | Diet[1] | | | | Contrasts | |
|---|---|---|---|---|---|---|
| Item | Control | PA | Phytase | SEM | Control vs PA | PA vs phytase |
| Soluble mineral content, ppm | | | | | | |
| Calcium | 951.4 | 935.8 | 999.1 | 151.8 | 0.945 | 0.779 |
| Potassium | 622.9 | 595.1 | 621.2 | 87.5 | 0.830 | 0.840 |
| Magnesium | 152.7 | 97.7 | 124.5 | 30.9 | 0.188 | 0.392 |
| Sodium | 559.8 | 829.5 | 882.2 | 102.6 | 0.187 | 0.730 |
| Total mineral content, g/kg DM | | | | | | |
| Calcium | 15.8 | 16.3 | 13.8 | 1.16 | 0.807 | 0.179 |
| Potassium | 1.9 | 2.9 | 1.9 | 0.3 | 0.058 | 0.066 |
| Magnesium | 2.5 | 2.7 | 2.6 | 0.6 | 0.793 | 0.889 |
| Sodium | 36.3 | 47.6 | 55.2 | 7.5 | 0.318 | 0.517 |

[1]Control = a casein-cornstarch-based diet without phytic acid and phytase, PA = control plus 2% phytic acid, Phytase = control plus 2% phytic acid plus phytase at 500 FTU/kg.

**Appendix 4.** Protocol for chronic catheterization of pancreatic duct in piglets weighing 10 kg.

Pigs are starved overnight before undergoing surgery. Pigs receive an injection of Excenel (a broad spectrum antibiotic, 3 mg/kg body weight administered intramuscularly) 12 hours before surgery. Banamine (Flunixin) (a 24 hour lasting analgesia, 1 mg/kg) is administered at the start of surgery. Banamine (Flunixin) and Ketoprofen are provided via intramuscular injection. During the surgery pigs are held under general anesthesia using iso-flurane. The pig is held in a left lateral recumbency position and shaved on the right hand side around the last ribs and then thoroughly cleaned within hibitane. The area is sterilized with 70% alcohol and 1% iodine. The pig is covered with disposable drapes. Once a surgical plane has been attained, a 4 to 5-cm-long incision is made between the two most caudal ribs on the right side, the muscle layers are separated by blunt dissection and the peritoneum is cut parallel to the incision. The dorsal curvature of the duodenum is located and retracted and the head of the pancreas is indentified. The pancreatic duct is carefully be separated from the adjacent tissue, and at 2-5 mm from the orifice, a catheter (0.76 mm inner diameter and 1.65 mm outer diameter) is inserted 5-10 mm into the duct; the catheter has two cuffs glued 5 cm from the end of the catheter and 3 cm apart and a movable ring between the cuffs. The distal pancreatic duct is securely ligated, and a cuff applied to the catheter 10 mm from the end is tied loosely to the ligated distal pancreatic duct. A second ligature is tied tightly around the pancreatic duct and the inserted catheter, proximal to the entry point of the catheter. In the duodenum directly distal to the pancreatic duct, digesta is gently squeezed from a segment 3-4 cm long, which is then isolated using intestinal clamps. A stab incision is

made in the antimesentric border of the intestine using a no. 11 blade, taking care to avoid direct penetration of blood vessels. A catheter (with a suture retention bead glued in place at the site of intraluminal placement and a silicon cuff preplaced approximately 1 cm above the bead) is inserted into the lumen in the direction of peristalsis; the catheter has also two cuffs and a movable ring between the cuffs like pancreatic catheter and its tip has a burp valve to avoid blockage of the catheter with intestinal contents. A purse string suture using 3-0 PDS is placed in the serosa between the suture bead, which is in the lumen of the intestine, and the silicone cuff. The silicone cuff is tacked in place with 2-4 serosal sutures using 3-0 non-absorbable suture material. Both the pancreatic duct and duodenal catheters are exteriorized through the wound between the ribs. During the closing of the surgical wound, the rings on the catheters are fixed between the muscle layer and peritoneum to protect them from being pulled out, allowing them to be mobile during the movement of the intestines and tissue growth. Post-surgical care is same as that for ileal cannulated pigs except that the pancreatic catheter is checked several times (after every two hours) daily to ensure continuous flow of pancreatic juice.