Effects of phytase with or without multi-carbohydrase supplementation on growth performance, nutrient digestibility, and bone traits in nursery pigs.

By

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ABSTRACT

The aim was to determine the effects of supplementing increasing doses of phytase (**PHY**) alone or in combination with a multi-carbohydrase (**MC**) blend to a **P-**deficient diet of nursery pigs on growth performance, nutrient digestibility, and metacarpal bone characteristics. A total of 192 weaned pigs (7.7 ± 0.05 kg BW) were allotted to 1 of 8 dietary treatments each with 8 replicates in a randomised complete block experimental design. Pigs were fed P-deficient diets in a 4 × 2 factorial arrangement based on phytase level (0, 250, 500,1000 FTU/kg) and **MC** level (0 and 0.1g/kg). Fecal samples (d 18, 19, and 20) and metacarpal bones (d 21) were collected to determine apparent total tract digestibility **(ATTD)** of energy and nutrients and bone mineralization, respectively. Data were analyzed using the MIXED procedure of SAS utilizing orthogonal polynomial contrast statements with a pen as the experimental unit for growth performance and digestibility and pig as experimental unit for bone traits. By d 14, PHY with MC interactive effects significantly increase ($P < 0.05$) gain to feed ratio **(G:F)** whereas by d 21, increasing doses of PHY with or without MC addition linearly and quadratically ($P < 0.05$) increased average daily gain (**ADG**). Overall, PHY supplementation alone or in combination with MC quadratically increased ($P < 0.05$) ADG. However, there was an interaction ($P < 0.05$) whereby PHY and MC in combination acted synergistically to increase the ATTD of ash. Phytase had linear ($P < 0.05$) and quadratic ($P < 0.05$) effects by increasing fat-free dry weight, ash content and percentage, and metacarpal bone P content. In conclusion, MC alone did not exert much beneficial effect while increasing levels of PHY alone and when combined with MC quadratically improved growth performance, ATTD of ash and P, and bone traits of pigs fed P-deficient diets.

Key words: bone characteristics, digestibility, growth, multi-carbohydrase, phytase, pig.

DEDICATION

This thesis is dedicated to my lovely mother Franca Ariyibi; my wife Janet Adeshakin; my children Ayobami Theodore and Araoluwa Amanda Adeshakin, my late father, Chief Oluwole Adeshakin and my siblings Busayo Adeshakin and the Ariyibis (Femi, Bukola, and Remi).

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FOREWORD

This thesis was written in a manuscript format, and it is composed of one manuscript. The manuscript was prepared from data obtained from a study conducted to achieve the objectives of the thesis research. Most of the data from this manuscript has been presented at the ASAS-CSAS periodic meeting in July 2021. Authors to the submitted and presented data are **O. Adeshakin, B. Koo, R. Patterson, and C. M. Nyachoti.** C. M. Nyachoti, O. Adeshakin and B. Koo are from Department of Animal Science, University of Manitoba. R. Patterson is from CBS Bioplatforms Inc. Calgary. AB Canada.

O. Adeshakin is the first and presenting author. I helped in designing the model and framework for the study, undertook the research work, analyzed, and interpreted the data, and wrote the manuscript. **B. Koo** contributed immensely to planning the experiments and the processing of the experimental data. **R. Patterson** supplied the test ingredients through his company, performed the numerical calculations for the posology of the test ingredients (enzymes) and provided critical feedback that helped in the interpretation of results. **C. M. Nyachoti** is the senior and corresponding author. He made substantial contribution in the conception and design of the work, approval and implementation of protocols, interpretation of data and proof reading of manuscripts, and final approval of version to be presented or published. He supervised the findings of this project.

The thesis was written according to the guidelines for the Journal of Animal Science manuscript preparation.

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LIST OF ABBREVIATIONS

ADFI	Average daily feed intake
ADG	Average daily gain
ATTD	Apparent total tract digestibility
BW	Body weight
BWG	Body weight gain
CP	Crude protein
d	Day
DDGS	Distillers dried grains with solubles
DM	Dry matter
EE	Ether extracts
EPU	Endo-pentosanase units
Exp	Experiment
FTU	Phytase units
G:F	Gain to feed ratio
GE	Gross energy

MC	Multi-carbohydrase
NC	Negative control
NSP	Non-starch polysaccharides
PHY	Phytase
P	Phosphorus
SBM	Soybean meal
U/kg	Units per kilogram
Wk	Week
XU	Xylanase units

1.0 GENERAL INTRODUCTION

One of the most essential mineral elements in animal nutrition is phosphorus (P) because of its requirement in large amounts than several other mineral elements. Phosphorus play major roles in numerous cellular and metabolic processes (Cromwell, 2015) and also participates in several physiological activities such as bone mineralization, blood buffering, and energy utilization (Oster et al., 2016). However, the P in common ingredients used to produce swine diets is poorly available and cannot be utilized because it is bound by phytate which serves as it's storage form in these feedstuffs (Gourley et al., 2018) . Phytic acid (myo-inositol-1,2,3,4,5,6,-hexakiphosphate) is the basic form of P in seeds constituting up to 75% P contents in seeds (Almeida et al., 2017). Consequently, inorganic P is supplemented in diets to meet requirements while considering economic and environmental implications. Moreover, the non-starch polysaccharides (NSP) are components of plant feedstuffs that exerts their effects by forming an encapsulating cell wall structure around nutrients thereby making them unavailable for digestion and utilization (Slominski, 2011a). In addition, the gastrointestinal tract of weaned piglets is poorly developed and cannot produce adequate endogenous enzymes for the digestion of these complex compounds.

Phytate and non-starch polysaccharides constitute anti-nutritional compounds that can reduce nutrient utilization or food uptake, which inherently leads to impaired gastrointestinal functions and metabolic performance. In view of this, phytase, a phosphodyrolytic enzyme that catalyzes the step-by-step removal of phosphate groups through the hydrolysis of the covalent bond between phytate and P for the release of phosphorus is supplemented in diets while carbohydrases are included for the degradation of the nutrient encapsulating cell wall barrier, reduction of viscosity problems associated with certain NSPs, and ultimately increasing the accessibility of phytase to its substrate phytate. The use of exogenous enzymes (phytase and carbohydrases) is a nutritional strategy applied to improve productivity and performance, reduce supplementation of P below nutrient requirements thereby decreasing the potential for environmental pollution from animal manure and reducing the cost of feed production. The concept of specificity in the activities of these enzymes by the degradation of target substrates allows simultaneous supplementation which could result in sub-additive, additive, or synergistic (less, equal or greater than respectively) outcomes (Adeola & Cowieson, 2011). The objectives of this study were to determine the effects of phytase supplementation with or without multi-carbohydrase on growth performance, nutrient digestibility, and bone traits in nursery pigs and to establish that multi-carbohydrase addition would enhance the efficacy of phytase and therefore change the matrix specifications for phytase.

2.0 LITERATURE REVIEW

2.1 Phytate and non-starch polysaccharides.

Phytate and non-starch polysaccharides (NSP) are the prime antinutritional constituents in plant based feed ingredients (Olukosi et al., 2007). Enhancing the nutritive value of these ingredients in swine diets can be accomplished through the following processes; phytate hydrolysis to release P; eliminating the nutrient encapsulating cell wall effect which could lead to improved energy utilisation; improving the solubility of non-starch polysaccharides(NSP) in the cell wall which often results in increased hindgut fermentation and energy metabolism; degradation of the carbohydrate-protein linkages resulting in improved amino acid utilization; and the annihilation of the antinutritive properties of some dietary NSP by their enzymatic hydrolysis to prebiotic type constituents which could in turn enhance gut health and development in monogastric animal (pigs and chickens) (Slominski, 2011a)

2.2 Phytate and phytic acid

Plant feedstuffs (cereals and oil nuts) contain organic and inorganic forms of phosphorous (P) (Almeida et al., 2013). Phytate represents the main organic P in these feedstuffs, and it is stored in plant seed protein vacuoles (the aleurone cell layer or seed embryo). They account for 60-70% total phosphorous content in cereals, legumes, and oil nuts. (Nunes & Kumar, 2018). The cyclic derivative of alcohol from glucose that endears a structural backbone for phytate is *myo-Inositol.* Due to the low level of phytase present in seeds and the gastro-intestinal tract of monogastric animals (Mullaney & Ullah, 2003; Singh & Satyanarayana, 2011), this negatively charged phytic acid (PA) under acidic, basic and neutral pH conditions (Woyengo & Nyachoti, 2013) in diet form insoluble complexes with cations (Ca, Mg, Zn, Cu, and Fe), proteins and digestive enzymes resulting in low bioavailability of minerals; decreased protein solubility, enzyme activity, proteolytic digestibility, and the deprivation of energy utilization (from lipid sources). Phytic acid also cause increased endogenous secretion of nutrients due to a negative feedback mechanism (Woyengo & Nyachoti, 2013). An eutrophic ecosystem usually develops resulting from the excretion of unutilized P from livestock production systems into the soil and water (Singh & Satyanarayana, 2015., Nunes and Kumar, 2018).

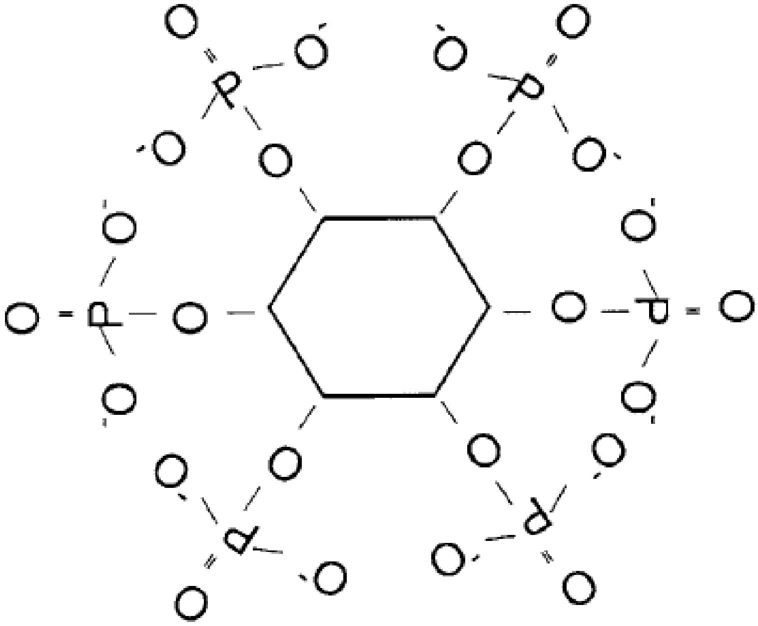

Figure 1: Structure of phytic acid (Cromwell, 2015).

2.3 Non-starch polysaccharides (NSPs)

Non-starch polysaccharides (NSP) are anti-nutritional factors present in large quantities in many plant-based feeds ingredients especially cereal grains often used in the production of swine diets. NSP are poorly utilized by pigs because they lack the endogenous enzymes required for their degradation. However, they (NSP) are fermented and used by pig intestinal microbes (Jha & Berrocoso, 2015). Some major NSP in plant cell wall comprises of cellulose (linear β-glucan chains), hemicellulose or non-cellulosic polymers (arabinoxylans, mixed-linked β-glucans, mannans, galactans, and xyloglucan) and pectic polysaccharides (polygalacturonic acids) (Recharla et al., 2019). Studies have revealed that high concentration of NSP in swine diets poses deleterious effects on nutrient digestibility and absorption. The soluble non-starch polysaccharides increase the viscosity of digesta thereby altering intestinal transit which could invariably lead to changes in the physiology (functions and activities) and ecosystem (environment) of the gut (Choct et al., 2010) while insoluble NSP are associated with fecal-bulking ability (Nunes & Kumar, 2018).

Corn, wheat plus soybean (SBM) diets are widely used in North America and North Asia (Ao et al., 2020). Ji et al. (2008) has shown that corn, wheat and SBM contain 7 - 9%, 11% and 22% NSP, respectively. Moreover, corn consist of mostly insoluble arabinoxylans while wheat is composed of both insoluble and soluble arabinoxylans and insoluble β-glucans (Willamil et al., 2012). However , the major carbohydrate in corn is starch and more than 95% of this can be digested serving as a source of glucose in swine diet, but the complex NSP cannot be utilized (Olukosi et al., 2007). Previous researchers have demonstrated that heat processing could be used to eliminate the antinutritional factors in SBM, but 5.6% α-1,6-galactosides and α-galactomannans remained which cannot be digested by pigs (Ao et al., 2020).

As enzyme substrates, soluble non-starch polysaccharides (NSP) in carbohydrates have antinutritional effects and cannot be degraded by pigs because they cannot secrete NSP enzymes (Ao et al., 2020).

Table 1. Contents of carbohydrate (g/kg) and major NSP in commonly used feedstuffs

Ingredient	Carbohydrate	Total NSP	Major NSP
Corn	830	119	Cellulose, arabinoxylans
Wheat	814	78-129	Arabinoxylans, xyloglucan
Barley (hulled)	823	167	Mixed-linked B-glucan
Oat (hulled)	787	232	Mixed-linked B-glucan
Soybean meal	400	148	Galacturonans, arabinans, galactomannan
Rapeseed meal	454	171	(Arabino) B-1,4-galactan
Flaxseed meal	493	271	Pectic polymers, arabinoxylans

(Kiarie et al., 2013)

2.3.1 Antinutritive effects of cellulose and arabinoxylans

The main plant cell wall polysaccharide component is cellulose. It is earth's most abundant organic material and composed of hydrogen-microfibriltic glucose monomer in links of 1-4 beta chains (O'Neill et al., 2014) . Cellulose is insoluble whereas the other types of NSP are soluble or partly soluble. Insoluble NSP comprises the bulk of the total fiber in diets which fulfills the satiety of the animals and exerts some beneficial property by promoting evacuation of gut contents/defecation thereby maintaining normal gut health (Nunes and Kumar, 2018). However, the most common cereal pentosan is arabinoxylan, which makes up the majority of cell wall NSP in corn. It consists of a ß-1-4 xylan backbone – the monomers being xylose – to which arabinose monomers commonly bind (Saulnier et al., 2012). Soluble NSP slow down the diffusion of digestive enzymes and the absorption of nutrients which consequentially leads to antinutritive effects in monogastric animals (Kumar et al., 2012) and thereby undergo rapid fermentation in the intestine, act as a source of energy for anaerobic microbes thereby helping to propagate harmful pathogens such as Clostridium perfringens which causes various diseases in animals (Nunes and Kumar, 2018). Corn is composed of 2.0% cellulose, 0.1% and 5.1% soluble and insoluble arabinoxylan respectively (Choct, 1997). The levels of NSP in corn is approximately 9–10% with insoluble arabinoxylans comprising over 40% (Fang et al., 2007). Arabinoxylan is the major endosperm cell-wall polysaccharide in wheat and makes up about 2–5% of the grain weight. Almost two-thirds of wheat-flour arabinoxylan is water insoluble. Moreover, both the soluble and the insoluble components have high water-absorbing properties. and can absorb about ten times their weight of water (Bedford & Partridge, 2010)

2.3.2 Antinutritive effects of galacturonans, arabinans and galactomannans.

It was reviewed by Ao et al. (2010) and Liener. (1994) that the antinutritional components in soybeans (Table 1) can be eliminated during heat processing in its conversion into SBM-meal but α-galactomannans and α-1,6-galactosides still persist. SBM is composed of considerable amount of α-1,6-galactosides (raffinose, 1.0% and stachyose, 4.6%) and β-galactomannans (1.2%) that cannot be digested and utilized by pigs (Ao et al., 2020). These antinutritional factors can caused flatulence, diarrhoea and impaired nutrient digestibility (Dadalt et al., 2017).

Soyabean meal also contains a particular hemicellulose, galactomannan, which is composed of D-mannose segments attached in a chain by β-1-4 linkages, including D-galactose units attached as sidechains by α-1-6 linkages. A study revealed that β-mannan decreases gastric emptying and prevents the release of insulin, glucose-dependent insulinotropic peptide (**GLP**), and insulin-like growth factor (IGF-I) (Pettey et al., 2002).

Therefore, dietary exogenous enzymes (such as phytase and carbohydrases) supplementation to animal diets has been widely adopted as an efficient nutritional strategy to improve the bioavailability of nutrients by increasing digestibility through the elimination of these anti-nutritional factors (Velázquez-De Lucio et al., 2021).

2.4 Phytases

Phytases also referred to the phosphohydrolytic enzyme myo-inositol (1,2,3,4,5,6)-hexakisphosphate phosphohydrolase catalyse the removal of phosphate groups from phytic acid. This occurs by the hydrolysis of the orthophosphate groups from the inositol ring of phytic acid to produce inorganic P and the inositol ring (Nunes and Kumar, 2018). Phytases can be produced from several sources such as plants, animals, and microorganisms but that of microbial origin are the most promising for commercial and large scale production (Rao et al., 2009).

Phytase activity is expressed as FYT, FTU, PU and U with all having the same denotation and defined as the amount of enzyme that will liberate 1µmol of inorganic-P per minute from 0.0015mol/L sodium phytate at pH 5.5 and 37˚C and this is used as a benchmark measurement for assay conditions such as pH, temperature, duration, mineral content and agitation (Simons et al., 1990). Classification of phytases into four major types has been done based on catalytic function and structure (Mullaney & Ullah, 2003) and these include histidine acid phosphatases (HAPs), ß-propeller phytases (BPPs), purple acid phosphatases (PAPs) and cysteine phosphatases (Jain et al., 2016). They can also be grouped as acidic, neutral, or alkaline phosphatases based on the optimum pH of activity usually with ideal activity recorded at pH 5.0, 7.0 and 8.0 (Baruah et al., 2007).

Table 2. Different microbial sources of phytases and their properties.

Microbial Sources	pH	Temp(˚C)	Specific Activity (U/mg) at 37˚C
Aspergillus caespitosus	5.5	80	-
Aspergillus fumigatus	5.0 -6.0	60	23-28
Aspergillus niger	5.0-5.5	55-58	50-103
Aspergillus oryzae	5.5	50	11
Aspergillus terreus	5.0-5.5	70	142-196
Emericella nidulans	6.5		29-33
Spilosoma castellii	4.4	77	418
Cladosporium	3.5	40	909
Klebsiella pneumoniae	5.5, 5.5	50,60	224,297
Klebsiella aerogenes	4.5, 5.2	68	
Peniophora lycii	5.5	58	1080
Bacillus subtilis	6.5-7.5	55-60	9.0-15
Citrobacter braakii	4	50	3457
Escherichia coli	4.5	55-60	811-1800
Klebsiella terrigena	5	58	205
Pantoea agglomerans	4.5	60	23
Pseudomonas syringae	5.5	40	769
Candida krusei	4.6	40	1210
Pichia anomala	4	60	-

(Bohn et al., 2008; Nunes & Kumar, 2018; Greiner & Konietzny, 2006)

2.4.1 Sources of phytases

Generally, there are four wellsprings of phytases which include- microbial, plant, phytase produced by the intestinal mucosa and gut-related micro floral derived phytases. Microorganisms (Table 1.1) and plants are the largest sources of phytases and to a lesser extent animals (Nunes & Kumar, 2018). Beforehand, phytases of microbial origin such as *A. niger*, *Neurospora crassa*, and Pseudomonas phytase were generally regarded to belong to the 3-phytases, while 6-phytases such as wheat bran phytase were thought to be restricted mainly to plant sources. The initial exclusion of the phytases made from *Paramecium, E. coli* and *A. oryzae* as 6-phytases has been reported (Almeida et al., 2013). Based on source and the expression of host, commercially produced phytases show variable efficacious characteristics resulting from their unique physical and biochemical properties (Almeida et al., 2013).

Microbial sources (bacteria, yeasts and fungi) in Table 2 are the most encouraging candidates for producing phytases on a commercial scale because of the cost-effective process of large scope manufacturing (Rao et al., 2009). Bacteria and fungi comprise the most important sources of phytase. The most common strains for commercial production of phytases among yeasts are *A. niger, Aspergillus ficuum, A. fumigatus,* and *S. cerevisiae*.

Dietary phytate hydrolysis by extrinsic microbial phytase was investigated first by Nelson et al. (1971). Isolation and characterization of phytases has been done from plant origins- wheat, corn, soybean, rice, rapeseed, and rye. Most plant phytases begin the hydrolysis of phytate at the C6 position of the myo-inositol hexaphosphate ring and thus considered 6 phytases but the soybeans phytase initiates hydrolysis at C3 position (Phillippy & Bland, 1988). Processing (steam-pelleting) during commercial production of feeds result in the loss of intrinsic phytase properties of some feed ingredients (Nunes and Kumar, 2018). The lower efficiency of plant phytases in comparison with their microbial counterparts is due to their narrower pH range of activity. Plant originated phytases have pH ranging from 5.0-6.0 whereby *A. niger, Escherichia coli* and *Peniphora lycii* – derived, also among commonly used microbial phytases have pH ranges of 2.0 to 5.0. (Woyengo et al., 2008)

The jejunum is the major site for intestinal mucosal phytase activity in pigs (Nunes and Kumar, 2018). When fed phosphorus deficient diets, several animals, and humans inclusive, were able to elevate intestinal phytase and phosphate activities (Zhang et al., 2005).

Genetically modified plant and microbial phytases' effects on health have been investigated using pigs and poultry when administered up to 20-fold higher doses than the recommended levels

(500U/kg), when followed by generalized necropsy and histological examination of the kidney, liver, and tibia, all these revealed no adverse effects. Consequently, performance, phosphorus availability and bone mineralization were elevated (Nunes and Kumar, 2018)

2.4.2 Sites of hydrolysis

The International Union of Pure and Applied Chemistry and International Union of Biochemistry and Molecular Biology (IUPAC-IUBMB) has proposed three classes of the enzyme based on the position of phytate dephosphorylation on the inositol ring and they include 3-phytase (EC 3.1.3.8 or myo-inositol hexakisphosphate 3-phosphohydrolase), 5-phytase (EC 3.1.3.72 or myo-inositol hexakisphosphate 5-phosphohydrolase) and 6-phytase (EC 3.1.3.26 or myo-inositol hexakisphosphate 6-phosphohydrolase) and they liberate P moiety at sites C3, C5 and C6, respectively, (Lei & Porres, 2003).

2.4.3 Factors affecting microbial phytase efficacy

Factors that influence the efficacy of phytase activities in vivo can be broadly divided into phytase, animal, and dietary related. Phytase influencing factors include type of phytases, expression organism, optimal pH, temperature, and resistance to the protease endogenous enzyme. Animal related factors comprises species, age of the animal and retention time while phytate content, calcium levels and ingredient composition (such as substrate type and intrinsic phytase activity) are the dietary related factors (Dersjant-Li et al., 2015a).

2.4.4 Phytase factors

For Bacterial or fungal phytases, some literature sources have proven that *E coli* phytases show more efficiency than fungal phytases (Dersjant-Li et al., 2015a) because most fungal phytases exhibit optimal activity at acidic pH and are sensitive to proteolytic enzymes but bacterial phytases posses several different biological properties such as phytate specificity, activity in neutral to alkaline pH, resistance to proteolysis and high catalytic efficiency (Jain et al., 2016). Under neutral to alkaline pH conditions, most bacterial phytases are optimally active (Singh & Satyanarayana, 2011).The optimal pH range of phytase will determine its effectiveness in the stomach or upper part of the intestine because the pH in the stomach is predominantly lower than 5.5 and it is used for the standardized measurement of phytase activity (Dersjant-Li et al., 2015a). Phytase from bacterial origin are optimally active in the temperature range of 25-70˚C because thermostability (Table 2) is very important to their utilization in animal feed production, especially during feed pelleting involving treatments of up to 80-85˚C for few seconds (Rao et al., 2009).

Phytases can also be divided into 3- and 6- phytases (Table 3) depending on the carbon site of hydrolysis of phytic acid (3 vs 6 position). The study conducted by Dersjant-Li et al., 2015 concluded that the position of first hydrolyzation has a considerable impact on protein and mineral binding. The results revealed that binding capacity decreased rapidly from inositol hexakiphosphate 6 (IP_6) to IP_3. $IP_{1–4}$ have very low protein binding capacities. An intermediate product of *E. coli* 6-phytase (IP5 (1,2, 3, 4, 5)) exhibited significantly lower protein aggregation than the 2 IP_5 isomers (IP_5 [1, 3, 4, 5, 6] and IP_5 [1, 2, 4, 5, 6]) generated by *A.niger* 3-phytase. It was discovered that the binding capacity to Fe^{3+} reduced proportionately from IP_6 to IP_3. To maximally alleviate pepsin inhibition, IP_6 must be broken down to IP_{1-2}. In addition, IP3 posses approximately 11% the binding affinity of IP_6. Consequently, the removal of IP_6 and IP_5 from the stomach will largely decrease the binding of Ca in the small intestine (Yu et al., 2012).

Morales et al. (2011) mentioned that the negative effect of IP6 on binding of proteins occurs mainly in acidic environment like the stomach. Thus, the early and critical hydrolysis of phytic acid by phytase in the digestive tract is vital for enhanced metabolism of phosphorus, Ca, protein, and other minerals. However, these revelations prescribe that phytate degradation by phytase in the upper part of the gut is a prerequisite for the annihilation of the negative effects of IP_6 (Dersjant-Li et al., 2015a).

Because bacterial phytases are very low in the wild strains, their genes have been cloned and expressed in homologous and heterologous hosts producing recombinant phytases. These recombinant enzymes display maximum activity at varied conditions in terms of thermostability (activity at higher temperature), wider pH range and resistance to proteolytic endogenous enzyme while still retaining a greater percentage of its original activity (Jain et al., 2016) However, in a particular study, there were variabilities between different *E. coli* phytase products due to different expression organisms used. *E. coli* expressed in *S. pombe* has higher activity than *E. coli* expressed in *P. pastoris* when using IP6 soy protein as substrate measured at pH 3 (Dersjant-Li et al., 2015a)

2.4.5 Animal factors.

These factors include species, age of animals site for phytase activity and endogenous phytase activity. Without microbial phytase inclusion in pig diets, the major phytase activity was seen in the colon (Dersjant-Li et al., 2015a) which is similar to a Nunes & Kumar, (2018) report that gut microfloral phytases are basically present in the large intestine. The main active site for supplemented microbial phytase activity is the stomach (highest in stomach digesta) because of more favourable pH and upper part of the small intestine (upper jejunum in piglets). The high endogenous phytase activity in the colon may be responsible for the low supplemental phytase activity (Dersjant-Li et al., 2015a).

In pigs, the predominant sites for phytase activities are the stomach and upper part of the small intestine while in poultry, the effects of phytase are demonstrated in the crop and upper part of the gastrointestinal tract. However, with the crop and proventriculus having a pH ranging from 5.2 to 5.8 and 2.8, respectively, phytase activity was anticipated to be higher, but on the contrary a decline was observed along the small intestine without any detectable activity in the ileum of broilers when exogenous phytase (*P lycii*) was supplemented. This diminishing activity in the lower part of the small intestine may be due to the activity of endogenous digestive protease which is able to break down exogenous phytase (Dersjant-Li et al., 2015a). It has been reported that increasing phytase supplementation in corn-SBM based-diets in broilers resulted in significant improvement in total tract phytate degradation within the range of 40.3 to 94.8%. Inclusion doses to a maximum of 12,000 FTU/kg led to increases in bird performance, nutrient retention, tibia ash and AME (Ravindran, 2013).

Age as a potential animal factor on the effect of phytase activity has been reported. Olukosi et al. (2007) documented that phytase supplementation improved growth performance in 10-kg pigs while there was no effect of the enzyme in 23-kg pigs both fed corn-SBM diets marginally deficient in total and non-phytate P. They attributed this to the high ability for phytate degradation possessed by the 23kg-pigs (older pigs) caused by increased jejunal alkaline phosphatase activity (Butler et al., 1985), which increases as pigs grow older (Adeola & King, 2006), However , a meta-analysis of the effect of phytase supplementation on P digestibility conducted by Rosenfelder-Kuon et al. (2020) showed similarity between weaners and growers but P digestibility was lower in finishers. It has been documented that weaned pigs have poorly developed digestive tract and immune system (Heo et al., 2013), and may therefore benefit more from the dietary supplementation of exogenous enzymes including phytase than growing-finishing pigs (Park et al., 2020). Similarly, Lan et al. (2017) have reported that the effect of dietary enzyme inclusion on nutrient digestibility decreases with increasing age especially because of enhanced capacity for digestion, development of the gut and improved microbial population as the animal ages.

2.4.6 Dietary factors

Dietary factors that influence phytase efficacy comprises phytate content, substrate type, intrinsic phytase activity, total P levels and Ca: P ratio (Dersjant-Li et al., 2015a). The rate of hydrolysis of phytate by phytase can be attributed to composition, level, and location of phytate (IP_6), and the impact of intrinsic phytase in different plant-based feed ingredients. Most phytate in corn can be found within the germ while that of sorghum and wheat is trapped within the aleurone layers. So it is probable that there is easier access by exogenous enzymes to the phytate in corn than the phytate located in fibrous matrix of wheat and sorghum (Almeida et al., 2017; Dersjant-Li et al,. 2015a). Morales et al. (2013) reported that the rate of protein solubility after phytase treatment is directly proportional to the protein fractions present. SBM contains storage proteins glycinin and conglycinin which have antigenic properties that can cause allergic responses and possibly resulting in intestinal damage in weaning piglets (Giesting et al., 2018). It can be presumed that enhanced protein digestibility of these soy proteins and decreased antigenic effect could result from increased solubility of glycinin and conglycinin (Dersjant-Li et al., 2015a).

Accurate dietary Ca: P concentrations is critical for performance in nursey pigs. Research studies have reliably proven that feeding excess dietary Ca impairs P absorption culminating in reduced growth performance and bone calcification in pigs (González-Vega et al., 2016). In a study by Wu et al. (2018) where nursery pigs were fed corn-SBM diets with various combination of Ca and P provided by inorganic sources or phytase, it was concluded that excess dietary Ca to P ratio negatively affected growth performance and percentage bone ash compared to when diets are deficient in STTD P. This occurs because free Ca binds with phytate-P in chyme forming insoluble salts leading to reduced digestion and absorption of dietary P. In this investigation, total Ca:P ratios ranging from 0.8:1 to 1.6:1 were fed without decrease in growth performance but reduction in performance was seen when total Ca:total P ratio exceeded 1.9:1. Although Qian et al. (1996) had shown improved growth performance in 9 to 23 kg pigs when total Ca:total P ratio was narrowed from 2.0:1 to 1.2:1 irrespective of dietary P concentration (0.36% or 0.45% total P). Also a linear reduction in ATTD of P from 56.9% to 46.2% when dietary Ca increased from 0.33% to 1.04% in growing pigs fed corn, corn starch, protein isolate and soya bean oil diets was reported by Stein et al. (2011).

Table 3. Examples of some available 3- and 6- phytases and their characteristics

Type	Protein origin	Expression	pH Optima	Temperature optima ˚C
3	*A, niger*	*A. niger*	2;5-5.5	65
3	*A. niger*	*A. niger, non-recombinant*	6.0	-
3	*A. niger*	*Trichoderma reesei*	2.5	-
6	*Escherichia coli*	*Schizosaccharomyces pombe* (ATCC5233*)*	4.5	55
6	*Escherichia coli*	*Pichia pastoris*	4.5	-
6	*Escherichia coli*	*Trichoderma reesei*	-	-
6	*Escherichia coli*	*Pichia pastoris*	3.3, 5.0	58
6	*Peniophora lycii*	*Aspergillus oryzae*	4-5, 5	50-55
6	*Buttiauxella spp*	*Trichoderma reesei*	3.5-4.5	60

(Dersjant-Li et al., 2015a)

2.5 Carbohydrases

Carbohydrases are enzymes that aid the degradation and digestion of carbohydrates and fibers especially non-starch polysaccharides (NSPs). Feed enzymes supplementation to weaning pig diets, is done because of poor nutrient utilization with respect to their immature digestive system and their inability to produce adequate endogenous enzymes (Ao et al., 2010). The effects of carbohydrase on growth performance and nutrient digestibility may be influenced by enzyme preparations and or physiological status of the animal and feed ingredient (Ao et al., 2020). Lu et al. (2016) has suggested that complex carbohydrase preparations are required to degrade NSP and enhance nutrient digestibility and utilization. In view of this, the type of enzyme supplemented in diets should be specific for the fiber present in the diet (Cromwell, 2009). However, the complexity of potential substrates has led a whole body literature advocating for the use of multienzyme preparations (O'Neill et al., 2014). Some examples of carbohydrases are amylase, cellulase, glucanase, invertase, mannanase, protease and xylanase.

2.5.1 Amylase

The degradation of cereal starch to dextrin and sugars is achieved by the application of the amylase enzyme. The two categories of amylases are endo and exo-amylases. The endoamylases catalyze hydrolysis of starch in the interior of the starch molecule resulting in the production of linear and branched oligosaccharides of different chain lengths. Exoamylases mode of action is the degradation of starch from the non-reducing portion leading to short end products (Bedford & Partridge, 2010). Park et al. (2020) reported that increasing levels of amylase at 150 U and 1,500 U supplemented with a multi-enzyme complex (containing 4,000 U of xylanase, 150 U of glucanase, 3,500 U of protease per kilogram of diet) and 1,000 FTU/kg of diet of phytase in nursery pigs fed corn-SBM-barley-based diets increased ADG from 262g to 290g and from 262g to 313g, respectively. Amylase supplemented at 150 U and 1,500 U also improved ATTD of fat from 30% to 36% and 37%, respectively. This improvement in growth performance is dose dependent and could be due to increased digestibility of starch in the small intestine resulting in efficient utilization of energy derived from starch.

2.5.2 Cellulase

Cellulases degrades cellulose to low molecular weight products and glucose to increase energy availability. The most employed microorganism for cellulase production is *Trichoderma reesi* (Nunes & Kumar ("Eds."), 2018). Emiola et al. (2009) reported that the supplementation of wheat DDGS based diet with a multi-carbohydrase enzyme comprising of cellulase, xylanase and glucanase activities improved growth performance and ATTD of nutrients (DM, N, GE and EE). Upadhaya et al. (2016) has shown that supplementation of increasing levels (0.05 and 0.10%) of cellulase to a corn based SBM diet fed to lactating sows had no effect on body weight or feed efficiency but improved the total tract digestibility of DM and N leading to better milk yield which ultimately increased the ADG of piglets.

2.5.3 Glucanase

Glucanase, especially the β-glucanase are enzymes that hydrolyses β-glucans to oligosaccharides and glucose to enhance feed utilization. One unit of β-glucanase is defined as the amount of enzyme that liberates 1 mg of total reducing sugar per 10 min. from 0.4% β-glucan at 30°C and pH 4.0.(Shim et al., 2004). Duarte et al. (2021) reported an improved nutrient digestibility (AID of crude protein and ATTD of gross energy) and growth performance (ADG) in nursery pigs fed corn-SBM diet with 30% corn distiller’s dried grains with solubles containing increasing levels of β-glucanase (0, 200, 400, and 600 U/kg) and xylanase (1,500 endo-pentosanase units [EPU/kg] feed). The improvement in nutrient digestibility and performance could be due to the hydrolysis of the β-glucan and xylan polymers in the diet thereby reducing their ability to increase digesta viscosity resulting in increased microbial fermentation.

2.5.4 Invertase

Invertase breaks down sucrose into glucose and fructose. It is produced from *Saccharomyces cerevisiae* and can be used in a multi-enzyme formula. It increases the digestion of starch, sugar, and other carbohydrates. Kpogo et al. (2021) observed minimal enzyme effect on performance and nutrient digestibility when invertase (150 U) and other carbohydrases (4,200 U of amylase, 1,900 U of cellulase, 300 units of glucanase and, 1,000 units of xylanase) were supplemented in a wheat-barley-SBM based diet where wheat millrun was included at the expense of wheat and fed to growing pigs. Multi-carbohydrase addition reduced G:F by d14 and did not affect BW, ADG or ADFI. Enzyme supplementation also decreased the ATTD of DM and energy and without any effect on the ATTD of N and P.

2.5.5 Mannanase

A specific hemicellulose present in the cell wall membrane of leguminous seed including soybeans is the galactomannan. This NSP has been described to reduce nutrient utilization and performance in pigs (Pettey et al., 2002) due to the lack of endogenous enzyme required for the degradation of the α-1,6-galactosyl and β-1-4-mannosyl bonds (Jang et al., 2020). However, the dietary inclusion of β-mannanase preparation (0 and 0,05%) to a corn-SBM diets in weanling and growing-finishing pigs enhanced feed efficiency, growth, and carcass performance without improvements in nutrient digestibility. *β*-mannanase could have enhanced improvements in growth performance not only via the hydrolysis of *β*-mannans leading to provision of additional energy, but possibly through an indirect response of regulatory hormones such as the IGF-I. (Pettey et al., 2002). On the other hand, Jang et al. (2020) observed no effects on growth performance but increased fat digestibility by the supplementation of β-mannanase in a corn-SBM based diets fed to weaning pigs. The lack of improvement in performance may be due to higher content of insoluble NSP and β-mannan in the diet (10.7% and 0.6%, respectively) in comparison with the diets of Pettey et al. (2002) and Kim et al. (2003) where there were increased growth performance.

2.5.6 Protease

Proteases are enzymes that digest proteins to peptides and amino acids (Nunes & Kumar ("Eds"), 2018) to increase protein digestibility and lower nitrogen excretion (Bedford & Partridge, 2010). Duarte et al. (2019) observed that protease supplementation (0 or 300,000 U/kg). individually improved feed efficiency and gut morphology in nursery pigs fed corn-SBM-corn-DDGS diet. Torres-Pitarch et al. (2018) reported that protease supplementation (75,000 protease units/g) to rapeseed meal and wheat-DDGS based-diet fed to growing-finishing pigs increased feed efficiency and ADG. These results indicate that supplementations with a combination of a protease, other carbohydrases, and dietary phytase can improve the use of several common ingredients in swine diets to sustain growth performance, carcass quality, and nutrient digestibility.

2.5.7 Xylanase

Xylanases are the most important type of carbohydrase used in wheat-based diets because they catalyze the hydrolysis of arabinoxylans to low molecular weight products and sugars thereby improving feed utilization. Arabinoxylans comprises 50–60% of the total NSPs in this viscous grain (wheat). (Bedford & Partridge, 2010). Xylanases are specific for the internal β-1,4 linkages. One unit of xylanase is defined as the amount of enzyme that liberates 1mg total reducing sugar/10 min. from 0.5% xylan at 30°C and pH 4.0 (Shim et al ., 2004). Furthermore, Passos et al. (2015) reported that increasing xylanase supplementation from 0 to 1400 XU/kg in a corn-SBM based-diet enhanced ileal digestibility of DM, OM, energy, NDF, and crude ash by 9.2, 8.5, 9.3, 12.4, and 10.7%, respectively However, Lan et al. (2017) reported that dietary supplementation of xylanase to a corn-SBM diet-based fed to weaned pigs improved growth performance (ADG and G:F) due to the increase in nutrient digestibility (ATTD OF DM, N and GE). Duarte et al. (2019) has shown that the dietary supplementation of xylanase (45,000 XU/kg of diet) in nursery pigs fed corn-SBM based-diets improved growth performance and gut morphology, decreased the viscosity of digesta, and all these outcomes are accomplished by the degradation of the cell wall component of NSP.

2.6. Phytases as additives in the pig feed industry

Because the activity of phytase is largely pH dependent, *A niger, E coli,* and *Saccharomyces cerevisiae* phytases all have a range between 2.0 and 5.8 and this is generally the optimal pH range of phytase activity (Yi & Kornegay, 1996). This is important because the dissociation of phytate to phytic acid, which increases its solubility and reduces the inhibitory interactions of related minerals to phytase, is promoted by low pH (Maenz et al., 1999). The predominant site of phytic acid hydrolysis is the stomach (Dersjant-Li et al., 2015a; Yi & Kornegay, 1996). In view of this, the perfect phytase for the porcine feed industry must be resistant to the acidic nature and proteolytic enzymes in the stomach and small intestines which are the sites for P absorption; must be economical to produce (cost-effective with large scale production); and be able to withstand high temperatures (65 to 80˚C), which is a requirement during steam pelleting (Woyengo et al., 2008). Consequent upon these, the factors that influence the efficacy of phytases include pH, duration/temperature, presence of P and Ca, and interactions with other enzymes (Dersjant-Li et al., 2015a; Naves et al., 2012; Newkirk & Classen, 1998).

2.7 Dietary phytase inclusion effects

The hydrolysis of phytic acid (PA) to improve growth performance by increasing nutrient digestibility and availability including optimizing bone mineralisation is anticipated by the dietary supplementation of phytase. The primary purpose for supplementing swine diets with phytases is to facilitate the breakdown of phytate molecules to release phosphorus, increase amino acid digestibility and energy efficiency (Dadalt et al., 2017). A recommendation in the range of 500-2000FTU/kg addition of phytase to diet to replace P supplementations has been suggested (Dersjant-Li et al., 2015a).

2.7.1 Phytase effects on growth performance

She et al. (2017) have shown the effects of adding increasing levels of phytase (250, 500 1000 and 2500 FTU/kg of feed) to P-deficient corn-SBM diets fed to weanling pigs which led to ADG, and body weight increases linearly and quadratically from day 0 to 6 and ADG, ADFI and G:F increased linearly for the overall experimental period (Day 0 to 27). However, there were no differences in final body weight (FBW), ADG, ADFI or G:F between pigs fed diets with 1000 and 2500 FTU/kg diets supplemental phytase.

Previous studies have also shown that supplementation of increasing doses of phytase from 500 – 1000 FTU/kg of diet individually in nursery pigs fed corn SBM diets deficient in P resulted in increased ADG and G:F ratio (Florh et al., 2016; Dersjant-Li et al., 2017; Gourley et al., 2018). A report by Gourley et al. (2018) also indicated a linear increase in ADFI in 21-d pigs fed diets with different levels of phytase inclusion.

Florh et al. (2016) and De Cuyper et al. (2020) recorded an increase in final body weight with phytase supplementation. An improvement in these performance parameters evaluated is due to an increase in the availability of phytate-P from phytic acid hydrolysis and a reduction in the inclusion of dietary inorganic P as a result of phytase addition (Bento et al., 2012) .

2.7.2. Phytase effects on nutrient digestibility and availability.

Dry matter digestibility is associated with the disappearance of all dietary components from the gastrointestinal system. (Acosta and Patience., 2019). The effects of phytase inclusion on nitrogen and energy digestibility have also been reported. In weanling pigs fed low P diets of corn-SBM, there were linear and quadratic increases (g or Mcal/d) in absorption, excretion of nitrogen (N) and gross energy (GE) with increasing concentration of *E coli* phytase supplementation at 2500 or 12500 FTU/kg. When Dicalcium phosphate was added with phytase supplementation, pigs had increased intake, absorption and excretion of N and GE (Veum et al., 2006). Zeng et al. (2014) has shown that there was a quadratic increase in total tract digestibility of nitrogen in weanling pigs fed a corn-SBM diet with low P supplemented with increasing concentration of *E coli* phytase supplementation. Dersjant-Li & Dusel. (2019) indicated that increasing the dose of dietary phytase (0, 2000 FTU/kg) added to a wheat, corn, barley, rapeseed and SBM diet deficient in energy and Ca without the inclusion of inorganic P increased the ATTD of N and energy in a dose dependent manner.

Adequate dietary Ca and P concentrations are important for performance of nursery pigs (Wu et al., 2018). She et al. (2017) affirmed a linear and quadratic increase in ATTD of P due to an inclusion of an *E coli* phytase in a low P corn-SBM diet fed to weanling pigs.

Almeida & Stein. (2010) discovered with microbial phytase supplementation an improvement in P retention in a corn-SBM diet fed to pigs from 56.03 to 71.48%, and the reduction of P concentration in feces from 1.98% to 1.15% in pigs fed corn and from 2.84% to 1.84% in those fed soybean meal (SBM). There was also a decrease in the daily fecal P output from 0.97 to 0.52g for corn and from 0.81 to 0.48 for SBM. Phytase supplementation at 500 FTU/kg also increased the standardized total tract digestible (STTD) P in corn and SBM from 26.4 to 64.4% and 48.3 to 74. 9% respectively, and the apparent total tract digestibility (ATTD) of P from 19.9 to 57.8% for corn and from 41.5 to 68.45% for SBM, which indicates increased release of P in the stomach and intestine through the degradation of phytate.

Almeida et al. (2013) similarly reported increased ATTD of P by 61.6%, 65.1%, 68.7% and 68.0% when *Aspergillus oryzae* phytase was supplemented to a corn SBM diet (without dicalcium phosphate) at 500, 1,000, 2,000 and 4,000 FTU/kg dose rates, respectively. The absorption of P also increased to 3.0, 3.3, 3.5, and 3.7 g/d, respectively. These shows the positive effects of phytase in pigs fed low P diets or without supplemental P.

The inclusion of microbial phytase at 470 FTU/kg to a basal diet of corn soy oil increased the amount of P intake, percentage absorbed and ATTD of P. The amount of P output and percentage in feces also decreased (Gonzalez-Vega et al., 2015).

In an experiment executed by Zeng et al. (2014) using low P corn-SBM diets in weaning pigs, dietary supplementation of *E coli* phytase resulted in linear increases in apparent ileal digestibility (AID) of Ca and total P. Ca and P digestibility, retention and utilization linearly increased with increasing dosage of phytase. Result obtained from Guggenbuhl et al. (2012) with a phytate rich corn-SBM without inorganic P supplemented with phytase fed to 60-day old ileorectal anastomosed pigs caused increased AID of P and Ca.

Woyengo et al. (2012) documented that the supplementation of phytase to an hulless low phytate barley-based diet fed to growing pig resulted in increased ATTD of nitrogen, P, and potassium. Dietary phytase supplementation improved the nutrient digestibility in pigs as a consequence of phytate hydrolysis.

Moreso, Kim et al. (2018) reported that the supplementation of phytase at 500 FTU/kg in a corn-SBM- hemp hull based-diet fed to growing pigs increased the ATTD and STTD of P in diets and hemp hulls test ingredient and decreased fecal P output from pigs. This could be due to the release of the phytate bound P by phytase. Similarly, Kim et al. (2017) documented that dietary phytase supplementation enhanced both ATTD and STTD of P and reduced fecal P excretion from pigs fed diets containing flaxseed meal. Lee et al. (2021) reported that dietary phytase inclusion at 500 FTU/kg to a corn-SBM based-diet containing 300g/kg high protein sunflower meal fed to growing barrows tended to increase the values of coefficient of apparent and standardized total tract digestibility of phosphorus in the high-protein sunflower meal from 0.18 and 0.19 to 0.37 and 0.38, respectively. Song et al.. (2022) has also documented that the supplementation of microbial phytase at 500 and 2,500 FTU/kg to a hybrid rye-sucrose based-diet fed to growing pigs improved the STTD of P in hybrid rye by 16.3% and 24.1%, respectively. These improvements in nutrient digestibilities by phytase can be attributed to the degradation of phytate thereby increasing the liberation of P, other minerals, and the utilization of other nutrients.

2.7.3. Phytase effects on non-phosphorus minerals availability

Stahl et al. (1999) reported that pigs fed a Fe deficient corn-SBM diet had lower hemoglobin (Hb) and parked cell volume (PCV) levels in blood than those fed Fe-supplemented diets but when fed the same basal diet supplemented with 1200 U of phytase/kg of feed, there was a better improvement than pigs fed Fe-supplemented diets without phytase. Consequently, dietary phytase supplementation of corn-SBM is effective in improving bioavailability of Fe and replenishing Hb in young anemic pigs. The ATTD of Ca increased from 69.65% to 80.42% when phytase was added to a corn SBM diet fed to growing pigs (Almeida & Stein, 2010).

Supplementation of low P corn soybean diet with phytase in weaned pigs caused linear increases in digestibility, retention, and utilization of Mg; the digestibility of Na and K; the digestibility and retention of Zn. There was also a quadratic increase in the digestibility, retention and utilization of Cu with increasing dose of *E coli* phytase (Zeng et al., 2014). The higher apparent total tract digestibility of cations such as Mg and Na can be attributed to the hydrolysis of phytic acid that forms insoluble complexes with these cations in the small intestine by phytase (Woyengo et al., 2012).

2.7.4. Effects of phytase on gut microbiota.

Phosphorus and Ca are important mineral nutrients involved in bacteria cell wall metabolism by being components of nucleotides, co-factors, teichoic acids and phospholipids (Heyer et al., 2015). Their availability alters cellulolytic activities (Metzler & Mosenthin, 2008) and bacterial composition along the gastrointestinal tract (Metzler-Zebeli et al., 2010, 2013) resulting in the modification of intestinal microbiota (Heyer et al., 2015; Mann et al., 2014). In a study conducted by (Metzler-Zebeli et al. (2010) in growing pigs fed corn SBM meal with ileal pectin infusion, phytase addition increased the ileal numbers of strictly anaerobic bacteria, such as the *C. coccoides* and the *C. leptum* clusters, the *Bacteroides-Prevotella-Porphyrmonas* group, with enhancement of those of *Enterobacteriaceae*. These outcomes might be connected with the increased availability of phytate–bound P in the small intestine (Metzler et al., 2008). The supplementation of monocalcium calcium phosphate (MCP) reduced mainly gram-positive bacteria such as lactobacilli, enterococci, and members of the *C leptum* cluster. Ca from MCP rather than P may act as a growth-inhibiting factor for peculiar bacteria whereby increasing Ca^+

ions may reduce their adhesion potentially culminating in decreased colonization of intestinal mucosal areas due to competition for binding sites with other bacteria as demonstrated by Larsen et al. (2007). Dietary phytase supplementation to corn-wheat-SBM based-diets resulted in dramatic alterations in prevalent families belonging to *Firmicutes* (Klinsoda et al., 2019).

2.7.5 Phytase effects on bone traits

One of the most efficient evaluation of P and Ca concentrations utilized and deposited from dietary sources are bone parameters. This is because bones are very sensitive to increasing proportions of these mineral elements (Walker et al., 1993). Phosphorus deposition in bones is at a relatively constant concentration and the total amount of bone ash increase with elevation in the quantities of available Ca and P (Petersen et al., 2011). She et al. (2017**)** observed an increase in fat-free dried bone weight, metacarpal bone ash weight, bone ash concentration, bone P and Ca in g in weanling pigs fed a P-deficient corn soya bean meal diet supplemented with graded doses of *E coli* phytase (250, 500, 1000, and 2500 FTU phytase/kg feed). Similarly, Zeng et al. (2014) discovered linear increases in metacarpal breaking strength, fat-free bone weight and ash weight with increasing dietary concentration of *E coli* phytase in weaned pigs fed a low P corn-SBM diet.

2.8 Effects of phytase and multi-carbohydrase supplementation

The impact of combining phytase and multi-carbohydrase on digestibility, bone mineralisation and performance has been documented in many studies as reviewed below

2.8.1 Effects of phytase with multi-carbohydrase supplementation on performance.

It has been documented that the simultaneous supplementation of phytase and multi-carbohydrase complex (xylanase, β-glucanase, and α-galactosidase) at recommended level of 0.1% in a corn SBM meal resulted in an improvement of G:F ratio (Shim et al., 2004). Lu et al. (2016)

reported that a combination of dietary metabolizable energy (ME), N, P, Ca reductions in a corn, SBM, and canola meal diet with added phytase *(E coli* expressed in *Schizosaccharomyces pombe*) and multi-carbohydrase mix (xylanase and β- glucanase) optimized production efficiency in nursery pigs by improving body weight and average daily gain (ADG) like the diet that meets and exceeds NRC (1988) requirements. These parameters increased with the addition of 0.05g /kg diet of enzymes. In an investigation done by Jang et al. (2017) using a corn SBM diet with different high-fiber phytate containing ingredients (i.e. corn DDGS, corn germ meal, and wheat middlings) supplemented with phytase and xylanase fed to growing pigs, phytase and xylanase inclusion resulted in increased feed efficiency and G:F ratio.

On the other hand, Woyengo et al. (2016) recorded lack of improvement in growth performance when multi-carbohydrase (cellulase, pectinase, mannanase, galactanase, xylanase, glucanase, amylase and protease) and phytase were supplemented in a corn-barley-SBM-wheat-DDGS diet without supplemental P fed to growing pigs, an outcome which they attributed to the lack of effect by exogenous enzymes addition on the digestibilities of energy and nutrients.
Torres-Pitarch et al. (2018) has shown that the supplementation of phytase (500 FTU/kg), xylanase and β-glucanase (22,000U and 15,200U, respectively), and protease (75,000U) to rapeseed- wheat DDGS based-diet did not improve growth and feed efficiency of grower-finisher pigs.
The improvement in growth performance could be ascribed to the effect of phytase and multi-carbohydrase enhancement of nutrient utilization where it was observed that the supplementation of a multienzyme cocktail containing (amylase[0.001%], glucanase[150 U], invertase [0.002%], protease[0.003%], xylanase[250 U], and phytase[400 U]) corn-wheat-SBM based diet fed to weaning pigs led to higher ADG and G:F ratios compare to those fed non-supplemented diets (Omogbenigun et al., 2004)

2.8.2 Effects of phytase and multi-carbohydrase supplementation on nutrient digestibility

Omogbenigun et al. (2004) reported that the combined supplementation of a multienzyme complex (amylase, glucanase, invertase, protease, xylanase, and phytase) in a corn-wheat-SBM based diet fed to weaning pigs resulted in increased ATTD of DM, starch, NSP, GE, CP, P and phytate.

An evaluation of the efficacy of a cocktail of xylanase, amylase, and protease (XAP) and or phytase used individually or in combination in corn SBM nursery pig diets that were marginally deficient in DE and P improved P and Ca digestibility and digestible energy (DE) content. This combination of XAP and phytase inclusion at 500 FTU/kg of diet also improved Ca digestibility (Olukosi et al., 2007). However, Woyengo et al. (2016) observed lack of effect of multi-carbohydrase on ATTD of energy and nutrients with dietary inclusion of these enzymes in corn-barley-SBM-wheat-DDGS diet without supplemental P fed to growing pigs.

It has been reported by Dadalt et al. (2017) that supplementation of a corn and micronized full fat SBM diet fed to 23-day old, weaned pigs with phytase (*Saccharomyces cerevisae*-derived which contained *E coli and Citrobacter braakii* genes) and multi-carbohydrase (700; 2200; 30,000 and 22,000 units of α-galactosidase, galactomannanase, xylanase and β-glucanase, respectively increased the ATTD of CP when the enzymes were used in combination compared with individual applications. When micronized full fat SBM was replaced with with textured soy flour in the same experiment, the ATTD of CP increased by 1.1 and 2.3% points with phytase and multi-carbohydrase supplementation. In addition, enzyme supplementation to the deficient diet compared to the positive which meets or exceeds NRC (1988) requirements increased apparent total tract digestibility of P and Ca by 11.4% and 17.1% for the 0.05 g kg^{-1} level and by 41.7% and

33.1% for the 0.075 g kg^{-1} level respectively. Huang et al. (2021) reported that the dietary inclusion of a multi-carbohydrase phytase complex in a corn-SBM-wheat based diet with reduced NE, STTD P and Ca fed to growing finishing pigs improved the ATTD of crude protein, crude fat, P and Ca. This implies that dietary inclusion of exogenous enzymes including NSP-degrading enzymes and phytase to a nutrient deficient diet resulted in the hydrolysis NSP and phytate thereby liberating more nutrients in the digestive tract. Similarly, in an investigation conducted by Kiarie et al. (2019) to determine the effects of a multienzyme supplement made up of phytase, protease, xylanase, and β-glucanase at 2,200, 8,300, 400, and 100 U/kg of feed, respectively, added to cornstarch and two soy products (full fat soybeans and SBM) fed to growing pigs, the inclusion of these enzymes increased the ATTD of DM, GE, CP, crude fat, P and Ca. The hydrolysis NSP and the release of encapsulated nutrients improved ATTD of NDF and ADF and other nutrients also.

2.8.3 Effects of phytase with multi-carbohydrase on bone traits.

Dietary supplementation of phytase and multi-carbohydrase (α-galactosidase, galactomannanase, β-glucanase and xylanase) to a corn-SBM based diet with low available P or (and) metabolizable energy and amino acids at 0.1%, respectively, increased P content in bone (Shim et al., 2004). Omogbenigun et al. (2004) reported an improvement in bone ash content in weaning pigs fed a corn-wheat-SBM based diet supplemented with dietary multienzyme complex containing carbohydrases and phytase.

Phytase and multi-carbohydrase enzyme supplementation rates also restored bone ash to the same level as in the positive control diet that met and exceeded NRC (1988) requirements in nursery pigs fed corn SBM canola meal diets (Lu et al., 2016). However, Torres-Pitarch et al. (2018) recorded lack of effect of phytase (500 FTU/kg) and protease (75,000U) on bone mineralization in growing-finishing pigs fed wheat-barley- wheat DDGS rapeseed based-diets with reduced P and Ca by 0.08 and 0.09%, respectively

2.9 CONCLUSION

Exogenous enzymes are supplemented in animal feed to increase digestibility, breakdown antinutritional components, to improve the availability of nutrients and prevent environmental pollution (Thacker et al., 2006) . Numerous studies have been conducted using various diet types to investigate the effects of increasing levels of phytase inclusion individually and or in combination with multi-enzyme complexes on digestibility, bone properties and performance with several different outcomes being established. Moreover, factors such as amount of enzyme used, substrate availability, and age of the animal have been proposed to be responsible for the inconsistent results obtained with enzyme supplementation. Synergism is an enhanced effect of two or more enzymes to facilitate the efficacy of one another (Bedford & Partridge, 2010). Notwithstanding, Omogbenigun et al. (2004) had confirmed increased digestibility of P and an efficient degradation of the phytate molecule when multienzyme preparations are supplemented instead of a single enzyme.

However, because of the variabilities in the response of pigs to the supplementation of carbohydrases individually (Cozannet et al., 2012) and the heterogenous nature in the composition of NSP in feedstuffs, enzymes with wider spectrum of activity (multi-carbohydrase) are required for the hydrolysis of these polysaccharides (Mathlouthi et al., 2002). Several studies have been conducted using different multi-carbohydrase blends, but there is little or no information on the effect of the supplementation of nursery pigs with carbohydrase blends like those used in this study. The multi-carbohydrase blend (amylase [80,000 U/g], cellulase [7,000 U/g, glucanase [3,000 U/g], invertase [4,000 U/g], mannanase [750 U/g], protease [6,500 U/g], and xylanase

[8,500 U/g] – expressed as units per gram of enzyme) used in this study is a product of a cutting-edge technology that is a departure from the traditional enzyme products which utilizes one or two activities to degrade indigestible feed components. This product was manufactured from different microbial strains and deploys seven different activities with each activity targeting a specific substrate of the diet. In addition, there is limited information about the effects of increasing levels of dietary phytase supplementation with multi-carbohydrase on digestibility, bone characteristics and performance in weaned pigs. Moreover, except an investigation conducted by Woyengo et al. (2008) where lack of synergistic effect was recorded between phytase and xylanase supplementation on performance and nutrient digestibility in growing pigs, at present, there is scarce information on the synergistic effects of phytase and multi-carbohydrase supplementation on performance, nutrient digestibility and bone traits. There is therefore the need to investigate the effects of products from this new multi-carbohydrase technology and get more information on the synergistic implications of supplementing phytase and multi-carbohydrase.

It was hypothesized that supplementation of increasing levels of phytase alone or in combination with multicarbohydrase will improve nutrient digestibility, bone traits, and thus enhance growth performance and that multi-carbohydrase inclusion with phytase will in synergy increase the efficacy of phytase.

The objectives of this study are:

To determine the effects of supplementing increasing doses of phytase alone or in combination with multi-carbohydrase in a nursery pig diet deficient in phosphorus on growth performance, nutrient digestibility, and metacarpal bone characteristics.

To determine the synergistic effects between phytase and multi-carbohydrase.

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3.0 MANUSCRIPT

Effects of dietary phytase with or without multi-carbohydrase supplementation on growth performance, nutrient digestibility, and bone traits in nursery pigs.

3.1 ABSTRACT

The aim was to determine the effects of supplementing increasing doses of phytase (**PHY**) alone or in combination with a multi-carbohydrase (**MC**) blend to a **P-**deficient diet of nursery pigs on growth performance, nutrient digestibility, and metacarpal bone characteristics. A total of 192 weaned pigs (7.7 ± 0.05 kg BW) were allotted to 1 of 8 dietary treatments each with 8 replicates in a randomised complete block experimental design. Pigs were fed P-deficient diets in a 4 × 2 factorial arrangement based on phytase level (0, 250, 500,1000 FTU/kg) and **MC** level (0 and 0.1g/kg). Fecal samples (d 18, 19, and 20) and metacarpal bones (d 21) were collected to determine apparent total tract digestibility **(ATTD)** of energy and nutrients and bone mineralization, respectively. Data were analyzed using the MIXED procedure of SAS utilizing orthogonal polynomial contrast statements with a pen as the experimental unit for growth performance and digestibility and pig as experimental unit for bone traits. By d 14, PHY with MC interactive effects significantly increase ($P < 0.05$) gain to feed ratio **(G:F)** whereas by d 21, increasing doses of PHY with or without MC addition linearly and quadratically ($P < 0.05$) increased average daily gain (**ADG**). Overall, PHY supplementation alone or in combination with MC quadratically increased ($P < 0.05$) ADG. However, there was an interaction ($P < 0.05$) whereby PHY and MC in combination acted synergistically to increase the ATTD of ash. Phytase had linear ($P < 0.05$) and quadratic ($P < 0.05$) effects by increasing fat-free dry weight, ash content and percentage, and metacarpal bone P content. In conclusion, MC alone did not exert much beneficial effect while increasing levels of PHY alone and when combined with MC quadratically improved growth performance, ATTD of ash and P, and bone traits of pigs fed P-deficient diets.

Key words: bone characteristics, digestibility, growth, multi-carbohydrase, phytase, pig.

3.2 INTRODUCTION

The supplementation of phytase and multi-carbohydrase in swine diets is adopted to alleviate the negative effects of phytate and non starch polysaccharides (NSP) which are the two major antinutritive components in plant-based feedstuffs. Most of the phosphorus (P) in plant-based feedstuff is bound by phytate which is poorly metabolized by nursery pigs (Pointillart et al., 1987; Veum et al., 2006) while NSP have a nutrient encapsulating effect by chelating phytate (J. C. Kim et al., 2005a; Slominski, 2011b). Consequently, because of the unavailability of phytin-P for utilization by weanling pigs, dietary P is often supplemented in swine diets (Pallauf & Rimbach, 1997; Keer et al., 2010) as an approach to correct this deficiency and achieve optimal bone mineralization. However, excessive inclusion often leads to dietary P exceeding age-specific requirements resulting in P overload in pig manure (Dourmad et al., 1999; Oster et al., 2016). To prevent this, a nutritional strategy of dietary P level restriction with phytase supplementation has been adopted.(Selle & Ravindran, 2008; Létourneau-Montminy et al., 2012). It has been reported that the addition of phytase to a diet deficient in P improved nutrient digestibility and growth performance (Mroz et al., 1994; Yáñez et al., 2013) and that the inclusion of suitable carbohydrases (multi-carbohydrase blend) decreased the antinutritive effect of NSP (Olukosi et al., 2007; Lu et al., 2016). Moreover, since inorganic P is added to diets to make up for the indigestible component, phytate-P, it must be supplemented carefully because high levels affects digestion and causes scouring (Varley et al., 2011). Furthermore, phytase supplementation has proven to mitigate high replacements rate of inorganic P without negatively impacting growth performance or bone traits. It has been reported that the effect of phytase inclusion in diets on growth performance is influenced by the level of inorganic P restriction in diet and is expected to be higher at low dietary

P concentrations (Braña et al., 2006). However, there is limited information on the synergistic effects of phytase supplementation in combination with multi-carbohydrase. The multi-carbohydrase that was used in this study was produced by CBS Bioplatforms from an evolving technology that identifies multiple strains which expresses multiple activities with complete coverage of wide spectrum of indigestible components present in feeds in contrast to the traditional process of blending single-sourced enzyme together. The objectives of this study were to determine the effects of supplementing different doses of phytase alone or in combination with multi-carbohydrase in a nursery pig diet deficient in phosphorus on growth performance, nutrient digestibility, and metacarpal bone characteristics and to evaluate the synergy between phytase and multi-carbohydrase.

3.3 MATERIALS AND METHODS

The protocol for this experiment was reviewed and approved by the University of Manitoba Animal Care Committee (Winnipeg, MB, Canada; AC11012), and the Canadian Council on Animal Care (2009) guidelines were used in managing pigs.

3.3.1 Animals, housing, treatments, and diet

A total of 192 pigs ([Yorkshire-Landrace] ×Duroc; Genesus, Oakville, MB, Canada) were obtained from Glenlea Swine Research Unit, University of Manitoba and housed in four rooms within the T. K. Cheung Centre for Animal Science Research. Pens consisted of plastic cover and expanded metal floors. Water and feed were provided ad libitum using nipple bowl drinkers and stainless-steel feeding troughs, respectively. Initial room temperature was set at 29 ± 1°C and decreased by 1°C every week. A 16-h light (0600–2200 h) and 8-h dark cycle were provided. After a 4-d adaptation period being placed on the control diet, pigs were weighed and randomly assigned into groups of 3 per pen and kept in 64 pens with 8 replicates per treatment involving eight blocks.

A randomized complete block design was used with the pig's initial body weight of (7.7 ± 0.3) considered as blocks. A 4 x 2 factorial treatment arrangement with phytase levels (0, 250, 500, 1000 FTU/kg) and multi-carbohydrase levels (0 and 0.1g/kg) were applied. The enzymes used in this study included Bio-phytase 5000G and a multi-carbohydrase blend, Superzyme (amylase [80,000 U/g], cellulase [7,000 U/g, glucanase [3,000 U/g], invertase [4,000 U/g], mannanase [750 U/g], protease [6,500 U/g], and xylanase [8,500 U/g] – expressed as units per gram of enzyme) brands supplied by CBS Bioplatforms respectively. One phytase unit (FTU) is defined as the amount of enzyme required to release 1 mmol of inorganic P/min from a 0.0015 M Na-phytate solution at pH 5.5 and 37°C. One xylanase unit was defined as the activity that releases 1 mol of xylose/min at pH 3.0 and 50°C. The enzymes were added at the expense of corn. The basal diet is a corn-SBM wheat based-diet in mash form, formulated based on STTD P of 0.24% (a 40% reduction relative to NRC (2012) requirement of 0.40%), and made to be deficient in P while other nutrients met the specifications of NRC (2012). Pigs were allotted randomly to one of the eight dietary treatments and fed for 21 days in a single-phase feeding regime. Diets contained 0.3% of titanium dioxide as an indigestible marker used to calculate the apparent total tract digestibility (**ATTD**) of energy and nutrients. Ingredient composition and nutrient content of the basal diet are illustrated in Table 4 (page 42). Body weight and feed disappearance was recorded weekly and used to calculate ADG, ADFI and G:F.

3.3.2 Sample collection

Fecal samples were collected in plastic sterile bags using the grab method after cleaning and washing the pens on days 18, 19 and 20 and stored in -20℃ for each collection day. The collected feces were pooled together into a single bag per pen, dried in a forced-air drying oven at

55°C for 5 days and ground using 1-mm screen with a Wiley Laboratory Mill (Model 3; Arthur H. Thomas Co., Philadelphia, PA) for chemical analyses.

One pig per pen close to average pen weight was euthanized on day 21 and the right front limb severed at the carpo-metacarpal joint, manually cleaned of all skin, muscle, and connective tissue to extract the fourth metacarpal bone which has been proven to be very sensitive to mineral changes in diet ((Varley et al., 2011; De Cuyper., et al 2020). Following removal, the metacarpals were defatted in Soxhlet extraction chamber using ethyl ether. Bones were dried to determine the fat free dry weight and ashed at 650°C in a muffle furnace for 24hours. The ash was digested in aqua regia (HCl/HNO3 mixture) and the sample product was used for the determination of metacarpal P and Ca concentrations using the inductively coupled plasma spectrophotometer (ICP-S).

3.3.3 Chemical analysis

Diet and fecal samples were analyzed for DM (method 990.03,AOAC.2006), TiO_2, ash (method 942.45, AOAC.2006), ether extract (method 920.39A; AOAC, 2006),), neutral detergent fiber and acid detergent fiber using Ankom Technology method (Ankom 2000 Fiber Analyzer, Ankom Technology, Macedon, NY), Calcium (Ca, method 968.08; AOAC, 2006), and P (method 946.06; AOAC, 2006). The gross energy contents of these samples were determined using an Isoperibol bomb calorimeter with benzoic acid as a standard (model 6400; Parr Instrument Co., Moline, IL, USA). Nitrogen (N) content was determined using the combustion method (984.13A-D; AOAC, 2006) with the LECO nitrogen analyzer (model CNS-2000; LECO Corp., St. Joseph, MI, USA) to calculate the CP content (N × 6.25). Phosphorus and Ca were analyzed in bone ash, diets and fecal samples using the inductively coupled plasma optical emission spectrometry (ICP-S).

3.3.4 Apparent total tract digestibility of energy and nutrients calculation

Apparent total tract digestibility (ATTD) of nutrients and energy were calculated using the following formula:

ATTD (%) = [1 - (Nf × Td)/(Nd × Tf)] x 100

where

Nf = nutrient concentration in feces (% DM)

Nd = nutrient concentration in diet (% DM)

Tf = TiO_2 concentration in feces (% DM)

Tf = TiO_2 concentration in diet (% DM)

3.3.5 Bone characteristics calculations

The percentage of bone ash was determined by dividing the weight of bone ash by the weight of fat free, dried bone, and multiplied by 100 while metacarpal bone P and Calcium contents in grams was calculated by multiplying bone P and Ca percentages by the quantity of bone ash and then divided by 100 (Blavi et al., 2019)

3.3.6 Statistical analysis

The MIXED procedures of SAS 9.4 (SAS Institute Inc., Cary, NC, USA) was used to analyze the data as a randomized complete block design with a 4 × 2 factorial treatment arrangement consisting of 4 and 2 levels of phytase and multi-carbohydrase respectively with 8 replicates per treatment. Treatments and their interactions were considered as main effects and initial body weight as random effect. Each pen was deemed as one experimental unit for growth performance and ATTD of energy and nutrients, while one pig per pen was taken as the experimental unit for bone parameters. Data were subjected to ANOVA with means reported as

LSMEANS. Linear and quadratic effects relative to phytase and multi-carbohydrase levels were tested using orthogonal polynomial contrasts. Interactive matrix algebra language (PROC IML) of SAS was adopted to generate the coefficients of unequally spaced contrasts. Tukey's method of comparison of means were used to separate interactive effects. Significance was set as ($P < 0.05$) and tendency ($0.05 \leq P \leq 0.1$).

Table 4. Ingredient composition, calculated and analyzed nutrient content of the basal diet

Ingredients	%
Corn	40.82
Wheat	23.00
Soybean meal, 48%CP	30.00
Vegetable oil	1.40
Limestone	1.72
Monocalcium phosphate	0.30
Salt	0.40
Vit-Min Premix[1]	1.00
Lys-HCL	0.60
DL-Met	0.21
L- Thr	0.19
L- Val	0.06
Titanium dioxide	0.30
Calculated nutrient composition %	
NE, kcal/kg	2,447
Crude protein, %	20.80
Ca, %	0.80
Total P, %	0.45
STTD P, %	0.24
SID Lys, %	1.36
SID Met, %	0.47
SID Met + Cys, %	0.75
SID Lys, %	1.36
SID Thr, %	0.80
SID Tryp, %	0.22
SID Val, %	0.86
SID Lys, %	1.36
SID His, %[1]	0.46
Analyzed nutrient composition %	
Crude protein	22.16
Ca	0.83
P	0.47

[1] Provided per kg of Premix: vitamin A, 2,200IU; vitamin D3, 220IU; vitamin E, 16IU; menadione, 0.5mg; thiamine, 1mg; riboflavin, 3.5mg; Calcium pantothenate, 10mg; choline, 500mg; niacin, 30mg; vitamin B6, 7mg; vitamin B12, 0.02mg; biotin, 0.05mg; folic acid, 0.3mg; iodine, 0.14mg; copper, 6mg; iron, 100mg; manganese, 4mg; selenium, 0.3mg; zinc, 100mg. There were 8 dietary treatments (4 diets supplemented with phytase alone and the other 4 contained phytase with multi-carbohydrase) with phytase levels 0, 250 ,500,1000 FTU/kg and multi-carbohydrase levels 0 and 0.1g/kg of diet. Standardized ileal digestibility (SID), net energy (NE), phytase units (FTU), multi-carbohydrase (MC)

4.0 RESULTS

The analyzed phytase activity for the treatment diets without multi-carbohydrase are 362, 488, 826 and 1,112 FTU/kg (expressed phytase units per kilogram of diet) respectively while those with multi-carbohydrase include 435, 493, 936, and 1,270 FTU/kg (expressed as phytase units per kg of diet). Moreover, analyzed xylanase activity for diets with only phytase inclusion are 39, 38, 41 and 45 U/kg (expressed as units per kg of diet) while those supplemented with phytase and multi-carbohydrase comprised of 753, 837, 513, and 709 U/kg (expressed as units per kg of diet) respectively.

4.1 Growth performance

The effects of enzyme supplementation on growth performance are presented in Table 6 (page 46). The supplementation of either phytase alone or with multi-carbohydrase did not significantly ($P > 0.10$) affect ADG, ADFI, G:F ratio and BW gain by d 7. There was no interaction between phytase and multi-carbohydrase for any of the growth parameters ($P > 0.10$) during this period.

However, by d 14 ($P = 0.070$) and d 21 ($P = 0.052$) of the trial, phytase with or without multi-carbohydrase addition tended to improve final body weight.

During the second week of this study, the interactive effects of phytase and multi-carbohydrase significantly ($P = 0.013$) increased G:F. In addition, there was a significant increase ($P = 0.031$) in ADG because of phytase addition. Also, there were linear and quadratic increases ($P < 0.05$) in ADG and G:F respectively due to phytase supplementation with or without multi-carbohydrase.

However, pigs fed the diet supplemented with 500 FTU/kg phytase individually or in combination with multicarbohydrase had the highest G:F during this period (d 14). More so, PHY supplementation alone or in combination with MC tended to improve BW (P = 0.070); and quadratically increased ADG (P = 0.011) and G:F (P = 0.037), respectively.

Moreover, by d 21, phytase alone or in combination with multi-carbohydrase had significant linear (P = 0.022) and quadratic (P = 0.019) effects on ADG, respectively.

However, multi-carbohydrase supplementation alone tended (P = 0.085) to improve ADFI during the second week of the experiment with no effect on other growth parameters throughout the trial when supplemented individually.

Over the entire trial, phytase supplementation alone tended (P = 0.050) to improve ADG, while orthogonal contrasts of inclusion of phytase alone or in combination with multi-carbohydrase showed statistically significant improvement (P = 0.018) for ADG and a tendency (P = 0.056) for G:F ratio quadratically, respectively.

Dietary treatments supplemented with phytase with or without multi-carbohydrase at 500 FTU/kg of diet yielded the highest ADG and G:F ratio compared to the other treatments.

4.2 Nutrient digestibility

Table 6 (page 47) shows the ATTD of energy and nutrients response to dietary phytase supplementation individually or in combination with multi-carbohydrase. Phytase alone or in combination with multi-carbohydrate did not affect the ATTD of DM and CP ($P > 0.05$). However, there was a tendency whereby phytase and multi-carbohydrase supplementation increased the ATTD of GE (P = 0.080) synergistically. A significant phytase and multi-carbohydrase interaction (P = 0.028) increased the ATTD of ash (mineral component of diet). Phytase alone significantly

($P <$ 0.05) increased the ATTD of ash, P and Ca while a significant multi-carbohydrase response was observed on the ATTD of ash and Ca.

More so, there were linear and quadratic responses ($P < 0.05$) to the supplementation of phytase alone or in combination with multi-carbohydrase on the ATTD of ash and P. Also, a significant linear effect ($P < 0.05$) of phytase inclusion alone or in combination with multi-carbohydrase enhanced the ATTD of Ca.

4.3 Metacarpal characteristics

Phytase significantly ($P < 0.05$) increased dry and crude ash weights, ash percentage, P and Ca contents of the metacarpus. (Table 7, page 48). The addition of phytase with or without multi-carbohydrase increased bone-dry weight, ash percentages and total amount of bone ash and P contents (linear and quadratic, P < 0.05); and bone Ca contents quadratically.

Additionally, the percentages of P and Ca the bone ash was not affected by dietary treatments. However, multi-carbohydrase inclusion individually did not exert any effect ($P > 0.05$) on any of the bone parameters measured and there was no interaction between phytase and multi-carbohydrase on bone characteristics.

Table 5. Effects of phytase and multi-carbohydrase on growth performance in nursery pigs fed a P-deficient corn-SBM diet.

	Without multi-carbohydrase				**With multi-carbohydrase (0.1 g/kg)**					***P*-value**				
	Phytase (FTU/kg)				**Phytase (FTU/kg)**									
Items	**0**	**250**	**500**	**1000**	**0**	**250**	**500**	**1000**	**SEM**	**Phy**	**MC**	**Phy*MC**	**Linear**	**Quad.**
BW, kg														
d 0	7.69	7.71	7.69	7.69	7.68	7.70	7.68	7.68	0.299	0.877	0.593	0.486	0.447	0.915
d 7	8.70	8.67	8.75	8.59	8.54	8.60	8.60	8.68	0.382	0.982	0.435	0.762	0.887	0.747
d 14	11.4	11.3	11.4	11.0	10.8	11.0	11.4	11.0	0.537	0.230	0.183	0.493	0.729	0.070
d 21	14.8	14.8	15.2	15.0	13.9	14.5	15.3	14.5	0.646	0.104	0.108	0.623	0.211	0.052
d 0 to 7														
ADG, g/d	145	137	151	131	122	132	130	143	21.71	0.986	0.504	0.781	0.844	0.790
ADFI, g/d	224	205	219	214	206	217	209	208	21.40	0.989	0.602	0.771	0.828	0.981
G: F, g/g	0.643	0.658	0.676	0.593	0.539	0.548	0.592	0.656	0.065	0.895	0.180	0.446	0.556	0.677
d 8 to 14														
ADG, g/d	381	376	384	343	321	341	406	331	27.70	0.031	0.123	0.195	0.521	0.011
ADFI g/d	480	456	484	426	396	432	445	451	36.51	0.663	0.085	0.175	0.943	0.281
G: F, g/g	0.798^{ab}	0.833^{ab}	0.792^{ab}	0.812^{ab}	0.823^{ab}	0.805^{ab}	0.919^{a}	0.743^{b}	0.030	0.090	0.507	0.013	0.272	0.037
d 15 to 21														
ADG, g/d	490	501	541	534	450	499	546	505	23.46	0.011	0.284	0.697	0.022	0.019
ADFI g/d	640	636	684	638	571	645	657	661	41.64	0.247	0.488	0.487	0.187	0.128
G: F, g/g	0.781	0.810	0.804	0.848	0.805	0.787	0.850	0.767	0.044	0.844	0.760	0.398	0.661	0.541
Overall														
ADG, g/d	339	338	359	336	298	324	361	326	19.12	0.050	0.140	0.529	0.348	0.018
ADFI g/d	448	432	463	426	391	431	437	440	29.81	0.523	0.231	0.317	0.496	0.214
G: F, g/g	0.741	0.767	0.757	0.751	0.723	0.713	0.787	0.722	0.023	0.164	0.202	0.200	0.752	0.056

Differences were considered significant at $P < 0.05$ and as tendency at $0.05 \leq P \leq 0.1$. Within a row, means without a common superscript differs. The basal diet was formulated to contain 0.24% standardized total tract digestible (STTD) P. There were 8 dietary treatments (4 diets supplemented with phytase alone and 4 contained phytase with multi-carbohydrase) with phytase levels 0, 250 ,500,1000 FTU/kg and multi-carbohydrase levels 0 and 0.1g/kg of diet. phytase (Phy), multicarbohydrase (MC), phytase units (FTU), body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), gain to feed (G: F) and quad. (quadratic).

Table 6. Effects of phytase and multi-carbohydrase on ATTD of nutrients in nursery pigs fed a P-deficient corn-SBM diet.

	Without multi-carbohydrase				**With multi-carbohydrase (0.1g/kg)**					***P*-value**				
	Phytase (FTU/kg)				**Phytase (FTU/kg)**									
Items	**0**	**250**	**500**	**1000**	**0**	**250**	**500**	**1000**	**SEM**	**Phy**	**MC**	**Phy*MC**	**Linear**	**Quad.**
DM, %	86.29	86.71	87.38	88.08	82.99	87.01	87.12	86.97	2.01	0.449	0.435	0.809	0.188	0.378
CP, %	83.34	83.58	83.74	85.77	86.37	84.48	83.85	84.79	1.17	0.473	0.313	0.294	0.558	0.144
GE, %	85.03	85.71	85.90	86.73	87.57	85.74	85.79	85.54	0.84	0.848	0.536	0.080	0.971	0.424
Ash, %	69.62^{bc}	68.62^{c}	76.20^{a}	75.50^{ab}	65.61^{c}	70.12^{bc}	70.63^{abc}	69.81^{bc}	1.61	0.001	0.006	0.028	0.001	0.019
P, %	50.02	63.73	69.52	71.38	53.98	59.08	66.10	65.79	2.51	<0.001	0.147	0.171	<0.001	0.002
Ca, %	75.27	72.93	82.49	81.55	72.54	69.90	76.79	78.15	2.11	0.002	0.016	0.892	0.004	0.737

Differences were considered significant at $P < 0.05$ and as tendency at $0.05 \leq P \leq 0.1$. Within a row, means without a common superscript differs. The basal diet was formulated to contain 0.24% standardized total tract digestible (STTD) P. There were 8 dietary treatments (4 diets supplemented with phytase alone and 4 contained phytase with multi-carbohydrase) with phytase levels 0, 250 ,500,1000 FTU/kg and multi-carbohydrase levels 0 and 0.1g/kg of diet. Phytase (Phy), multicarbohydrase (MC), phytase units (FTU), dry matter (DM), crude protein (CP), gross energy (GE) and quad (quadratic).

Table 7. Effects of phytase and multi-carbohydrase on metacarpal characteristics in nursery pigs fed a P- deficient corn-SBM diet.

	Without multi-carbohydrase				**With multi-carbohydrase (0.1 g/kg)**					***P*-value**				
	Phytase (FTU/kg)				**Phytase (FTU/kg)**									
Items	**0**	**250**	**500**	**1000**	**0**	**250**	**500**	**1000**	**SEM**	**Phy**	**MC**	**Phy*MC**	**Linear**	**Quad.**
Bones														
Dry, g	1.99	2.07	2.18	2.16	1.86	2.14	2.17	2.12	0.111	0.002	0.607	0.511	0.005	0.006
Ash., g	0.846	0.924	0.977	0.950	0.774	0.941	0.964	0.933	0.051	<0.001	0.318	0.494	0.003	<0.001
P, g/metacarpal	0.083	0.091	0.088	0.086	0.081	0.086	0.088	0.088	0.002	0.003	0.439	0.405	0.040	0.003
Ca, g/metacarpal	0.173	0.190	0.184	0.178	0.170	0.179	0.183	0.183	0.005	0.007	0.395	0.259	0.101	0.005
Ash, %	42.6	44.7	44.8	44.1	41.8	43.9	44.3	44.1	0.748	0.007	0.326	0.922	0.029	0.007
P, %	19.4	20.3	19.7	19.5	19.3	19.7	19.9	19.9	0.371	0.234	0.948	0.356	0.505	0.104
Ca, %	40.7	42.5	41.1	40.3	40.7	40.8	41.3	41.5	0.741	0.430	0.894	0.155	0.992	0.204

Differences were considered significant at $P < 0.05$ and as tendency at $0.05 \le P \le 0.1$. There was a total of 8 dietary treatments. The basal diet was formulated to contain 0.24% standardized total tract digestible (STTD) P. There were 8 dietary treatments (4 diets supplemented with phytase alone and 4 contained phytase with multi-carbohydrase) with phytase levels 0, 250,500,1000 FTU/kg and multi-carbohydrase levels 0 and 0.1g/kg of diet. Phytase (Phy), multicarbohydrase (MC) and quad. (quadratic).

5.0 DISCUSSION

Several factors have been established that affect the effectiveness of exogenous enzymes. These include age of the animal, enzyme activity and substrate availability as a result of diet composition (Dersjant-Li et al., 2015, Dadalt et al., 2017). In addition, the level of P restriction in diets has been documented to influence the responses to phytase supplementation and available evidence shows that this is more pronounced at lower dietary P concentrations (Yi et al., 1996, Braña et al., 2006). Thus, the basal diet used in this study was formulated to be deficient in P (STTD 0.24%) with a 40% reduction relative to NRC (2012) recommendations. The objective of adding phytase to swine diets is to meet P requirements through the release of phytate-P. Carbohydrases are usually supplemented with phytase to decrease the encapsulating effects of NSP thereby enhancing the efficacy of phytase.

5.1 Growth performance

In this study, dietary phytase (250, 500 and 1000 FTU/kg) supplemented individually or in combination with multi-carbohydrase (0.1g/kg) to a corn, wheat, and soybean meal diet improved ADG on d 21 compared to the negative control (0 FTU/kg) diet. A similar trend was observed by Torrallardona & Ader (2016) and De Cuyper et al (2020) who supplemented phytase alone in a corn soybean and corn, barley and soybean meal diets, respectively. However, the improvements in ADG observed in this trial with the combined supplementation of phytase and multi-carbohydrase in weaned pigs were also reported by Shim et al (2004) who supplemented phytase in combination with an enzyme complex (xylanase, galactomannanase, glucanase, beta-glucanase and alpha-galactosidase enzymes) in a corn-SBM diet with reduced available P. Over the entire trial, the similarity in ADG and final BW among treatments supplemented with phytase individually at 250 and 1000 FTU/kg and a lack of improvement among diets with phytase and multi-carbohydrase combined (250 and 100 FTU/kg) compared to the negative control could be due to the impact of increased endogenous phytase production in the intestinal mucosa, which usually increases with age resulting in a phytase-balance thereby enhancing the ability of pigs to degrade phytic acid (Da Silva et al., 2019).

The lack of significant dietary enzyme effect on ADFI recorded in this trial is in agreement with Braña et al (2006) and Acosta & Patience (2019). However, report of significant effects of phytase inclusion on ADFI individually has been documented (De Cuyper., et al 2020) and this could be due to the differences in enzymes and feed composition used in these studies (Lu et al., 2016).

Results from this experiment observed a synergistic effect between phytase and multi-carbohydrase by d 14 on G:F and this suggests a complementary action between these enzymes in improving performance. The absence of significant effect on G:F in the overall result could have resulted from the large consumption of diets by the pigs caused by the fiber degrading enzyme (multicarbohydrase) increased digesta flow rate (Lyberg et al., 2008). A tendency to increase G:F by phytase supplementation as reported in this study have been previously reported (Shim et al., 2004).

Except for the trending effect of multi-carbohydrase supplementation on ADFI during the second week of the trial, the lack of response to multi-carbohydrase inclusion individually by any of the growth parameters agrees with the findings of Olukosi et al (2007) which stated that dietary supplementation with a carbohydrase cocktail did not improve performance of weaned piglets fed corn, wheat, wheat middlings, canola meal and soybean-based diets. However, this lack of effect in pigs fed diets supplemented with multi-carbohydrase alone could be due to the pigs having the lowest feed intake compared to the negative control and other treatment diets. This suggest that dietary nutrients released by multi-carbohydrase individually resulting from the lesser feed intake was not adequate to increase ADG.

Consequently, this study has shown that the supplementation of phytase alone or in combination with multicarbohydrase alleviated the effect of P deficiency on growth performance in a dose dependent manner with diminishing responses with increasing dosage and the magnitude of this improvement was greatest in diets containing 500 FTU/kg. The maximal growth recorded by pigs fed diet with phytase supplementation at 500 FTU/kg with multi-carbohydrase although with lesser digestibility of P and Ca could be because the requirement for P to optimize body weight gain is lower than required to maximize bone ash (Blavi et al., 2019)

5.2 Nutrient digestibility

Non-starch polysaccharides (NSPs) may encapsulate nutrients within cell walls, cause antinutritive effect by increasing viscosity leading to decreased digestibility with the proliferation of unfavourable microflora (O'Neill et al., 2014). However, multi-carbohydrase reduces the antinutritive effects of NSPs by the partial hydrolysis of soluble and insoluble NSP, decreasing the viscosity of digesta, and degradation of NSP-containing cell walls, thereby making the contents available for digestion. Other effects include modifications in the population of gut microflora (Diebold et al., 2004)

In this study, the supplementation of phytase individually or in combination with multi-carbohydrase had no effect on the ATTD of DM and CP and this is in agreement with the research conducted by Varley et al. (2011) and Arredondo et al. (2019) where increasing levels of phytase were supplemented to a wheat barley and corn soybean meal-based diets contrary, Omogbenigun et al. (2004) and Olukosi et al. (2007) used multi-carbohydrase supplementation alone or with phytase which significantly increased the ATTD of DM, CP and GE. The absence of significant phytase effect on ATTD of energy and protein could be that the bonding between phytic acid and protein and(or) starch is not adequate to affect the digestibilities of CP and energy in weanling pigs (J. C. Kim et al., 2005b). The lack of improvement in the digestibility of CP and energy associated with DM could also be because CP and energy are non-limiting nutrients (Diebold et al., 2004). In addition, lack of improvement in some nutrients digestibilities could be due to high ratio of between insoluble and soluble NSP in corn-SBM diets whereby the soluble components interferes more with digestibility (Duarte et al., 2019) and could also be that the dietary fibre in the diet is not enough to result in a significant increase in digestibility in response to these enzymes (phytase in combination with multi-carbohydrase)(Zhang et al., 2020)

The synergistic outcome seen on the ATTD of ash shows beneficial effects of the simultaneous inclusion of both enzymes by increasing substrate accessibility thereby reducing the antinutritive dietary components, especially for the pigs fed treatment diets supplemented with 500 FTU/kg. The addition of phytase alone or in combination with multi-carbohydrase to the NC diet resulted in significant positive factorial, linear and quadratic responses for the ATTD of P, and linear effect for Ca ATTD. These dose-dependent increases in P digestibility compared with the negative control (0 FTU/kg) agree with other studies (Varley et al., 2011; Almeida et al., 2013). The increase in digestibility of P and Ca by our treatment diets compared to the negative control confirms the ability of the exogenous enzymes added in this study to liberate and increase the availability and utilization of phytin bound P resulting in improved growth performance and bone mineralisation. Also, the improved digestibility of ash which signifies enhanced metabolism of other minerals beside P and Ca could have contributed to the observed performance results. The lack of maximum effectiveness of multi-carbohydrase in our experiment was possibly due to the low content of non-starch polysaccharides in the diet (Atakora et al., 2011).

5.3 Bone traits

Bone characteristics have been identified as being very sensitive to increasing P and Ca availabilities. The increased absorption of P and Ca resulted in more bone formation and more bone ash retention (She et al., 2017). The result of selected bone traits in this study agrees with findings of several other researchers (Varley et al., 2011; Yanez et al., 2013). There were no treatment effects on metacarpal P and Ca percentages because bones maintain their composition and are usually unaffected by increased dietary P and Ca concentrations (González-Vega et al., 2016; Blavi et al., 2019). However, the lack of multi-carbohydrase effect and phytase and multi-carbohydrase interactive effects could be that the nutrient provided by phytase supplementation had already met bone requirements and the effects of added multi-carbohydrase could be saturated (Dong et al., 2018). It could be deduced that the additional P liberated from phytate hydrolysis from phytase and multi-carbohydrase supplementation was utilized for bone development (Omogbenigun et al., 2004)

6.0 SUMMARY AND CONCLUSIONS

Phytase increases the digestibility of phytate and the release of P, thereby improving availability. It is utilized as a feed additive in swine to reduce the requirement for inorganic phosphorus supplementation. Phosphorus utilization improvement and reduced inorganic P in the diet result in less phosphorus excretion from pigs. It has been documented that the addition of combinations of carbohydrases with different substrate specificity and mode of action targeting various components of cell wall polysaccharides in feed have improved nutrient digestibility and availability (Meng et al., 2005). It has been reported that multi-carbohydrase blends would improve digestion and performance more than individual carbohydrases (Zeng et al., 2018). Moreso, the multi-carbohydrase used in this study is a comprehensive blend with seven enzyme activities. However, supplementation of phytase alone or together; with multi-carbohydrase may be applied to complement low inclusion levels of inorganic phosphates in diets, improve digestibility and availability thereby reducing feed cost and environmental pollution (Dersjant-Li & Dusel, 2019). The growth improvement caused by phytase, and multi-carbohydrase can be attributed to the better utilization of plant-origin P, the degradation of dietary fibre and the release of starch and protein bound to phytic acid (Da Silva et al., 2019; Lu et al., 2016). It can be concluded that the phytase and multi-carbohydrase used in this study demonstrated their efficacies by improving the ATTD of P and Ca thereby enhancing performance and bone mineralization in weaned pigs fed diets deficient in P. In addition, phytase and multi-carbohydrase supplementation in this study may have promoted growth by improving the availabilities and utilization of other minerals other than P and Ca.

7.0 GENERAL CONCLUSION

Feed cost is an important aspect in pork production because of it’s impact on efficiency and profitability (Woyengo et al., 2014). Inefficient utilization of nutrients supplied through diets can result in unfavourable economic and environmental consequences (Bento et al., 2012). The limited ability of monogastric animal to metabolise certain dietary components (phytate and dietary fibers) due to inadequate amount of endogenous enzymes is a great concern to the animal nutritionist. Consequently, metabolism can be enhanced by the supplementation of exogenous dietary enzymes (especially phytase and NSP depolymerases) which degrades the anti-nutritional factors that are present in these plant feed ingredients (Lu et al., 2016). Most of these anti-nutritional components disrupts nutrient digestibility and availability which ultimately results in poor growth (Sun et al., 2020) and reproductive performance. The simultaneous inclusion of phytase and multi-carbohydrase in this study was in anticipation that MC will breakdown the encapsulating fibre component allowing phytase to access phytate for more effective breakdown (Huang et al., 2021).

With regards to decreasing inclusion costs together with the increasing prices of feed ingredients, phytase and multi-carbohydrase addition have become cost effective. Moreover, results of the current study indicated that the supplementation of increasing levels of phytase with or without multi-carbohydrase improved growth performance, nutrient digestibility, and bone characteristics but the effectiveness of muti-carbohydrase inclusion was possibly reduced by the low level of NSP in the diet (Atakora et al., 2011).

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