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Investigating methods of evaluating swine feed additives: Phytase and alternatives to antibiotic growth promoters

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Investigating methods of evaluating swine feed additives: Phytase and alternatives to antibiotic growth promoters

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

To my parents, Wayne and Janell Hansen, for raising me to know I can do anything I set my mind to and work hard for. I know your unwavering support and confidence in me is how I got to where I am.

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ABSTRACT

Feed additives are often included in swine diets to increase digestibility of certain nutrients, improve intestinal function, and prevent or mitigate illness, ultimately with a goal of improved productivity. Whenever additives are introduced into diets, it is imperative that their efficacy be demonstrated with thorough research, and the elucidation of information such as proper inclusion rate and effects on the pig is necessary for their inclusion to be practical and economically favorable. Thus, proper evaluation of feed additives is critical. The objective of this thesis was to investigate the methods with which phytase and alternatives to antibiotic growth promoters (AGP's) are evaluated. Phytase is commonly added to swine diets to improve availability of dietary phosphorus (P), and its P releasing efficacy is usually determined using pigs fed diets deficient in P. Chapter 2 used 72 growing barrows (BW = 22.95 ± 1.87 kg) and 8 dietary treatments to test the effects of adding phytase to a P adequate diet compared to a P-deficient diet on P and Ca digestibility and balance, and to generate a P release curve for phytase. Phytase improved ATTD and STTD of P (quadratic P) and absorbed P (linear, quad). Urinary P excretion increased linearly with phytase inclusion; retention of P also increased (P). Phytase was predicted to release 0.049% STTD P for 200 FTU/kg added to P-adequate diets, and this number may be lower than release values observed in P-deficient diets (STTD P release was estimated to be 0.059% for 200 FTU/kg; $P < 0.05$); this corresponded to a 28% increase in P digestibility in the P-deficient diet whereas there was only a 12% improvement in the P-adequate diet. The results demonstrated that urine P excretion could not be used as a predictor of phytase P release, and evaluation of phytase in P-adequate diets, rather than P-deficient diets, may be advantageous to making precise estimates of P release values. Results of AGP alternative studies have thus far been inconsistent, and part of this may be due to inconsistencies in experimental methodology

and lack of helpful information being reported in individual studies. In Chapter 3, the objective was to model a framework for studies evaluating AGP alternatives and investigate the impact of AGP alternatives, pig group size, and their interaction on nursery pigs. A total of 1,300 weaned pigs (6.14 ± 0.18 kg) were assigned to 8 different treatments: 4 diets evaluated across 2 group sizes. The 4 dietary treatments were: negative control (NC), positive control (PC; NC + in-feed antibiotics), pharmacological levels of zinc oxide plus a dietary acidifier (blend of fumaric, citric, lactic and phosphoric acid, ZA; NC + ZnO + acid), and a *bacillus*-based direct-fed-microbial plus resistant potato starch (DR; NC+DFM+RS). The 2 group sizes were 31 or 11 pigs/pen. Collection and testing of oral fluid and serum samples, and necropsy of deceased pigs allowed for characterization of pig health status and identified specific pathogens as potential influential factors in the study, including a natural porcine reproductive and respiratory syndrome virus challenge in wk 4-6. The PC diet improved ADG, ADFI, and G:F ($P < 0.05$) regardless of group size. The ZA diet improved ADG and ADFI when pigs were housed in large groups, but not in small groups ($P < 0.05$). This indicates that group size may be a contributing factor to outcomes of AGP alternative experiments. Careful study design, protocol implementation, sample collection, and recording of important information allowed for characterization of the health status of this group of pigs and determination of treatment effects on growth performance and morbidity. Similar methods of collecting and reporting crucial information in future studies evaluating AGP alternatives may lead the industry toward quicker progress in identifying and implementing effective alternatives to AGPs.

CHAPTER 1

LITERATURE REVIEW: EVALUATION OF SWINE FEED ADDITIVES WITH A FOCUS ON PHYTASE AND ALTERNATIVES TO SUB-THERAPEUTIC ANTIBIOTIC GROWTH PROMOTERS

Introduction

Feed additives can be included in swine diets to increase the digestibility of certain nutrients, improve intestinal function and prevent or mitigate illness. The ultimate goal is improved growth performance and productivity and animal well-being. Examples of common additives used in the swine industry include antibiotics, zinc oxide, exogenous enzymes, milk products, and plasma. Whenever feed additives are introduced into diets, it is important that their efficacy be demonstrated with thorough research, and the elucidation of information such as proper inclusion rate, interactions with other dietary components, and effects on the pig is necessary for their inclusion to be practical and economically favorable. Thus, their proper evaluation is critical for effective use.

Two common classes of feed additives employed by the U.S. pork industry are phytase and sub-therapeutic levels of antibiotics. The inclusion of phytase has become increasingly common; because it involves manipulation of essential nutrients in the diet (mainly phosphorus), it is important that information available on its implementation is as accurate as possible. On the other hand, sub-therapeutic antibiotics are becoming less common in the industry due to consumer demand and 2017 regulatory changes; therefore, other feed additives are being tested to serve as potential replacements for, or alternatives to, antibiotics. Thus far, consistently effective alternatives have not been found. Inconsistent results lead researchers to question in

which situations specific alternative ingredients are most effective, but inconsistent experimental methodology makes it difficult to make comparisons across studies. In the case of both phytase and growth-promoting antibiotic alternatives, proper evaluation will improve their use. Thus, an investigation into the methods of evaluating both of these additives may be advantageous to their most effective implementation in swine diets.

Phytase and phosphorus metabolism in the pig

Phosphorus

Phosphorus (P) is an essential major mineral in swine diets. P is a critical component of bone, where 80% of P in the body is found as hydroxyapatite in combination with calcium (Ca) (Breves and Schröder, 1991). The other 20% of P in the body exists as components of phospholipids, nucleic acids, low and high-energy phosphate bonds (ex. glucose-6-phosphate; adenosine triphosphate), coenzymes such as nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD), and other enzymes involved in metabolic processes (Weremko et al., 1997; Cromwell, 2005). Phosphorus is also critical for the body's acid-base balance via its role as an intracellular buffer (Cromwell, 2005) and as a component of the calculation of dietary undetermined anion (Patience and Chaplin, 1997).

Phosphorus absorption occurs primarily in the jejunum through both paracellular and transcellular pathways, with the transcellular route generally predominating (Lee et al., 1986; Breves and Schröder, 1991; Eto et al., 2006). Phosphorus transcellular absorption is mediated by sodium-dependent phosphate transporters on the apical side of the enterocytes in the jejunum (Berndt et al., 2005). Similarly-structured sodium-linked transporters exist in the kidney proximal convoluted and straight tubules that serve to resorb P from filtrate in order to maintain

a normal P balance in the body (Berndt et al., 2005). There are two major hormones that govern P absorption and excretion in the body: parathyroid hormone (PTH) secreted by the parathyroid gland and 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) synthesized by the action of 1-α hydroxylase in the kidney. In response to low levels of serum P, 1α,25$(OH)_2D_3$ is secreted and acts on the intestine as well as the kidneys to up-regulate luminal intestinal absorption and increase reabsorption, respectively. In response to high levels of serum P, PTH is secreted and acts largely on the kidney to decrease P reabsorption in the renal tubules, thus decreasing P retention and increasing P excretion in the urine. In addition to these two important hormones, compounds called phosphatonins contribute to the decreased retention of P in the kidney in response to increased P levels (Berndt et al., 2005). Phosphorus can also be mobilized from the skeleton into body fluids when necessary, also under the control of PTH and 1α,25$(OH)_2D_3$ (Cromwell, 2005). While it is accepted that P balance is regulated in part by alterations in intestinal uptake, P balance studies in pigs have suggested that the modulation of renal reabsorption plays a larger role in governing P balance than the role of the intestine, especially when dietary P exceeds the pig's requirement (Gutierrez et al., 2015). In this regard, Gutierrez et al. (2015) demonstrated that as dietary P is increased from below requirement to above requirement, P is absorbed and used for growth and bone development until maximum growth and bone development occur, then excess P is excreted in the urine. This study showed that when P is fed at levels exceeding the pig's requirement, absorption of P in the intestine was not a limiting step (Gutierrez et al., 2015).

Calcium and its interactions with phosphorus

Because Ca and P combine in bone at a 2.1:1 ratio as hydroxyapatite, the utilization of Ca and P in bone is dependent on each other's concentration. Additionally, this ratio varies little during times of Ca/P deficiencies, suggesting that the utilization of one depends on the presence of the other at the proper ratio (Cromwell, 2005). Calcium balance is also regulated in part by PTH and $1\alpha,25(OH)_2D_3$; thus the interaction of Ca and P within the body is important to consider when studying either of these minerals. Furthermore, high amounts of dietary Ca have been reported to decrease P absorption, which has been attributed to Ca and P binding to form insoluble tricalcium phosphate within the pig's gastrointestinal tract (Cromwell, 2005) This has been shown to decrease growth performance with diets high in Ca (Letourneau-Montminy et al., 2012). Data from González-Vega et al. (2016) and Heaney and Nordin, (2002) also support this theory, showing that increasing Ca intake was related to increased fecal P, and thus decreased P digestibility. However, Gonzalez-Vega (2016) also showed that as Ca levels in the diet increased, fecal P excretion increased, but P retention was also improved due to the increased amount of Ca available for bone formation, thus more of the P that was absorbed was utilized by the pig and less was excreted in the urine. This supports the notion that the ratio of Ca to P is crucial for the effective use of both of these minerals in the body, and thus is a critical consideration for diet formulation.

Unlike P, the Ca balance is regulated largely by the digestive tract rather than the kidneys. This is supported by evidence that urinary excretion of Ca is very low except at very low Ca and P intake (Fernández, 1995; Gutierrez et al., 2015). In addition, Gutierrez et al. (2015) demonstrated linear decreases in apparent total tract digestibility of Ca as Ca was increased in

the diet and a “constant curvilinear relationship between intake and absorption or retention of Ca” (Gutierrez et al., 2015).

Phytate

Phytic acid (myoinositol (1,2,3,4,5,6) hexakisphosphoric acid) has the molecular formula $C_6H_{18}O_{24}P_6$ and a molecular mass of 660.04 g/mol. It is the main storage form of P in plants, serving as a source of P during seed germination (Selle and Ravindran, 2007; Kumar et al., 2010). Phytic acid’s polyanionic structure gives it the ability to chelate cations and form insoluble salts. Known as phytates (or simply, phytate), these salts of phytic acid are commonly present as salts of potassium (K^+), magnesium (Mg^+) and calcium (Ca^{2+}) (Selle and Ravindran, 2007; Kumar et al., 2010). This characteristic also makes phytate a storage form for other minerals within the plant, such as Ca, Mg, zinc, iron, and manganese (Weremko et al., 1997). Phytates are found in large quantities in the aleurone layer of cereals such as wheat, barley, and oats (Weremko et al., 1997), and is found largely in the germ portion of corn (O’Dell et al., 1972). Viveros et al. (2000) reported that in corn, 78% of P is bound to phytate, compared with 45% in soybean meal. Phytate P is largely unavailable to the monogastric animal due to their inability to hydrolyze the phosphate groups from the inositol ring. This results in a large portion of P found in swine diets being unavailable for absorption. For this reason, the pig’s P requirement is often met by inorganic P supplementation in the form of mono- or di-calcium phosphate, or the inclusion of P-rich feedstuffs such as meat and bone meal (Selle et al., 2009). To correct for the portion of P that is unavailable to the pig, swine diets are often formulated on the basis of available or digestible P, rather than total P. Available P refers to the content of P in and ingredient or diet that is not bound to phytate (total P – phytate bound P), and digestible P is

measured with digestibility experiments which quantify fecal losses of P for a given ingredient or diet. Also of extreme interest is the issue of phytate's chelation with Ca, which causes a decrease in the availability of Ca, compounding the issue of phytate and phytate-bound P in swine diets (Bohlke et al., 2005).

In addition to the negative impacts on P digestibility, phytate may also bind nutrients such as amino acids and fatty acids, causing a decrease in their availability (Bohlke et al., 2005; Vigors et al., 2014). Bohlke et al. (2005) observed that, when comparing a low-phytate corn to normal corn, ileal and total tract digestibility of P and Ca were significantly higher, and ileal digestibility of several amino acids was greater, indicating that phytate does indeed have the ability to bind and decrease the availability of amino acids. This again points to the importance of the polyanionic property of phytate, giving it the ability to bind to positively charged molecules (like Ca and amino acids) (Angel et al., 2002; Bohlke et al., 2005). However, the practical significance of phytate's interaction with amino acids has been questioned (Bohlke et al., 2005).

Due to the large amount of P in swine diets that is unavailable to the pig, there are concerns about high levels of P in manure. Because of the degree of fermentation in the cecum and large intestine, most of the P excreted in feces is actually not bound to phytate (Selle and Ravindran, 2008). Excessive P in manure has led to concerns about the potential negative implications on water quality due to movement of P from animal production operations to water resources (Lanyon, 2005). These environmental concerns, coupled with desires to increase the dietary P efficiency utilization, have fueled the development of exogenous phytases for use in swine diets (Beaulieu et al., 2007; Selle and Ravindran, 2007).

Phytase

Phytase (myo-inositol hexakisphosphate phosphohydrolase) is an enzyme that hydrolyzes phytate and releases inorganic phosphate and myo-inositol (Lei and Stahl, 2000; Cromwell, 2005). This occurs in a step-wise manner, beginning with one phosphate on the myo-inositol ring (IP-6), and continuing through a progression of lower myo-inositol phosphate esters (ie. IP-5 through IP-1) ultimately yielding six phosphate groups and inositol (Selle and Ravindran, 2007; Holloway, 2016). According to Selle and Ravindran (2007), the axial phosphate group on the C2 carbon of inositol is particularly resistant to hydrolysis; thus phytase's hydrolysis of phytate is more likely to yield five phosphate groups and IP-1. While there is inherent phytase activity in both plants and the pig's gastrointestinal tract, it is considered to be insignificant in terms of its ability to mediate the negative impact of dietary phytate-P on P availability (Eeckhout and De Paepe, 1994). Thus, animal scientists have been motivated to look for other sources of phytase that are beneficial to the pig by improving P availability. The first instance of an exogenous phytase source becoming commercially available for inclusion in swine diets was a preparation of phytase isolated from *Aspergillus niger* (*A. niger*) in 1991 (Natuphos; Kumar et al., 2010). There have been many microbial sources used to produce commercially available phytase including yeasts, like *A. niger*, *A. Ficuum*, and *A. fumigatus*, and bacteria like *Escherichia coli* (*E. coli*), and *Peniophora lycii* (Augspurger et al., 2003; Kumar et al., 2010; Dersjant-Li et al., 2015). In addition to their microbial origin, phytases can be categorized by the position on the myo-inositol ring at which they initiate hydrolysis, typically either the 6 or 3 position (Augspurger et al., 2003; Selle and Ravindran, 2007). Furthermore, phytases can be categorized based on the pH at which they are most active (i.e. their pH optima) as either acidic or alkaline

phytases, having pH optima around 5 or 8, respectively; phytases used in animal nutrition are generally acidic (Kumar et al., 2010).

In numerous studies, phytase has been shown to be effective in improving dietary P availability and reducing fecal P excretion (Fan et al., 2005; Beaulieu et al., 2007; Augspurger et al., 2009; Letourneau-Montminy et al., 2012; Vigors et al., 2014; She et al., 2017). This has been effective in studies with nursery pigs as well as growing and finishing pig (Lei and Stahl, 2000). In a 2000 review of exogenous enzymes in monogastric nutrition, Bedford referred to phytase as a "ubiquitous" feed additive due to the clear benefits to the environment and to feed costs (Bedford, 2000).

Evaluation of phytase

In order to effectively formulate diets with exogenous phytase, accurate P release values must be assigned to specific sources and inclusion levels of phytase. Traditionally, release values have been determined by feeding diets well below the pig's requirement for P. Then, standard curves have been developed using regression analysis to predict the P release of phytases based on dietary phytase level and growth performance parameters (i.e. ADG, G:F) and/or bone mineral (ash) characteristics (Augspurger et al., 2003; Jones et al., 2010). In general, bone characteristics like weight, ash weight, percent ash, and breaking strength have been the most sensitive measurements to determine P status in pigs, and thus are logically used to measure P status as it relates to phytase P-release (Cromwell, 2005). For example, Jones et al. (2010) fed a P-deficient diet as a negative control and added inorganic P via monocalcium phosphate to create a standard curve. Using this curve, available P release was predicted from increasing levels of *E. Coli*-derived phytase based on improvements in ADG, G:F, ash weight, and percentage bone ash.

Percentage bone ash proved to be the best variable to predict P release, and their prediction equation yielded similar release values to manufacturer's recommendations (Jones et al., 2010). Kerr et al. (2010) also developed regression equations to predict P release from different sources and levels of phytase; P-deficient diets were also fed in these experiments. Here, improvements in apparent total tract digestibility of phosphorus were used to predict P release (Kerr et al., 2010). Results from the Kerr et al., study showed lower P-release for the same phytases evaluated in the Jones et al., study; whereas the Jones study resulted in P-release values close to manufacturer's recommendations.

However, there are multiple disadvantages to this approach. First, pigs in these trials must be fed diets severely deficient in dietary P, which can cause reductions in growth performance and bone mineralization and lead to well-being issues (Cromwell, 2005; Jones et al., 2010; Vigors et al., 2014). Hypercalciuria (excessive excretion of Ca in urine) may also be observed at very low levels of dietary P (Gutierrez et al., 2015). Additionally, pigs may respond to low levels of P intake by increasing intestinal P uptake (Berndt and Kumar, 2009) and mobilizing bone P (Cromwell, 2005). Therefore, the improvements in P utilization due to phytase in a low-P diet may not be the same as those employing high-P or P adequate diet. For example, Saddoris et al. (2010) observed increases in Na-dependent phosphate uptake in the small intestine in response to decreasing dietary P and concluded that this was likely due to increased translocation of the transporter NaPi-IIb to the apical membrane, rather than to increased expression of the transporter itself. Other studies have also shown smaller improvements in P digestibility due to phytase when diets are adequate in P compared to when they are deficient (Fan et al., 2005; Almeida et al., 2013). Thus, it is possible that the values currently existing for P release by

specific phytases may not be entirely accurate when applied to a commercial pork production setting where diets are not P-deficient.

It has been demonstrated that when pigs are fed diets containing P levels above their P requirement, excess P is accumulated in bones until it reaches a plateau and is then excreted in the urine (Gutierrez et al., 2015). Therefore, a plausible alternative for evaluating the P releasing capabilities of phytase may be feeding diets above the pig's P requirement and quantifying urinary P excretion. By adding phytase to the diet, increases in urinary P can be attributed to increased P made available for absorption by the action of phytase.

Antibiotic growth promoters in swine diets

In the late 1940's, the growth promoting benefits of feeding aureomycin (now known as chlortetracycline) were discovered by chance as researchers were trying to find a new source for Vitamin B_{12} in poultry diets (Gustafson and Bowen, 1997). The livestock industry quickly realized the implications to commercial production, and soon after, more studies showed growth benefits due to relatively small amounts of aureomycin and other antibiotics in swine, cattle, and poultry (Gustafson and Bowen, 1997). Since that time, antibiotics have been included in swine diets at "sub-therapeutic" levels, meaning levels far lower than the doses generally used to treat disease, to improve growth performance, feed efficiency, and reduce mortality and morbidity (Cromwell, 2002). Antibiotics in this context are often referred to as "antibiotic growth promoters" or AGP's. In his 2002 review of antibiotics in swine production, Cromwell summarized over 1,000 experiments conducted in the U.S. between 1950 and 1985 and showed improvements due to AGP addition (including a wide range of antibiotics such as chlortetracycline, tiamulin, lincomycin, carbadox, and more) ranging from 4.2-16.4% for ADG,

and 2.2-7% reductions for feed-to-gain ratio. As is commonly noted when AGP's are discussed, the improvement was largest when nursery pigs were studied (Cromwell, 2002). Considering the amount of time that has passed since these data were generated and the fact that pork production practices have changed substantially in that time, it is possible that the improvements due to the addition of AGP's may not be as large today; perhaps closer to 5% improvement in ADG and 2% improvement in feed efficiency may be expected (Dritz et al., 2002) . Nonetheless, their inclusion in diets has persisted, especially in nursery pigs, and more recent data supports their utility as growth promoting agents (Jacela et al., 2009). It is important to note that, in addition to their growth promoting effects, AGP's also decreases the incidence and severity of some diseases such as swine dysentery and *Clostridium perfringens*, and their ability to reduce mortality has also been a key contributor to their widespread use (Zimmerman, 1986; Doyle, 2001).

For almost as many years as antibiotics have been included in swine diets, the safety of this practice has been questioned. Antibiotics suppress bacterial growth via interference with the bacteria's replication mechanisms, thus limiting proliferation (Levy, 1998). This can be through binding of the bacteria ribosome to limit protein synthesis or blocking of synthesis of the new cell wall (Levy, 1998). Due to the ability of bacteria to adapt quickly and to pass on their genetic information, survivors of antibiotics (i.e. resistant strains) can pass on their resistance to other bacteria; resistant strains of several bacteria have been found on farms in multiple regions of the world (Doyle, 2001). Resistance genes enable bacteria to inhibit the action of antibiotics; these genes may code for enzymes that break down antibiotics, molecules that alter antibiotic's cellular targets, or alterations in the antibiotic's mode of entry into the cell (Levy, 1998). Bacteria can obtain resistance genes through inheritance, and spontaneous mutations can create or strengthen

traits of resistance genes (Levy, 1998). Furthermore, bacteria can “take up” genes from the resistant bacteria around them to gain resistance. As an antibiotic kills a susceptible group of bacteria, resistant bacteria survive, have reduced competition, and proliferate (Levy, 1998).

What is unclear is whether resistant strains pose a threat to human or animal health. The largest concern about resistance caused by antibiotics has been around penicillin and the tetracycline classes of antibiotics, as they are important in human medicine (Cromwell, 2002). While no direct ties to antibiotic usage in animals and human health issues have been made (Cromwell, 2002), many countries have banned the use of antibiotics for growth promotion in animal feed (Højberg et al., 2005). Beginning January 2017, new regulations from the U.S. Food and Drug Administration went into effect. These changes attempt to eliminate the use of “medically important antibiotics” for the purposes of improving growth performance and to increase veterinary supervision over antibiotic usage. In addition many antibiotics that were previously available over-the-counter and used as AGP’s now require a Veterinary Feed Directive and are to be used for disease treatment or prevention, rather than growth promotion (National Pork Board, 2016). These changes in the ways producers can employ certain antibiotics and the push for the industry to reduce antibiotic usage in general have driven a search for effective feed additives that can serve as alternatives to antibiotics (i.e. AGP alternatives).

There is a long list of specific antibiotics that have been included in swine diets including chlortetracycline, tiamulin, carbadox, neomycin and oxytetracycline. Discerning the specific mode of action of AGP’s when it comes to improving growth has been elusive (Pluske et al., 2007). While their chemical characteristics and target bacterial population vary, in general, antibiotics used in swine production all possess the ability to suppress microorganism growth

(Cromwell, 2002). There are several proposed theories regarding the specific mechanism for improvements in pig performance, including the bactericidal effect itself, beneficial alterations of the gut microflora, nutrient-sparing effects, and improvements in intestinal function (Zimmerman, 1986; Doyle, 2001). Lack of or lower-magnitude improvements in growth performance in germ-free pigs and in "clean" environments (in contrast to commercial farms) suggest that the bactericidal effect of feeding AGP's is the main contributor to growth promotion (Zimmerman, 1986; Doyle, 2001; Cromwell, 2002; NRC, 2012). It has also been suggested that the alterations in the natural microbiota of the pig's intestinal tract may lead to more beneficial populations that contribute to improved performance (Doyle, 2001). Bhandari et al. (2008) observed that a nursery pig diet containing an AGP (chlortetracycline, sulfamethazine, and penicillin at 200, 200, and 100 g/ton, respectively) fed during an *E. Coli* challenge resulted in increased microbial diversity in the gut, and while no differences in growth performance were observed, mortality was decreased, suggesting possible health benefits of increases in microbial diversity. The possible nutrient-sparing effect of AGP inclusion has been attributed to the energy otherwise lost to microbial fermentation becoming more available to the pig due to antibiotic-driven alterations in the gut microbiota (Doyle, 2001). While the specific growth-promoting mode of action of AGP's has not been entirely elucidated, it seems clear that the microbiota of the pig's intestinal tract plays a vital role. Thus, the microbiota has become a primary target in the development of AGP alternatives.

In order to discuss AGP alternatives and their potential modes of action, a brief discussion of the microbiota and its importance to the pig will be useful. The microbiota is the natural population of microorganisms that exists in the pig's gastrointestinal tract, with the highest numbers being in the distal small intestine, cecum, and colon (Stensland and Pluske,

2017). The most common groups include *Lactobacillus, Streptococcus, Peptococcus, Eubacterium, Clostridium, Bifidobacterium, and Bacteriodes* (Stensland and Pluske, 2017). In general, *Lactobacillus* and other lactic-acid producing bacteria are regarded as beneficial species. By colonizing the intestinal tract, the microbiota aids in prevention of pathogen adhesion, synthesis and secretion of natural antimicrobial compounds such as organic acids, hydrogen peroxide, and bacteriocins and support more optimal intestinal barrier function and anti-inflammatory responses (Stensland and Pluske, 2017). Richness refers to the number of species present and diversity takes into account richness as well as species distribution (Gotelli and Chao, 2013). Stability and diversity of species in the microbiota are generally regarded as "healthy", but abrupt changes in environment, such as weaning, are known to cause shifts in the microbiota, and these changes can sometimes allow the adhesion and proliferation of pathogenic species (Stensland and Pluske, 2017). A healthy microbiota is essential for a healthy pig; thus feeding strategies that beneficially alter the microbiota may be important tools in maintaining high levels of health and production.

Alternatives to growth promoting antibiotics

Numerous feed additives have been evaluated for their abilities to serve as alternatives to AGP's. In general, targeted modes of action of proposed AGP alternatives include improving immune function, modulating the pig's gut microbiota, and enhancing digestion (de Lange et al., 2010). Alternatives have largely been studied in the context of the nursery phase of production, which is where the bulk of this review will focus.

Pharmacological levels of zinc as an AGP alternative

Zinc (Zn) is an essential mineral for swine, generally regarded as a trace mineral due to low inclusion levels in feed. In the body of the pig, Zn is found as a component of many enzymes, including metalloenzymes, transferases, and many digestive enzymes. Because of Zn's importance in many enzymes, it is critical for lipid, protein, and carbohydrate metabolism (Pluske, 2012). While the pig's dietary Zn requirement is around 100 ppm, much higher "pharmacological levels" 1000-3000 ppm, have been included in swine diets, most often for young pigs, to promote growth and decrease the negative impacts of post-weaning diarrhea caused by enteric disease (Pettigrew, 2006; Pluske, 2012). High levels of Zn are usually accomplished by the addition of zinc oxide (ZnO), although other forms such as tribasic zinc or forms of organic zinc are also available. Many studies have demonstrated both growth-promoting and diarrhea-reducing benefits. For example, Pérez et al. (2011) conducted a series of experiments and found that supplementing 3,000 ppm Zn as ZnO improved nursery pig growth performance in two of the three experiments. In the presence of a natural pathogenic *E. Coli* infection, they observed an increase in growth and feed intake due to Zn, reduced morbidity and fewer pigs requiring therapeutic antibiotic treatments. Heo et al. (2010) also observed a decrease in diarrhea scores/incidence after an experimental challenge with enterotoxigenic *E. Coli* due to the inclusion of 2,500 ppm ZnO in the diet.

The mode of action responsible for these improvements has not been entirely established. Increased secretion of antimicrobial peptides, improvements in microbiota stability, increased secretion of growth factors, reductions in intestinal electrolyte secretion, and alterations in digestive enzyme activity are proposed mechanisms (Pluske, 2012). Data from Carlson et al. (1999) suggest that increased amounts of Zn held in intestinal cells may contribute to intestinal

health via increased protein synthesis and cell proliferation (Carlson et al., 1999). Wang et al. (2007) studied the influence of pharmacological levels of Zn (3,000 ppm) on the antimicrobial peptide PR-39; Zn-finger proteins are thought to be a component of the nuclear transcription factor that regulates the expression of this protein. The results of this study showed increases in ADG and ADFI accompanied by increases in the mRNA expression of PR-39 (Wang et al., 2007). Furthermore, *in vitro* data from Carlson et al. (2006) suggest that Zn may be able to inhibit the action of secretagogues in the intestine and decrease intestinal permeability (Carlson et al., 2006). Further supporting the potential of Zn to improve gut function, Li et al. (2006) demonstrated the ability for 3,000 ppm Zn supplied as ZnO to improve ADG, ADFI, and G:F and observed increased intestinal villus height as well as increased IGF-1 and IGF-1 receptor in the intestine (Li et al., 2006b).

While Zn supplementation has proven to be useful in commercial production (Tokach and Dritz, 2000) and evidence supports its inclusion as an effective alternative to antibiotics, it is worth mentioning that concerns of accumulation of some metals in the environment (Stensland and Pluske, 2017) have led to restrictions on using Zn at such high levels in animal diets. Additionally, the potential for Zn-induced antibiotic resistance (Slifierz et al., 2015) may lead to future conversations about reducing the addition of pharmacological levels of Zn.

Dietary acidifiers as AGP alternatives

The addition of acidifiers to feed is another commonly studied AGP alternative strategy, and is thought to enhance enzyme activity in the small intestine and provide nutrients specifically for the intestinal tissue to enhance intestinal function (Pettigrew, 2006; de Lange et al., 2010). Additionally, there may be beneficial antimicrobial activity of some acids (de Lange et al., 2010)

The acidifiers are commonly organic acids (such as butyric acid), inorganic acids (such as phosphoric acid), or salts of either organic or inorganic acids (such as sodium butyrate). There are a wide variety of acids and their forms that can be added to swine diets, and acids are often included in the diet alone or in blends with other acids. Some acidifiers are encapsulated in a lipid matrix in order to maintain acidic conditions along the gastrointestinal tract, to help the acid reach the hind gut, and to mitigate palatability issues (Tung and Pettigrew, 2006).

The original theory behind the acidification of feed is centered around the idea that piglets when first weaned exhibit sub-optimal acid production in the stomach for proper break down of proteins and activation of other digestive enzymes, resulting in inefficient digestion (Ravindran and Kornegay, 1993). Consequences of insufficient acid production can include: incomplete activation of pepsinogens and pancreatic enzymes (like trypsin and chymotrypsin) incomplete stimulation of bicarbonate secretion from the pancreas, and increased coliform proliferation due to increased substrate (undigested food) (Ravindran and Kornegay, 1993). For these reasons, acidifying the feed has been a proposed strategy for improving growth performance and feed efficiency, especially in the nursery period. Some observed benefits of acidifiers have been increased gain and feed efficiency (Canibe et al., 2005; Walsh et al., 2007; Li et al., 2008), increased feed intake (Upadhaya et al., 2016; Wang et al., 2016) decreased diarrhea (Fang et al., 2014; Wang et al., 2016), and improvements in nutrient digestibility (Wang et al., 2016).

In their 1993 review, Ravindran and Kornegay highlighted that the addition of acidifiers to pig diets has not consistently lowered stomach pH as expected (Ravindran and Kornegay, 1993). Thus, other possible mechanisms for the benefits of acids have been discussed. Acids have been thought to alter microbial populations throughout the pig's gastrointestinal tract, and

furthermore, acids may be able to enter bacterial cells in their undissociated form and then dissociate, killing or damaging the cell. Therefore, acids included in their undissociated form may have more effective antimicrobial properties (Pettigrew, 2006; Upadhaya et al., 2016). Organic acids, specifically, are intermediates in the tricarboxylic acid cycle; thus it is proposed that these may serve as additional energy sources to the cells of the GIT, potentially leading to increased cell proliferation, which may improve gut function (Ravindran and Kornegay, 1993; Mroz, 2005; de Lange et al., 2010).

Wang et al. (2016) demonstrated decreased diarrhea in pigs fed a blend of calcium formate, calcium lactate, citric acid and medium-chain fatty acids when compared to a negative control diet but did not observe significant differences in performance. In a separate experiment, Wang reported improvements in ADFI, increased ileal Ca, DM, and energy digestibility, higher concentrations of *lactobacillus* in the ileum, and lower total ileal bacterial counts (Wang et al., 2016). Increased *lactobacillus* populations were also reported in conjunction with improvements in ADG and ADFI by Upadhaya et al. (2016) after the feeding of a protected organic acid blend (fumaric, citric, malic, capric and caprylic acids), further supporting the hypothesis that the addition of acids to the diet can beneficially modulate the pig's gut microbiota. Li et al (2008) utilized two nursery experiments, with and without an experimental *E. Coli* challenge. In the first experiment, without *E. Coli* challenge, ADG and feed efficiency were significantly improved by the addition of an organic acid blend (Ca-2-hydroxy-4-(methylthio) butanoic acid, fumaric acid, and benzoic acid) to the diet. Potassium diformate resulted in gain and feed efficiency in between that of the control and the acid diet. In a follow-up experiment, pigs were challenged with K:88 *E. Coli* on d14 post-weaning. While no differences were observed overall, in the post-challenge period the acid blend improved ADG and F:G. Again, trends towards increased *lactobacillus* and

decreased *E. Coli* counts in the small intestine support the hypothesis of beneficial modulation of the microbiota (Li et al., 2008).

While improvements in growth and animal health have been shown in many studies, there are also quite a few studies that do not show improvements due to the addition of acidifiers. For example, Boas et al. (2016) fed diets containing a blend of organic acids, sodium butyrate, or their combination to nursery pigs and found no improvements in growth performance or digestibility. The authors noted that there was no diarrhea during this experiment, which could indicate a high health status in this group of pigs. Additionally, all diets in this study contained an AGP, ZnO, and copper sulfate, which may have resulted in superior growth performance. The authors also discuss the potential negative effect of a high dietary buffering capacity (theoretically caused by high levels of dicalcium phosphate and limestone in these diets) on the effectiveness of dietary acidifiers (Boas et al., 2016). These factors could lead to less noticeable effects of the acid products; this highlights diet composition as a key consideration when acidifiers are being evaluated. Buffering capacity, inclusion of other additives (such as antibiotics, zinc, and milk products), and complexity of the diet have all been proposed, but not clearly defined, as dietary factors that may influence the effectiveness of acidifiers (Ravindran and Kornegay, 1993).

While acidifiers have certainly been regarded as a potential replacement or alternative to in-feed antibiotics, their effectiveness has not been consistent (Ravindran and Kornegay, 1993). Proposed reasons for inconsistences include differences in experimental methodology, types of acidifiers, inclusion rate, diet composition, health status of pigs, age of pigs, and time on treatment (Ravindran and Kornegay, 1993; Mroz et al., 2000; Mroz, 2005; Biagi et al., 2007; Boas et al., 2016). However, these have largely been speculation, and none have been

conclusively studied as to how they come into play when acidifiers are being evaluated, and ultimately, included in commercial swine diets.

Probiotics as AGP alternatives

Probiotics, more recently referred to as direct-fed microbials (DFM), are "selected and concentrated" sources of viable bacteria, usually lactic acid producing strains (Kyriakis et al., 1999). Common species added to swine diets include strains of *Lactobacillus, Bacillus, Enterococcus, Bfidobacterium* and yeasts, with strains of *Lactobacillus* and *Bacillus* probably being the most widely studied (Stein and Kil, 2006). Some studies have shown benefits including improved growth performance (Kyriakis et al., 1999; Gebru et al., 2010; Lee et al., 2012), decreased diarrhea (Bhandari et al., 2008; Lee et al., 2012), decreased mortality (Kyriakis et al., 1999), and decreased bacterial shedding during an *E. coli* (Lee et al., 2012) or *Salmonella* (Gebru et al., 2010) challenge. DFM may be useful particularly after weaning, because populations of lactic acid bacteria tend to decrease after weaning (Stensland and Pluske, 2017). This decrease is likely associated with an increase in coliform bacteria populations, such as *E. Coli*, some strains of which are known to cause diarrhea in post-weaning piglets (Doyle, 2001). Therefore, the direct supplementation of viable bacteria via probiotics may be a tool to increase the population of commensal bacteria and lower the population of harmful, pathogenic bacteria. DFM are thought to accomplish this through a mechanism known as competitive exclusion: the bacteria contained in probiotics may adhere to the mucosal layer of the intestine, compete with pathogenic bacteria for nutrients in the intestinal tract, and cause a reduction in the pH of the intestinal lumen (Stensland and Pluske, 2017). These actions are thought to limit the proliferation of potentially pathogenic bacteria, such as coliforms, thus establishing a more "healthy" and

"beneficial" microbiome (Stensland and Pluske, 2017). In addition, bacteria provided by DFM are thought to be capable of producing antimicrobial compounds such as organic acids, hydrogen peroxide, bacteriocins (which are peptides used to compete with other bacteria; Sang and Blecha, 2008), modulating the pigs intestinal immune system, and improving the barrier function of the intestine (Zimmermann et al., 2001; Stensland and Pluske, 2017). Some studies have also shown that DFM can improve digestibility of certain nutrients (Liao and Nyachoti, 2017).

Growth promoting effects of DFM are less consistently than for Zn and acidifiers. Bhandari (2008) observed that, during a pathogenic *E. Coli* challenge, a DFM (*Bacillus subtilis*) had no impact on growth performance of nursery pigs, but reduced scouring and mortality compared to a control group. While there was no detectable difference in mucosal-associated *E. Coli*, an increase in intestinal microbiota richness and diversity in the probiotic-containing treatments indicates that the modulation of the microbiome may be partially responsible for the improvements in piglet health in this study (Bhandari et al., 2008). Similarly, in a 28 d nursery trial, ADG and G:F were significantly improved by feeding $20x10^9$ cfu/kg feed and $4x10^9$ cfu/kg of *Bacillus subtilis*, but not by feeding a lower inclusion rate of $2x10^9$ cfu/kg. *Bacillus subtilis* at all three inclusion levels was successful in reducing diarrhea, and an increase in *Lactobacillus* and decrease in *E. coli* was also observed (Hu et al., 2014). Kyriakis et al. (1999) also showed improvements in performance and mortality with the addition of a *bacillus* probiotic (*Bacillus lichenformis*). In this study, the changes also appeared to be dependent on the inclusion rate of the probiotic, as gain and feed efficiency were impacted more at 10^7 cfu/kg than at 10^6 cfu/kg (Kyriakis et al., 1999). Lee et al. (2012) observed that a *Lactobacillus* DFM improved growth performance, decreased rectal temperatures, and decreased diarrhea and *E. Coli* shedding during an *E. coli* challenge. This study also showed a dose-dependent response to the DFM, where the

highest inclusion level (10^{10} CFU/kg compared to 10^{8} and 10^{9}) appeared to be the most effective (Lee et al., 2012). Improvements in morbidity, diarrhea, and growth performance of nursery pigs with a known history of post-weaning diarrhea caused by *E. coli* were also observed by Papatsiros et al. (2011) due to the addition of a *bacillus* DFM.

Currently, the variation in DFM strains, inclusion rates, length of treatment and "husbandry practices" makes it difficult to draw conclusions about the usefulness of DFM in modern pork production (Kenny et al., 2011; Liao and Nyachoti, 2017). A meta-analysis conducted by Zimmerman (2016) aimed to address factors that may impact probiotic efficacy relating to growth performance. Their results suggest that number of animals is a common limiting factor in probiotic studies, and stage of production also appeared to be a factor (Zimmermann et al., 2016). This meta-analysis attempted to shed light on inconsistencies in results of probiotic studies. However, because of their inclusion parameters, a relatively small number of studies were included, and a major drawback is their exclusion of any studies in which pigs were sick. This prevents any discussion of how health status may be influencing results and ignores the demonstrated benefits of probiotics to piglet health during disease challenge. Presently, it would be very difficult to conduct a meaningful meta-analysis on probiotics due to the variation in experimental components (Kenny et al., 2011). Additionally, analysis of probiotic products and experimental diets for the presence of viable probiotic organisms is rarely reported, which could be a contributing factor to inconsistent responses.

Resistant starch as a prebiotic

Similar to probiotics, prebiotics are occasionally included in swine diets with the intention of establishing and maintaining a healthy microbiome in the gastrointestinal tract. A

prebiotic is defined as a non-digestible ingredient that can beneficially modulate the microbiota in the gut (de Lange et al., 2010) Usually, prebiotics are types of non-digestible carbohydrates, and some common sources are oligofructose, fructooligosaccarides, and inulin (Jacela et al., 2010). Prebiotics act as a substrate for certain microorganisms, thus enhancing their growth and/or activity in the intestinal tract (Zimmermann et al., 2001). Prebiotics may also interfere with the ability of some pathogenic bacteria to adhere to the intestinal wall (Stensland and Pluske, 2017). Recently, resistant starch has been gaining support as a prebiotic and potential alternative to antibiotics (Giuberti et al., 2015). Resistant starch is regarded as a component of dietary fiber and is defined by the American Association of Cereal Chemists as being "resistant to digestion and absorption in the human small intestine"(AACC, 2001). There are different categories of resistant starch depending on how it is created and it's physical and chemical characteristics. This section of the review will focus on the effects of resistant potato starch (RPS), which falls under the second category of resistant starch (RS2): resistant granules (Englyst et al., 1992). This type of resistant starch contains native granules that are naturally resistant to enzyme breakdown due to their physical shape or structure (Giuberti et al., 2015) As demonstrated by Tatsumi et al. (2007), the size of the starch granules in potato starch is relatively large as compared to other starches, such as cereals. This characteristic of potato starch has been suggested as the reason for its resistance to hydrolysis in the small intestine, and thus more starch reaches the large intestine for fermentation (Krause et al., 2010; Giuberti et al., 2015). As compared to probiotics, zinc, and acidifiers, far fewer studies exist that investigate resistant starch as an alternative to antibiotics. However, the prebiotic effects of RPS have been demonstrated to decrease diarrhea in nursery piglets, and thus have generated interest (Bhandari et al., 2009; Krause et al., 2010; Heo et al., 2014).

Bhandari et al. (2009) investigated the inclusion of RPS in a 3-wk nursery study. While they did not observe any meaningful differences in growth performance, they found that including RPS at 7% of the diet reduced fecal scores (thus, decreased diarrhea) so that they were similar to the positive control (AGP). Diets containing 14% RPS resulted in higher fecal scores than the 7% RPS diet, similar to the negative control. This they observed in combination with a decrease in microbial species diversity and richness in the 14% RPS diet. Their results suggest that a more diverse and "rich" microbiome may be beneficial to the pig (Bhandari et al., 2009). Heo et al. (2014) fed diets containing much lower levels of RPS (0.5 or 1% of the diet) to nursery pigs. While growth performance was unaffected, RPS at both inclusion levels improved fecal consistency in the second week of the experiment, and the 1% RPS diet resulted in firmer feces than the 0.5% RPS treatment when averaged over the first two weeks of the study. They also observed increases in total volatile fatty acids, acetate, and propionate in the cecum, and decreases in branched chain fatty acids when pigs were fed RPS, perhaps due to increased substrate for "carbohydrate-utilizing" bacteria. In addition, ileal and cecal digesta pH was also reduced in the RPS treatments. Branched chain fatty acids can be a harmful product of fermentation, and their reduction may be related to the improved fecal scores of the RPS treated pigs (Heo et al., 2014).

In addition to probiotics and prebiotics, an emerging idea is the combination of pre- and probiotics, or multiple prebiotics or probiotics; these combinations are known as synbiotics (Stensland and Pluske, 2017). This is thought by some to be more beneficial as the prebiotics could provide a source of substrate for the probiotic bacteria. (Stensland and Pluske, 2017). Krause et al. (2010) investigated the addition of RPS under conditions of an intentional K88 *E. Coli* challenge. RPS was included at 14% of a nursery diet alone and in combination with an *E.*

Coli derived probiotic, which was derived from a strain specifically selected to inhibit pathogenic K88 *E. Coli*. The RPS and probiotic-containing diets were compared against a positive control diet, which contained antibiotics. Both before and after the challenge, pigs fed RPS alone had lower ADG, but pigs fed the combination RPS/probiotic treatment had the highest ADG. In addition, the RPS/probiotic combination tended to improve feed intake. The RPS/probiotic treatment resulted in fecal scores similar to the positive control, and these were lower than the treatments containing RPS and the probiotic alone. In colon digesta, they observed the highest levels of richness and diversity when pigs were fed the diet containing both RPS and the probiotic (Krause et al., 2010). The results of this experiment begin to justify the combination of prebiotics and probiotics to improve growth performance and reduce diarrhea. However, the absence of a negative control diet in this experiment limits the conclusions that can be drawn about the effectiveness of RPS and the probiotic when they were included alone in the diets (Krause et al., 2010).

Before resistant potato starch can be accepted as an AGP alternative, more studies demonstrating benefits to growth performance and pig health are certainly needed. Proper inclusion rate as well as the impacts of factors such as the age of the pig, interactions with other components of the diet, and disease have yet to be thoroughly investigated.

Importance of consistent evaluation of AGP alternatives

While the benefits of pharmacological levels of ZnO are the most documented and consistent for ingredients discussed thus far in this review, it is true that the proposed benefits of AGP alternatives are inconsistent, and factors that influence their efficacy have not been fully determined. Evidence that products such as organic acids may be more or less effective in the

presence of certain dietary components (Boas et al., 2016) points to the importance of clear and detailed reporting of diet composition. Additionally, attempts to analyze final experimental diets for the AGP alternative being studied are rare, and, since diet mixing, pelleting, transport, and storage can introduce error and impact the viability of some products (such as probiotics) it is possible that the final diet does not always have the intended amount of product. Some evidence exists suggesting that antibiotics are more effective on commercial farms than in academic-type research settings (Cromwell, 2002; Dritz et al., 2002), and this has been hypothesized to be partially due to lower pathogen load and incidences of "sub-clinical" disease in such facilities (Zimmerman, 1986). If it is indeed the case that differences in pathogen presence and disease incidence impact the effectiveness of antibiotics, then it is logical to wonder if these could influence the effects of AGP alternatives as well. However, comments relating to health status are often missing in AGP alternative studies. Many studies exist that evaluate alternatives in situations such as an experimental *E. Coli* challenge, and these have been useful in demonstrating benefits of AGP alternatives to pig health. However, there have been few attempts to assess AGP alternatives in the context of diseases such as porcine reproductive and respiratory syndrome virus, porcine epidemic diarrhea virus, or influenza, which are all common and relevant in today's industry. Evidence of decreases in mortality due to AGP alternatives are of great interest to the swine industry, but reports of mortality and/or morbidity are often absent in published papers. As research continues, a standardized approach to the evaluation of AGP alternatives will increase the consistency of studies, make each study more informative, and will facilitate the comparison of results across studies and to commercial conditions. In general, more research on the effects of AGP alternatives in commercial settings is also necessary to fully understand the role of these ingredients as antibiotic use is reduced.

Effect of group size on pig performance and implications for AGP alternative studies

When looking at the effects of antibiotic alternative ingredients, a possible reason for differences in results obtained across studies is the variation in environment and management of the experimental pigs. One factor that makes up the environment in which pigs are housed is the size of the groups pigs are kept in. This is also a factor that differs between academic research settings and commercial facilities. While group sizes of 20-30 pigs per pen are common in U.S. confinement systems, some modern wean-to-finish facilities are moving towards larger groups of up to 60 to 100 pigs, especially during the nursery phase, in order to maximize space usage and optimize facility cost (Wolter and Ellis, 2002; Ellis and DeDecker, 2010). However, there have been concerns that performance may be reduced when pigs are housed in larger group sizes.

In a 9-wk study with pigs starting at 5.3 kg of body weight, Wolter et al. (2000) found that pigs in groups of 100 had lower ADG and ADFI for the first 4-wk of the study, and lower ADG for wk 4-9 of the study compared to groups of 20. In a commercial wean-to-finish study, Wolter et al. (2001) found that, for the first 8 wk after weaning, pigs in groups of 25 had higher ADG and better feed efficiency than pigs housed in groups of 50 or 100. However, when the overall wean-to-finish period was considered, there were no observable differences in growth performance due to group size (Wolter et al., 2001). Lack of performance differences due to group size in larger pigs has also been reported in other studies (McGlone and Newby, 1994; Nielsen et al., 1995; Schmolke et al., 2003), although increases in morbidity were observed when pigs were kept in groups of 40 rather than 10 or 20 (McGlone and Newby, 1994). Observations of feeding behavior in one study revealed that pigs in groups of 20 made fewer trips to the feeder, but at each visit ate longer and larger quantities, thus maintaining growth and feed intake of pigs housed in smaller groups (Nielsen et al., 1995). A possible explanation for the negative

effects of larger group size on nursery pig performance may be an inability for younger pigs to adapt their feeding behaviors to maintain growth. Hyun and Ellis (2002) showed decreases in growth performance in 26 kg pigs as group size increased from 2, 4, 8, or 12 pigs per pen, and that pigs in groups of 12 had a limited ability to alter their feeding behaviors to maintain performance similar to pigs housed in smaller groups (Hyun and Ellis, 2002). This contrasts the studies that showed no differences in growth for pigs at this stage of growth or bigger; however it is important to note that the largest group size in this study is actually closer to the smallest group size in some of the other studies that did not show a response (McGlone and Newby, 1994; Nielsen et al., 1995; Wolter et al., 2001; Schmolke et al., 2003). In addition, the smallest group sizes in this study (2 and 4) are much smaller than groups in some of the other studies, and are not likely to be found in commercial pork production. However, it would not be uncommon to find group sizes this small in academic research settings, and many of the studies discussed earlier in this review regarding AGP alternatives would have utilized small group sizes such as these.

The results of these studies do not strongly suggest a large influence of group size on performance, and decisions about group size will likely continue to be made based on optimal use of facility space, costs, and management preferences. However, the issue of group size may be relevant in the search for effective alternatives to antibiotics. Many studies that have been done thus far on antibiotic alternatives have taken place in academic-type research facilities, generally utilizing smaller numbers of pigs per pen than typically found in commercial production. While this is understandable due to the cost and labor constraints of many research projects, this may actually hinder our ability to interpret the results of studies in the context of larger, commercial production systems. If nursery pigs are indeed more stressed and experience

depressed growth when housed in larger groups, there may be a greater opportunity for an AGP alternative to exert its beneficial effects than when pigs are housed in small groups. Studies that assess the impact of group size on the efficacy of AGP alternatives are needed.

Conclusion

There is a large volume of research supporting the use of many feed additives in swine diets. While phytase already has a clear role in the swine industry, improvements in its evaluation will allow nutritionists to more accurately formulate diets, thus making feed more economically beneficial for the producer and further reducing the impact of phosphorus excretion on the environment. It appears that AGP alternatives will also play an important role in the industry; indeed, they already are. However, a better understanding of their modes of action and how they interact with other factors such as diet, health, and environmental conditions is needed for their role to be clearly defined and their effectiveness maximized. Overall, careful consideration of experimental methodology and meticulous recording and reporting of details will improve our understanding of additives, allowing for improved diet formulation and ultimately leading to superior performance and profitability in pork production.

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CHAPTER 2

EVALUATING PHOSPHORUS RELEASE BY PHYTASE IN DIETS FED TO GROWING PIGS THAT ARE NOT DEFICIENT IN PHOSPHORUS

A paper in preparation for submission to the *Journal of Animal Science*

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Abstract

Microbial phytase is widely used to enhance digestibility of phytate-P. By tradition, P-deficient diets are used to quantify phytate-P release by phytase, but P-adequate diets may be more physiologically relevant. The objective of this study was to investigate the effects of phytase on P digestion and metabolism and develop a P release curve for phytase in P-adequate diets, and to compare these effects in a P-deficient diet. Three replicates of 24 barrows each (BW = 23.0 ± 1.8 kg) were randomly assigned to 1 of 8 dietary treatments, housed in individual pens for 21 d, then moved to metabolism stalls for 5 d urine and fecal collections. A basal corn-soybean meal diet was formulated at 0.36% standardized total tract digestible (STTD) P and total Ca:STTD P of 1.83. Phytase was added at 200, 400, 600, and 800 phytase units (FTU)/kg. A positive control diet was formulated using monocalcium phosphate (MCP) to increase STTD P by 0.16% to 0.52%, the expected STTD P release of 800 FTU/kg. A P-deficient diet was formulated by reducing MCP to achieve 0.21% STTD P and 200 FTU phytase/kg was added to the P-deficient diet for the eighth treatment. Pig was the experimental unit, and replicate and dietary treatment were fixed effects. Orthogonal polynomial contrasts tested linear and quadratic effects of phytase within the 5 P-adequate diets. Phytase increased percent apparent total tract digestibility (ATTD) and STTD (quadratic $P < 0.001$), and quantity of absorbed P (linear $P < 0.001$; quadratic $P = 0.069$). Urinary P increased linearly with phytase ($P < 0.001$) and retained P also increased (linear $P = 0.001$, quadratic $P = 0.094$). Phytate- P release by adding 200 FTU phytase/kg was predicted to be 0.049% STTD P, respectively. It appears that the effect of phytase may be slightly lower in P-adequate diets as compared to P-deficient diets. whereas there was only a 12% improvement in the STTD P-adequate diet and a 28% improvement in STTD P in the P-deficient diet. In conclusion, phytase improved P digestibility and retention in P-

adequate diets, and P digestibility was used to estimate P release from phytase. Further research investigating phytase P release in P-adequate diets, rather than P-deficient diets, may be needed.

Introduction

The use of microbial phytase to increase P availability has become increasingly common in commercial pig production (Selle and Ravindran, 2008). Previous studies have predicted the release of P by phytase, and dose-response curves have been developed using growth performance or bone characteristics as response criteria (Augspurger et al., 2003; Jones et al., 2010). Bone characteristics, such as ash weight or percent ash, have generally been the most sensitive measurements with which to determine P status in pigs, and are often used to measure P status in relation to phytase concentration in the diet (Cromwell, 2005). Almost all dose-response curves have been developed using diets well below the pig's requirement for P (Augspurger et al., 2003; Jones et al., 2010; Kerr et al., 2010; Gourley et al., 2018). Low P intake as a result of a P-deficient diet may result in greater efficiency of dietary P utilization, due to enhanced absorption, or due to stimulation of P release from bones (Cromwell, 2005; Berndt and Kumar, 2009). Additionally, pigs deficient in P suffer impaired growth and chronic health issues, which affects animal well-being. Thus, phytase release curves developed under conditions of P sufficiency may be more representative of normal physiological conditions and result in improved well-being of experimental animals. Gutierrez et al. (2015) demonstrated that pigs fed diets above their requirement for P excrete excess P in the urine in a linear fashion, meaning that urinary P may be an indicator of P release by phytase.

The primary objectives of this experiment were to evaluate P digestibility and balance in response to phytase addition to a basal diet that meets the pig's requirement for P and to generate

a P release curve for phytase. The secondary objective was to compare phytase effects on P and Ca metabolism and nutrient digestibility in both a P-adequate and a P-inadequate diet. The hypotheses tested were that phytase would improve P digestion and absorption in the P-adequate diets, resulting in increased excretion of P in urine, and that P release by phytase would be lower in the P-adequate diet than the P-deficient diet.

Materials and methods

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Iowa State University (11-16-8379-S).

Animals, Housing, and Management

This experiment was conducted at the Iowa State University Swine Nutrition Farm (Ames, IA). Seventy-two crossbred barrows (Genetiporc 6.0 × Genetiporc F25, PIC, Inc., Hendersonville, TN) with a starting BW of 23.0 ± 1.8 kg were used in 3 replicate trials of 30 d each. Twenty-four barrows were included in each of the 3 replicates and the same procedures were used in each replicate. Pigs were randomly assigned to 1 of 8 dietary treatments and fed the assigned diets for the duration of the 30-d experiment. Within each replicate, pigs were assigned to dietary treatments based on a completely randomized design. For the first 21 d of the experiment, pigs were housed in individual pens (1.8 m x 2.7 m) with slatted floors, equipped with a self-feeder and nipple waterer. Pigs were given feed and water *ad libitum* until d 19, at which point pigs were limit fed at 2.85 times the daily maintenance energy requirement for the average body weight of each replicate (197 kcal of ME/kg $BW^{0.60}$; NRC, 2012). The average BW on d 19 was estimated from d 14 BW to calculate the feed allowance for d 19 and 20; when

pigs were weighed on d 21, the feed allowance was recalculated using the d 21 average BW for each replicate. The daily ration of feed was given in two equal meals at 0800 and 1600 h. If a pig did not consume its entire meal, orts were collected 1 h after feeding and weighed. Pigs were moved to metal metabolism stalls equipped with slatted floors and feeders for the last 9 d of the experiment. In the metabolism stalls, water was given at a 2.7:1 ratio to feed 1 h after initial feeding. Water was controlled in order to limit luxury water consumption (Fraser et al., 1993), but water requirements were met as described in Shaw et al. (2006). Pigs were allowed a 6-d adaptation to the limit feeding schedule and a 4-d adaptation to metabolism stalls. Thus, the total adaptation time to the dietary treatments was 25 d (21 d in pens, 4 d in metabolism stalls).

Experimental Design and Diets

Eight corn-soybean meal-based diets were formulated based on NRC requirements for growing pigs (NRC, 2012). Basal ingredients were assayed for Ca and P content to ensure precision in final diet composition. A basal diet was formulated to meet or exceed all NRC nutrient requirements, including P and Ca. The basal diet was formulated to contain 0.36% standardized total tract digestible (STTD) P, which was above the requirement of 0.31% (NRC, 2012). To achieve the objective of estimating P-release by phytase in P-adequate diets, a microbial phytase (Quantum Blue 5G, 5,000 phytase units (FTU)/g, AB Vista, Marlborough, UK) was added to the basal diet at the expense of corn to achieve levels of 200, 400, 600, and 800 FTU/kg. One FTU is defined as the amount of enzyme activity that liberates 1 μmol inorganic orthophosphate per minute from 0.0051 mol/L sodium phytate at pH 5.5 and 37°C. The sixth diet was formulated as a positive control to compare with the highest level of phytase in the P-adequate diet: monocalcium phosphate (MCP) was added to achieve STTD P equivalent

to the expected STTD P release capabilities of 800 FTU phytase/kg (800 FTU was expected to release 0.16% STTD P; thus, this diet was formulated to contain 0.52% STTD P). To compare the effects of phytase supplementation in a P-deficient diet with phytase supplementation in a P-adequate diet, the seventh diet was formulated to be slightly deficient in STTD P, at 0.21%. This diet was designed to demonstrate potential differences in phytase efficacy due to lower dietary P but not to be deficient enough to cause lameness. To maintain a constant total Ca: STTD P ratio with the P-adequate basal diet, total Ca was also reduced. Phytase was added at 200 FTU/kg to the seventh diet to achieve the eighth diet. The bag of phytase was pre-analyzed for phytase activity and was determined to contain 7,600 FTU/g. This analyzed activity was used to determine the amount of phytase to add to each diet. Titanium dioxide (TiO_2) was added at 0.4% to all the diets as an indigestible marker. To ensure uniformity of diets, ingredients with low inclusion rates including vitamin and trace mineral premixes, MCP, limestone, salt, synthetic AA, TiO_2, and phytase were weighed on an analytical scale and premixed in a small batch mixer (approximately 57 L capacity, Hobart Corporation, Troy, OH) before being added to the bulk ingredients in the main batch mixer. In addition, scales were validated with a standard check weight and precise weights of all ingredients added were obtained and recorded during mixing. All these procedures were followed to ensure the precision of the diet formulation and mixing procedures.

Sample and data collection

To give pigs extra adaptation time to limit feeding prior to entering metabolism stalls, limit feeding began on d 19. Thus, feed disappearance was determined on d 14 and 19. Pigs were weighed on d 0, 14, and 21, before being moved to metabolism stalls, to determine ADG, ADFI,

and G:F. Pigs were also weighed at the end of the metabolism period (d 30). Representative diet samples were collected during mixing, homogenized, and stored at -20°C until further analysis. Samples of MCP, limestone, corn, and soybean meal were also obtained at the time of mixing and stored; samples of the vitamin and trace mineral premixes were taken after mixing, but were the same source and batch used at the time of mixing. Total urine and fecal grab samples were collected twice a day during the last 5 d of the experiment. Urine was collected in acid washed containers pre-loaded with 20 mL of HCl. Urine was filtered through glass wool, subsampled, and stored in acid washed plastic containers at -20°C until further analysis.

Chemical Analysis

After each replicate was completed, urine and fecal samples were thawed at room temperature, homogenized for each pig, and subsampled. Fecal samples were dried in a convection oven at 75°C until a constant weight was achieved. Urine samples were re-stored at -20°C. Diet and dried fecal samples were ground through a 1 mm screen and stored in desiccators. Urine subsamples were thawed, mixed, and filtered through Whatman 41 filter paper (GE Healthcare Life Sciences, Chicago, IL, USA) prior to analysis. Diet and fecal samples were analyzed for DM (method 990.03, AOAC 2007), TiO_2 (Leone, 1973), ash (method 942.45, AOAC, 2007), GE, N, and acid hydrolyzed ether extract (AEE). Gross energy was determined using an isoperibolic bomb calorimeter (Parr 6200 calorimeter; Parr Instruments Co., Moline, IL); benzoic acid (6,318 cal GE/kg; Parr Instruments Co.) was used as the standard for calibration and was determined to be 6,316 ± 7 cal GE/g. Acid hydrolyzed ether-extract (method 2003.06, AOAC 2007) was determined using a SoxCap SC 247 hydrolyzer and a Soxtex 255 semiautomatic extractor (FOSS North America, Eden Prairie, MN). Total N (method 990.03,

AOAC 2007) was analyzed with a TruMac apparatus (Leco Corporation, St. Joseph, MI). Ethylenediaminetetraacetic acid (EDTA; 9.56% N) was used for calibration and was determined to contain 9.58 ± 0.04% N. Crude protein was calculated as N x 6.25.

Diet, fecal, and urine samples were analyzed for P and Ca by inductively coupled plasma optical emission spectrometry (ICP; Optima 7000 DV; PerkinElmer, Waltham, MA) as described by Pogge et al. (2014). Monocalcium phosphate, limestone, corn, soybean meal, and vitamin and mineral premixes were analyzed for Ca and P in the same manner. Prior to ICP analysis, ingredients, diets, and fecal samples were prepared by acid digestion as described by Richter et al. (2012), and urine samples were diluted 1:10 in 1% nitric acid. Analyses were performed in duplicate. A maximum of 1% CV between duplicates was required for GE, N, DM, and ash. A maximum CV of 5% was required for TiO_2 and AEE, and 10% for Ca and P. If the CV between duplicates of a sample exceeded these maximums, the sample was re-run in duplicate. Diets were also analyzed for phytase activity (method 2000.12; AOAC, 2007) and phytate-P content using the method described in the Megazyme phytic acid/total phosphorus kit (K-PHYT; Megazyme, Wicklow, Ireland).

Calculations

To determine the daily mean values for total DM intake and urine output of P and Ca, 5 d totals were recorded and divided by d within each collection period (g/d). Apparent total tract digestibility (ATTD) of nutrients and fecal DM output were calculated according to Oresanya et al. (2008). Standardized total tract digestibility (STTD) of P was calculated as described in NRC (2012), assuming basal endogenous losses of P (EPL) to be 190 mg/kg DMI. Mineral absorption and retention were calculated on a DM basis (g/d) as:

Absorption = mineral intake – mineral in feces

Retention = mineral intake – mineral in feces – mineral in urine

Retention of P, as a percentage of P intake and of P absorbed, were also calculated:

Retention, % of intake = retention / P intake x 100

Retention, % of absorbed = retention / absorbed P x 100

Statistical Analysis

Data were analyzed in a model including the fixed effects of dietary treatment, replicate, and their interaction. The interaction term was removed from the model when not significant ($P > 0.05$). Least-squares means were separated using Tukey's method. Prior to final analyses, outliers (defined as Studentized residuals greater than 3 standard deviations from zero) were identified and removed and normality of the residuals was verified using the Shapiro-Wilk's test. Residual plots were examined to confirm the assumptions of equal variances were met. Orthogonal polynomial contrasts were performed on the first 5 treatments (P-adequate basal diet with 0, 200, 400, 600, or 800 FTU phytase/kg) to test the linear and quadratic effects of phytase level on selected response variables. For these 5 treatments, the dietary treatment x replicate interaction was not significant ($P > 0.05$), so the interaction term was excluded from the model statement used to test linear and quadratic effects of phytase and to generate the appropriate regression equations. Regression estimates were obtained by regressing the response variable against phytase units in the P-adequate diets. Differences were considered significant if $P < 0.05$, and trends if $0.05 < P < 0.10$. SAS 9.4 (SAS Inst. Inc., Cary, NC) was used for all analyses with

GLM and UNIVARIATE procedures used for statistical analyses and outlier identification, respectively.

Results

Across treatments, pigs performed as expected and did not exhibit any signs of skeletal weakness due to P-deficiency. Irrespective of treatments, for the *ad libitum* feeding period, ADG (d 0-21) was 0.74 ± 0.09 kg and ADFI (d 0-19) was 1.61 ± 0.19 kg. Pig BW on d 21 was 38.9 ± 3.0 kg; d 30 BW was 44.3 ± 3.3 kg. A few instances of diarrhea were observed prior to the metabolism period, and pigs were treated with tylosin phosphate; whenever affected pigs were treated, all pigs in that rep were treated as well. Analyzed values for Ca and P in ingredients and diets and phytase in diets confirmed expected formulated values, except for higher Ca content in the vitamin and trace mineral premixes than expected (Tables 1, 2 and 3). This made the final total Ca:STTD P ratio 2.03 in the basal P-adequate diet, and 2.14 in the basal P-deficient diet.

Effect of phytase on P digestibility

In the P-adequate diets, phytase improved ATTD of P in a quadratic fashion ($P < 0.001$, Table 4). In the P-deficient diet, the addition of 200 FTU phytase/kg also improved ATTD of P ($P < 0.05$). The highest ATTD of P was observed in the P-adequate diets with 400, 600 and 800 FTU/kg phytase and the positive control diet. As expected, STTD of P also increased with phytase addition in a pattern similar to ATTD of P (treatment $P < 0.001$).

Effect of phytase on P balance in P-adequate diets

Absorbed P increased with phytase in the P-adequate diets (linear $P < 0.001$, quadradic $P = 0.069$; Table 4). Urinary P excretion increased linearly in response to phytase ($P < 0.001$).

However, retained P also increased in response to phytase (linear $P = 0.001$, quadratic $P = 0.094$). Retention of P as a percentage of intake also increased (quadratic $P = 0.009$) and reached a maximum of 53% in the diets containing 400, 600, and 800 FTU phytase/kg. Fecal and total excretion of P decreased with phytase addition (quadratic $P = 0.001$; quadratic $P = 0.001$ for fecal and total P excretion, respectively).

Effect of phytase on Ca balance in P-adequate diets

Phytase improved ATTD of Ca in the P-adequate diets (linear $P < 0.001$; Table 5), although mean Ca ATTD was similar for diets containing 200, 400, 600, and 800 FTU phytase/kg, as well as for the positive control diet ($P > 0.05$). This corresponded to a linear increase in absorption of Ca ($P < 0.001$). Retained Ca also increased with phytase inclusion (linear $P < 0.001$, quadratic $P = 0.072$). Pigs appeared to excrete basal levels of urinary Ca around 0.14-0.30 g/d for all P-adequate diets, including the positive control ($P > 0.05$). Fecal and total Ca excretion decreased with phytase addition (fecal excretion: linear $P < 0.001$; total excretion: linear $P < 0.001$, quadratic $P = 0.0935$

Effect of phytase on P balance in P-deficient diets

As in the P-adequate diets, 200 FTU phytase/kg improved absorption and retention of P ($P < 0.05$; Table 4) when added to the P-deficient diet. Pigs fed P-deficient diets appeared to excrete basal levels of P in urine, which did not increase when phytase was added to the P-deficient diet ($P > 0.10$). The addition of 200 FTU phytase/kg to the P-deficient diet tended to reduce fecal P excretion ($P = 0.063$) and significantly reduced total excretion of P ($P < 0.05$).

Effect of phytase on Ca balance in P-deficient diets

When added to the P-deficient diet, 200 FTU phytase/kg numerically, but not significantly, improved ATTD of Ca and absorbed Ca ($P > 0.10$; Table 5). The addition of phytase significantly improved retention of Ca ($P < 0.05$), likely through the slight increase in absorption and a reduction in urinary Ca losses ($P < 0.05$). Both P-deficient diets resulted in elevated urinary Ca when compared to the P-adequate diets ($P < 0.05$), which is typical for pigs fed below their requirement for P.

Results of regression analysis for effect of phytase in P-adequate diets

Because retained P, as well as urinary P, increased when phytase was added to the diet, it was determined that urinary P alone would not suffice as a predictor for phytase-P release in this scenario. The increase in ATTD and STTD of P was used to predict P release by phytase. The improvement in P digestibility for a given phytase level, as indicated by the corresponding regression equation (Table 6), was multiplied by the total P content of the diet to determine P release. It was estimated that an additional 0.049, 0.08, 0.093, and 0.09 % STTD P would be released for 200, 400, 600, and 800 FTU phytase/kg, respectively. The improvement in STTD P from 200 FTU phytase/kg added to the P-deficient diet was used to estimate P release and resulted in an estimated 0.059% STTD P release. In the same manner, Ca release by phytase in the P-adequate diets was estimated using ATTD of Ca; it was estimated that an additional 0.022, 0.045, 0.062, and 0.091% ATTD Ca would be released for 200, 400, 600, and 800 FTU/kg, respectively.

Effect of diet on apparent total tract digestibility of energy and other nutrients

In the P-adequate diets, there was a significant phytase effect ($P < 0.001$) on ATTD of DM, with the highest DM ATTD being observed in the diets containing 400, 600, and 800 FTU/kg (Table 7). There was also an effect of phytase in P-adequate diets on ATTD of GE ($P = 0.004$), with the diet containing 400 FTU phytase/kg having the highest while 200, 600, and 800 FTU/kg had intermediate GE ATTD between this diet and the basal P-adequate diet. For ATTD of N, there was a significant effect of phytase in the P-adequate diets with phytase-containing diets having slightly higher ATTD of N than the basal P-adequate diet ($P = 0.038$). The highest ATTD of N was observed in the positive control diet, although only differing significantly from the basal P-adequate diet ($P < 0.05$). Ash ATTD was significantly improved by phytase in the P-adequate diets ($P < 0.001$), and in the P-deficient diet ($P < 0.05$). There was a tendency for phytase to improve ATTD of AEE in the P-adequate diets ($P = 0.06$). The P-deficient diet with 200 FTU phytase/kg had significantly higher ATTD of AEE than the P-deficient diet without phytase ($P < 0.05$). The positive control diet had the highest ATTD of AEE (overall treatment $P < 0.001$).

Discussion

A portion of the P in most pig diets is almost always bound as myo-inositol hexakisphosphate (IP_6), or phytate, and is therefore largely unavailable for absorption by the pig (Selle and Ravindran, 2008). For this reason, the inclusion of microbial phytase, which can hydrolyze phytate to release P, has become common practice in commercial swine diets. To effectively formulate diets with phytase, precise estimates for P release are necessary. The traditional approach to evaluating P release involves feeding P-deficient diets and quantifying responses in bone characteristics or growth performance to graded levels of increased phytase

(Selle and Ravindran, 2008). Feeding diets severely deficient in P can cause reductions in growth performance and bone mineralization and lead to welfare issues (Cromwell, 2005; Jones et al., 2010; Vigors et al., 2014). Hypercalciuria (excessive excretion of Ca in urine) may also be observed at very low levels of dietary P (Gutierrez et al., 2015). Therefore, feeding P-adequate diets when evaluating phytase may be more representative of normal physiological conditions. Therefore, the objectives of this study were to investigate the effects of microbial phytase on P and Ca balance in pigs fed P-adequate diets, to investigate urinary P as a predictor of phytate P release, to develop a P-release curve for phytase, and to compare the effects of phytase on P and Ca metabolism and nutrient digestibility in a P-adequate diet to a P-deficient diet.

As expected, phytase increased ATTD, STTD, and absorbed P, and decreased fecal excretion of P. Although phytase caused a linear increase in urine P when pigs were fed P-adequate diets, retained P was also increased. Letourneau-Montminy et al. (2012) demonstrated that retained P increased with increased P availability when a proper Ca:P ratio was used. Results from Gutierrez et al. (2015) indicated that, although pigs began excreting more P in urine after requirement for growth was met (at 4.96 g/d STTD P intake), femur mineral content continued to increase suggesting that pigs can retain more P and Ca in bone than required for growth. The established requirement for P is largely based on growth performance, rather than bone development. In other words, while 0.31% STTD P may be the amount required to maximize growth (NRC, 2012) it may not necessarily be the amount required to maximize bone development and mineral retention. Stein et al. (2008) observed a similar response for retained P supplied as MCP in the diet and Ca:P ratios remained constant, suggesting that pigs have a higher capacity to retain P in the body than what is necessary for growth. Dietary Ca was also made more available by phytase in the current experiment (ATTD improved from 48.4 to 60.6%

with 800 FTU phytase/kg), and all excess Ca appears to have been retained with no increase in urinary Ca excretion. Thus, while urinary excretion of P was increased due to phytase, some of the excess P made available by phytase was also retained in the body through a simultaneous increase in the availability of Ca.

Because retained P also increased with phytase inclusion in the adequate diets, the quantification of urinary P alone was not sufficient to estimate P release by phytase. Improvements in digestibility of P as ATTD % and STTD % were used to estimate P release (Table 6). The present estimates for STTD P release were lower than the manufacturer's STTD P suggestions of 0.08, 0.12, 0.14, and 0.16% for 200, 400, 600, and 800 FTU/kg, respectively.

When 200 FTU/kg was added to the P-deficient diet, STTD of P was improved by 28%, but only by 12% when added to the P-adequate diet. Based on the increase in STTD P, 200 FTU/kg phytase released 0.059% STTD P in P-deficient diet, whereas this release value in the P-adequate diets, as estimated by the prediction equation, to be 0.049%. The P-deficient diet in this study was formulated at 68% of NRC requirement, whereas many studies evaluating phytase use basal diets with P as low as 20-40% of requirement (NRC, 1998; Augspurger et al., 2003; Jones et al., 2010; Kerr et al., 2010; Gourley et al., 2018). Lowered phytase efficacy when P-adequate diets are used has been previously reported (Fan et al., 2005; Almeida et al., 2013; Rodehutscord, 2016). Several hypotheses for decreased phytase activity in adequate or high-P diets have been proposed. To meet the P requirement, inorganic P in the form of MCP or dicalcium phosphate is often added. Since inorganic P is the final hydrolysis product of phytase, it is possible that inorganic P inhibits phytase activity (Greiner et al., 1993; Rodehutscord, 2016). Pigs may respond to P-deficiency by increasing the efficiency of P-uptake in the intestine (Berndt and Kumar, 2009; Saddoris et al., 2010), or by increasing the mobilization of P from bone

(Cromwell, 2005). Additionally, a buffering effect from increased Ca, usually present in the form of limestone or MCP in P-adequate diets, has been proposed as a reason for reduced efficacy of phytase in P-adequate diets (Almeida et al., 2013); it seems likely that pH in the gastrointestinal tract influences the action of phytase (Selle et al., 2000). While the actual difference in STTD P release for 200 FTU/kg phytase added to the P-adequate compared to P-deficient diet in this study was not large, it does appear that phytase was more effective when added to the P-deficient diet than to the P-adequate diet. It is possible that this difference could be exaggerated in diets that are more limiting in P. Therefore, further research developing phytase release curves with P-adequate diets and comparing the effects of phytase in P-adequate diets and P-deficient diets is warranted.

Urine P excretion increased as expected due to phytase addition in the P-adequate diets, confirming that the diets were, in fact, above P requirement (Gutierrez et al., 2015). The urine P excretion data may be an indication that the P releasing capabilities of phytase were not as high as expected, as the positive control diet resulted in much higher urine P excretion than the diet containing 800 FTU/kg phytase. This was further confirmed, as previously mentioned, by the ATTD, STTD and absorbed P data, and ultimately by the resulting prediction curve. Pigs fed P-deficient diets appeared to exhibit basal urinary P losses, and these pigs retained 99% of absorbed P, compared to 96% or less when pigs were fed P-adequate diets. The stagnation of urine Ca excretion as P absorption increased also indicates that the P requirement was satisfied, although the basal losses of Ca in this study appeared to be slightly lower than the estimate of 0.4 g/d reported by Gutierrez et al. (2015). Excess excretion of Ca in urine can occur when pigs are fed below their requirement for P, due to the lack of P available to combine with Ca in bone (Stein et al., 2008; Gutierrez et al., 2015). Pigs fed P-deficient diets had higher levels of urine Ca

excretion, which was slightly decreased when P was made more available by using phytase, further confirming that the design of P-adequacy and slight P-deficiency was achieved in the respective diets.

A few other possible explanations for P-release values below manufacturer suggestions in P-adequate diets may also be the result of dietary Ca or substrate limitations. As the substrate for phytase, phytate-P must be present at adequate quantities for phytase to be effective in improving P availability. Phytate-P should not have been a limiting factor in this study (Dersjant-Li et al., 2015; Zeng et al., 2016), although it is recognized that phytase efficacy is likely a function of dietary phytate content (Selle and Ravindran, 2008). It is believed that high dietary Ca: P ratio negatively impacts phytase efficacy and P digestibility (Selle et al., 2000). The Ca:P ratios used in the present study were likely not wide enough to cause issues (Beaulieu et al., 2007; González-Vega et al., 2016). Furthermore, the issue of wide Ca:P seems to be less critical when diets are at or above the pig's requirement for P (Wu et al., 2018). However, this does not necessarily rule out the potential for higher total Ca, regardless of the ratio, to inhibit phytase as previously discussed.

The formation of Ca-phytate complexes in the pig's gastrointestinal tract reduces the availability of Ca, and roughly one third of dietary Ca may be present in these complexes (Selle et al., 2009). Improvements in ATTD of Ca due to phytase are therefore expected and have been observed in many other experiments (González-Vega et al., 2013; González-Vega et al., 2015; Zeng et al., 2016; Blavi et al., 2017). Relative to P, there are few studies that estimate phytase's Ca releasing capacity. Using ATTD of Ca as the response variable, the results predicted 0.022% Ca release for every 200 FTU/kg phytase added to the P-adequate diet. This value corresponded to similar improvements in Ca digestibility reported by Selle et al., (2009) and González-Vega et

al., (2015). One phytate molecule may bind up to 5 or 6 Ca molecules (Selle et al., 2009), and most phytases are assumed to release Ca and P in a ratio of 1:1 or higher since IP6 and IP5 have greater affinities for Ca than IP4 or IP3 (Cowieson et al., 2011). The present results show release of Ca and P in a ratio of roughly 0.5:1 for lower phytase inclusions, although this ratio nears 1:1 as phytase inclusion increases to 800 FTU/kg since the response was linear for Ca but quadratic for P. These results challenge the assumptions of Selle et al., (2009) and suggest that in vivo Ca release may differ from the theoretical models described. They also indicate that more Ca than expected may be bound to lower esters and for phytase to release Ca from IP4 or IP3, higher concentrations of phytase are required to rapidly and nearly completely degrade phytate to inositol (Holloway, 2016). In addition, Ca as well as P, are reported to significantly reduce phytate P hydrolysis by phytase (Rodehutscord, 2016) further supporting the use of a Ca matrix with phytase supplementation.

When phytase was added to the P-deficient diet, there was only a numerical improvement in Ca ATTD. Inorganic Ca from limestone and MCP can bind phytate found in corn and soybean meal (González-Vega et al., 2015); thus lower amounts of inorganic Ca may result in fewer Ca-phytate complexes. The observation of higher Ca ATTD in the P-deficient diet compared to the P-adequate basal diet may be a result of this effect, possibly lowering the impact of phytase.

Phytate can also bind and decrease the availability of other nutrients including amino acids, starch (either through hydrogen bonds with the phosphate group or binding starch-associated proteins (Thompson, 1993)) and fatty acids (Angel et al., 2002; Johnston et al., 2004; Bohlke et al., 2005). Thus, it is not surprising to see improvements in GE and N ATTD due to phytase addition, and is supported by Selle et al. (2000), Adeola and Cowieson (2011), Almeida et al. (2013), Zouaoui et al. (2018). Improvements in energy utilization as reviewed by Adeola

and Cowieson (2011) are variable, and most of the mechanistic effects have been observed in poultry, rather than swine. It has been suggested that phytate decreases fat digestibility, and that phytase should mitigate this effect (Camden et al., 2001; Selle et al., 2003; Vigors et al., 2014). The present results agree with this theory, and have also been demonstrated by Jang et al., (2017). Interestingly, although data from Almeida et al. (2013) suggest phytase only improves N and GE digestibility when P-deficient diets are used, the current data demonstrate that phytase can improve digestibility of N and GE in P-adequate diets as well. Conversely, the present data show no improvement in DM, GE, or N ATTD when phytase was added to the P-deficient diet. The P-deficient diet without phytase had slightly higher DM, GE, and N digestibility than the P-adequate diet without phytase; perhaps there were fewer anti-nutritional phytate complexes (such as phytate-protein complexes) formed in this diet, thus less potential for improvement due to phytase (Selle et al., 2000). This observation is consistent with Johnston et al. (2004) who reported higher ileal and hind-gut digestibility of N and GE in diets with reduced P and Ca content. Phytase's improvement of AA digestibility in swine diets seems to be inconsistent (Selle et al., 2012). Nonetheless, improvements in amino acid and energy availability due to phytase are more widely recognized (Dersjant-Li et al., 2015).

In general, nutrient digestibility was higher for the positive control diet than for the basal P-adequate diet, even though the only difference between these two diets was an increase in MCP and a decrease in limestone, resulting in higher P and the same total Ca content. González-Vega et al. (2015) reported higher ATTD of Ca in MCP than in limestone, suggesting that the Ca from limestone is less soluble, less digestible, or more easily bound to phytate than the Ca from monocalcium phosphate. The reason for greater digestibility of AEE and N in this diet is not entirely known; a possibility is that less Ca from limestone may have led to lower levels of

insoluble Ca-soaps or Ca-phytate complexes binding fatty acids and amino acids (Almeida et al., 2013; Tancharoenrat and Ravindran, 2014).

In conclusion, these data support the growing body of literature demonstrating that phytase can positively impact digestion and utilization of P, Ca, and other nutrients. In P-adequate diets, phytase increased digestibility, absorption, retention, and urinary excretion of P. A prediction equation for STTD P release by phytase in P adequate diets was developed; it indicated that an *E. coli* phytase released slightly less STTD P than previously determined from P-deficient diets. There appears to be a difference in the P-releasing capacity of phytase when P-adequate, rather than P-deficient, diets were used for evaluation. Therefore, P-release values determined using models with P-deficient diets should be validated in P-adequate diets or diets marginally deficient in P. Furthermore, the Ca releasing capacity of phytase should be considered when formulating diets to achieve a proper Ca:STTD P ratio, recognizing that adding phytase to a diet will likely improve utilization of both P and Ca. Most importantly, these matrix values need verification in longer term growth and bone metabolism studies in order to confirm their value in the field.

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Table 2.1. Ingredient and nutrient composition of experimental diets (as-fed basis)

	Dietary Treatment[1] (STTD P, %)		
	0.36[1]	0.52	0.21[2]
Ingredient, %			
Corn	72.38	71.88	73.56
Soybean meal (47.5% CP)	23.20	23.20	23.20
Soybean oil	0.80	0.80	0.80
Limestone	0.92	0.51	0.60
Monocalcium phosphate	1.12	2.02	0.26
L-Lys HCl	0.30	0.30	0.30
DL-Met	0.06	0.06	0.06
L-Thr	0.07	0.07	0.07
Vitamin Premix[3]	0.20	0.20	0.20
Mineral Premix[4]	0.15	0.15	0.15
Titanium dioxide	0.40	0.40	0.40
Salt	0.40	0.40	0.40
Calculated nutrients, %			
STTD P	0.36	0.52	0.21
Total P	0.63	0.81	0.46
Total Ca	0.66	0.66	0.38
Ca: STTD P[5]	1.83	1.27	1.83
NE, Mcal/kg	2.49	2.47	2.52
SID Lys	0.98	0.98	0.98
SID Met	0.30	0.30	0.30
SID TSAA	0.50	0.50	0.50
SID Thr	0.59	0.59	0.59
SID Trp	0.17	0.17	0.17

[1]There were 5 diets containing 0.36% standardized total tract digestible (STTD) P. Phytase was added at the expense of corn in the following amounts: 0, 200, 400, 600, 800 phytase units (FTU/kg; (Quantum Blue, AB Vista, Marlborough, U.K.))

[2]There were 2 diets containing 0.21% STTD P. Phytase was added at the expense of corn at 0 or 200 FTU/kg phytase (Quantum Blue, AB Vista, Marlborough, U.K.)

[3]Premix provided per kg of complete diet: 6,125 IU vitamin A, 700 IU vitamin D3, 50 IU vitamin E, 3 mg vitamin K, 11 mg riboflavin, 56 mg niacin, 27 mg pantothenic acid, 24 mg vitamin B_{12}

[4]Premix provided per kg of complete diet: 165 mg Fe (ferrous sulfate), 165 mg Zn (zinc sulfate), 39 mg Mn (manganese sulfate), 16.5 mg Cu (copper sulfate), 0.3 mg I (calcium iodate), 0.3 mg Se (sodium selenite)

[5]Estimated STTD Ca of basal diet was 0.51%

Table 2.2 Analyzed nutrient composition of experimental diets (as-fed basis)

	Dietary Treatment[1]							
Diet STTD P, %	Adequate, 0.36%					PC,0.52%	Deficient, 0.21%	
Phytase, FTU/kg	0	200	400	600	800	0	0	200
Nutrient, %								
DM	88.68	88.40	88.51	88.57	88.60	88.40	88.42	88.29
GE, Mcal/kg	3.91	3.92	3.95	3.93	3.93	3.93	3.99	4.01
CP	15.26	15.64	16.15	15.95	14.97	15.71	16.02	16.08
AEE[2]	4.16	4.14	4.12	4.20	4.06	4.09	4.04	4.30
Ash	4.66	4.84	4.86	4.79	4.81	5.22	4.00	3.94
Total Ca[3]	0.73	0.73	0.73	0.73	0.73	0.73	0.45	0.45
Total P[3]	0.63	0.63	0.63	0.63	0.63	0.80	0.46	0.46
Phytate-P	0.23	0.24	0.24	0.23	0.23	0.24	0.24	0.23

[1]There were a total of 8 dietary treatments. Diets 1-5 were P-adequate diets: (0.36 % standardized total tract digestible (STTD) P) with 0, 200, 400, 600, or 800 phytase units (FTU) phytase/kg (Quantum Blue, AB Vista, Marlborough, U.K.), respectively. A sixth diet (positive control, PC) was formulated with increased monocalcium phosphate to reach 0.52% STTD P. Diets 7 and 8 were P-deficient diets (0.21% STTD P) with 0 or 200 FTU/kg phytase, respectively. Phytase was added at the expense of corn.

[2]AEE=Acid hydrolyzed ether extract

[3]Ca and P were calculated based on analysis of ingredients for Ca and P

Table 2.3. Analyzed total P and Ca content of ingredients[1] (as-fed basis)

Ingredient	P, %	Ca, %
Corn	0.31	0.01
Soybean meal	0.78	0.50
Limestone	-	38.90
Monocalcium phosphate	19.80	17.80
Vitamin premix	0.06	19.70
Mineral premix	0.06	7.60

[1] Samples were analyzed for P and Ca by inductively coupled plasma optical emission spectrometry (ICP; Optima 7000 DV; PerkinElmer, Waltham, MA) as described by Pogge et al. (2014)

Table 2.4. Least square means for the effect of dietary treatment on digestibility (ATTD) and balance of P in growing pigs

	Dietary treatments[1]												
Diet STTD P, %	Adequate, 0.36					0.52, PC	Deficient, 0.21			*P*-value			
FTU/kg	0	200	400	600	800	0	0	200	SEM	TRT[2]	PHY[3]	Lin[3]	Quad[3]
P intake, g/d	9.45^{b}	9.58^{b}	9.27^{b}	9.40^{b}	9.56^{b}	12.05^{c}	6.66^{a}	6.97^{a}	0.121	<0.001	-	-	-
ATTD, %	46.42^{b}	52.53^{c}	60.12^{d}	60.47^{d}	60.40^{d}	60.51^{d}	41.21^{a}	53.77^{c}	1.12	<0.001	<0.001	<0.001	0.001
STTD[4], %	49.94^{a}	56.08^{b}	63.58^{cd}	63.97^{d}	63.96^{d}	63.25^{cd}	45.75^{a}	58.56^{bc}	1.15	<0.001	<0.001	<0.001	0.001
Absorbed, g/d	4.40^{c}	5.04^{cd}	5.43^{de}	5.70^{de}	5.79^{e}	7.29^{f}	2.75^{a}	3.57^{b}	0.156	<0.001	<0.001	<0.001	0.069
Retained, g/d	4.20^{bc}	4.69^{cd}	4.95^{cd}	5.04^{d}	5.03^{d}	5.08^{d}	2.73^{a}	3.55^{b}	0.167	<0.001	0.004	0.001	0.094
Urine, g/d	0.19^{ab}	0.35^{bc}	0.48^{cd}	0.66^{de}	0.76^{e}	2.28^{f}	0.02^{a}	0.03^{a}	0.055	<0.001	<0.0001	<0.001	0.782
Fecal, g/d	5.05^{a}	4.54^{a}	3.84^{b}	3.70^{b}	3.77^{b}	4.76^{a}	3.90^{b}	3.40^{b}	0.116	<0.001	<0.001	<0.001	0.001
Total excreted, g/d	5.25^{b}	4.89^{cb}	4.32^{de}	4.36^{cde}	4.53^{cd}	6.97^{a}	3.93^{e}	3.26^{f}	0.132	<0.001	<0.001	<0.001	0.001
Retention, % of absorbed	95.67^{de}	93.13^{cd}	90.83^{bcd}	88.44^{cb}	86.62^{b}	69.76^{a}	99.19^{e}	99.08^{e}	1.093	<0.001	<0.001	<0.001	0.744
Retention, % of intake	44.35^{bc}	48.90^{cd}	53.07^{c}	53.51^{c}	52.41^{c}	42.21^{a}	40.87^{a}	53.47^{c}	1.56	<0.001	0.007	<0.001	0.009

[1] There were a total of 8 dietary treatments. Diets 1-5 were P-adequate diets: (0.36 % standardized total tract digestible (STTD) P and 0.73% total Ca) with 0, 200, 400, 600, or 800 phytase units (FTU)/kg phytase (Quantum Blue, AB Vista, Marlborough, U.K.), respectively. A sixth diet (positive control, PC) was formulated with increased monocalcium phosphate to reach 0.52% STTD P and 0.73% total Ca. Diets 7 and 8 were P-deficient diets (0.21% STTD P and 0.45% total Ca) with 0 or 200 FTU/kg phytase, respectively. Phytase was added at the expense of corn.

[2] *P*-value for overall effect of dietary treatment.

[3] Orthogonal polynomial contrasts were performed to test the effect of phytase level in the 5 P-adequate diets (0.36% STTD P). PHY represents the overall *P*-value for effect of phytase in these diets. Lin and Quad represent the *P*-values for the linear and quadratic effects of phytase level.

[4] STTD P was calculated assuming 190 mg endogenous P losses/kg DMI based on NRC (2012).

[a-f] Means lacking the same superscript are statistically the same ($P > 0.05$) based on Tukey's Method.

Table 2.5. Least square means for the effect of dietary treatment on apparent total tract digestibility (ATTD) and balance of Ca in growing pigs

	Dietary treatments[1]												
Diet STTD P, %	Adequate, 0.36					0.52, PC	Deficient, 0.21			*P* - value			
Phytase, FTU/kg	0	200	400	600	800	0	0	200	SEM	TRT P[2]	PHY[3]	Lin[3]	Quad[3]
Ca intake, g/d	10.71^{b}	10.86^{b}	10.52^{b}	10.66^{b}	10.82^{b}	10.70^{b}	6.30^{a}	6.60^{a}	0.124	0.001	-	-	-
ATTD, %	48.36^{a}	52.93^{ab}	59.89^{bc}	58.87^{bc}	60.64^{bc}	59.57^{bc}	58.64^{bc}	66.53^{c}	2.195	<0.001	0.001	<0.001	0.110
Absorbed, g/d	5.18^{bc}	5.76^{cd}	6.39^{d}	6.32^{cd}	6.57^{d}	6.37^{d}	3.70^{a}	4.41^{ab}	0.238	<0.001	0.001	<0.001	0.139
Retained, g/d	4.87^{c}	5.57^{cd}	6.24^{d}	6.12^{d}	6.43^{d}	6.22^{d}	2.33^{a}	3.62^{b}	0.225	<0.001	<0.001	<0.001	0.072
Urinary, g/d	0.30^{a}	0.19^{a}	0.14^{a}	0.20^{a}	0.14^{a}	0.15^{a}	1.37^{c}	0.78^{b}	0.048	<0.001	0.535	0.197	0.440
Fecal, g/d	5.53^{a}	5.10^{ba}	4.46^{b}	4.34^{b}	4.26^{b}	4.33^{b}	2.60^{ca}	2.19^{ca}	0.231	<0.001	0.001	<0.001	0.165
Total excreted, g/d	5.84^{a}	5.28^{ba}	4.61^{bc}	4.54^{bc}	4.40^{bc}	4.48^{bc}	3.96^{dc}	2.97^{d}	0.224	<0.001	<0.001	<0.001	0.094

[1] There were a total of 8 dietary treatments. Diets 1-5 were P-adequate diets: (0.36 % standardized total tract digestible (STTD) P and 0.73% total Ca) with 0, 200, 400, 600, or 800 phytase units (FTU)/kg phytase (Quantum Blue, AB Vista, Marlborough, U.K.), respectively. A sixth diet (positive control, PC) was formulated with increased monocalcium phosphate to reach 0.52% STTD P and 0.73% total Ca. Diets 7 and 8 were P-deficient diets (0.21% STTD P and 0.45% total Ca) with 0 or 200 FTU/kg phytase, respectively. Phytase was added at the expense of corn.

[2] *P*-value for overall effect of dietary treatment.

[3] Orthogonal polynomial contrasts were performed to test the effect of phytase level in the 5 P-adequate diets (0.36% STTD P). PHY represents the overall *P*-value for effect of phytase in these diets. Lin and Quad represent the *P*-values for the linear and quadratic effects of phytase level.

[a-d] Means lacking the same superscript are statistically the same ($P > 0.05$) based on Tukey's Method.

Table 2.6. Regression coefficients of the effect of phytase units (FTU/kg) on P and Ca balance in P-adequate diets[1]

	Intercept		Linear		Quadratic			
Trait	Estimate	SE	Estimate	SE	Estimate	SE	*P*-value[2]	$R^{2\,3}$
P, g/d								
Absorbed	4.58	0.17	0.0017	0.00036	-	-	<0.001	0.30
Retained	4.38	0.17	0.0009	0.00035	-	-	0.001	0.15
Urine	0.20	0.05	0.0007	0.0001	-	-	<0.001	0.48
Fecal	5.11	0.11	-0.00416	0.0007	0.0000031	0.0000008	0.001	0.53
Total	5.30	0.11	-0.00336	0.00072	0.000003	0.0000009	0.001	0.31
ATTD[4], %	46.16	1.31	0.0458	0.0078	-0.000035	0.000009	<0.001	0.60
STTD[5], %	49.54	1.33	0.0457	0.0080	-0.000035	0.00001	<0.001	0.59
Ca, g/d								
Absorbed	5.38	0.25	0.00167	0.00057	-	-	<0.001	0.16
Retained	5.05	0.28	0.00183	0.00057	-	-	<0.001	0.23
Fecal	5.40	0.20	-0.00166	0.00040	-	-	<0.001	0.24
Total	5.86	0.23	-0.00385	0.00138	-0.0000026	0.0000017	0.094	0.31
ATTD[4], %	50.02	1.87	0.01524	0.00381	-	-	<0.001	0.21

[1] Regression on phytase units (FTU/kg) in the 5 P-adequate diets (0.36% standardized total tract digestible (STTD) P and 0.73% total Ca)

[2] *P*-value for the highest-order regression component

[3] R^2 was calculated by taking the sum of squares for the selected regression model divided by the total sum of squares minus the sum of squares for the three dietary treatments not included in the regression analysis.

[4] Apparent total tract digestibility.

[5] STTD P was calculated assuming 190 mg endogenous P losses/kg DMI based on NRC (2012).

Table 2.7. Effect of dietary treatment on apparent total tract nutrient digestibility (ATTD)

	Dietary treatments[1]										
Diet STTD P, %	Adequate, 0.36					0.52, PC	Deficient, 0.21			*P* - value	
Phytase, FTU	0	200	400	600	800	0	0	200	SEM	TRT P[2]	PHY[3]
DM, %	85.00[a]	86.07[ab]	87.13[b]	86.71[b]	86.89[b]	87.33[b]	86.22[ab]	87.19[a]	0.356	<0.001	<0.001
GE, %	84.69[a]	85.60[ab]	86.52[b]	86.05[ab]	86.14[ab]	87.34[b]	85.72[ab]	86.45[ab]	0.389	<0.001	0.004
N, %	82.07[a]	84.85[ab]	85.55[ab]	84.89[ab]	85.24[ab]	86.54[b]	84.19[ab]	84.37[ab]	0.856	0.032	0.038
Ash, %	42.17[a]	49.40[b]	56.98[d]	53.95[cd]	55.19[d]	54.16[d]	50.54[bc]	55.19[d]	0.846	<0.001	<0.001
AEE[4], %	50.92[a]	51.30[a]	51.66[a]	54.19[ab]	51.05[a]	59.30[c]	53.00[a]	57.58[bc]	0.934	<0.001	0.060

[1] There were a total of 8 dietary treatments. Diets 1-5 were P-adequate diets: (0.36 % standardized total tract digestible (STTD) P and 0.73% total Ca) with 0, 200, 400, 600, or 800 FTU/kg phytase (Quantum Blue, AB Vista, Marlborough, U.K.), respectively. A sixth diet (positive control, PC) was formulated with increased monocalcium phosphate to reach 0.52% STTD P and 0.73% total Ca. Diets 7 and 8 were P-deficient diets (0.21% STTD P and 0.45% total Ca) with 0 or 200 FTU/kg phytase, respectively. Phytase was added at the expense of corn.

[2] *P*-value for overall effect of dietary treatment.

[3] Orthogonal polynomial contrasts were performed to test the effect of phytase level in the 5 P-adequate diets (0.36% STTD P). PHY represents the overall *P*-value for effect of phytase in these diets.

[4] AEE = Acid hydrolyzed ether extract.

[a-d] Means lacking the same superscript are statistically the same ($P > 0.05$) based on Tukey's Method

CHAPTER 3

THE EFFECTS OF GROUP SIZE AND SUB-THERAPEUTIC ANTIBIOTIC ALTENRATIVES ON GROWTH PERFORMANCE AND MORBIDITY OF NURSERY PIGS: A MODEL FOR FEED ADDITIVE EVALUATION [1]

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Abstract

The objectives of this experiment were to evaluate the effects of alternatives to antibiotic growth promoters (AGP), two group sizes, and their interaction on nursery pig performance to serve as a model for future AGP alternative studies. A 41-d experiment was conducted in a commercial wean-to-finish barn; 1,300 piglets weaned at 21-d of age (weaned 2 or 4 days prior to experiment; 6.14 ± 0.18 kg BW; PIC 1050 sows and multiple sire lines) were blocked by sire, sex, and weaning date, then assigned to 8 treatments: 4 dietary treatments each evaluated across 2 group sizes. The 4 dietary treatments were: negative control (NC), positive control (PC; NC + in-feed antibiotics), zinc oxide plus a dietary acidifier (blend of fumaric, citric, lactic and phosphoric acid) (ZA; NC + ZnO + acid), and a *bacillus*-based direct-fed-microbial plus resistant potato starch (DR; NC+DFM+RS). The 2 group sizes were 31 or 11 pigs/pen; floor space was modified so area/pig was equal between the group sizes (0.42m^2/pig). There were 7 pens/diet with 11 pigs/pen and 8 pens/diet with 31 pigs/pen. Data were analyzed as a randomized complete block design with pen as the experimental unit. Diagnostic assessment of oral fluids, serum, and tissue samples was used to characterize health status. Pigs experienced natural challenges of acute diarrhea and septicemia in week 1 and porcine reproductive and respiratory syndrome virus (PRRSV) in weeks 4-6. There was a significant interaction between diet and group size for ADG ($P = 0.012$). PC increased ADG in large and small groups ($P < 0.05$) and ZA increased ADG only in large groups ($P < 0.05$). Small groups had improved ADG compared to large groups when fed NC or DR diets ($P < 0.05$). Similarly, PC increased ADFI ($P < 0.05$). Compared to NC, ZA improved ADFI in large groups only ($P < 0.05$; diet*group size: $P = 0.015$). Pigs fed PC had greater G:F than NC ($P < 0.05$), and small groups had greater G:F than large groups ($P < 0.05$). There was no effect of ZA or DR on G:F. Pigs fed PC required fewer individual medical

treatments than NC and pigs fed ZA were intermediate ($P = 0.024$). More pigs were removed from large than small groups ($P = 0.049$), and there was no effect of diet on removals ($P > 0.10$). In conclusion, careful study design, protocol implementation, sample collection, and recording of important information allowed us to characterize the health status of this group of pigs and determine treatment effects on growth performance and morbidity.

Keywords: antibiotic growth promoter (AGP), pen size, porcine reproductive and respiratory syndrome virus (PRRSV), swine

Introduction

Consumer interest in pork raised without antibiotics or with limited antibiotics and the introduction of the Veterinary Feed Directive in the United States have encouraged producers to look for alternatives to antibiotic growth promoters (AGP) in feed. There are many products already available that may be considered alternatives to AGPs. However, the efficacy of AGP alternatives in commercial pork production has not been clearly defined, and the results of AGP alternative studies are often inconsistent (Jacela et al., 2009; Jacela et al., 2010; Thacker, 2013; Liao and Nyachoti, 2017). This may be due to inconsistent experiment methodology, including differences in health status, genetics, experimental conditions, and diet composition (Allen et al., 2013). This leaves a significant gap in knowledge about the effectiveness of AGP alternatives and the ability to make comparisons or observe trends across studies. To most efficiently identify useful AGP alternatives and apply them in production, it is necessary first to increase the consistency with which studies evaluating AGP alternatives are conducted. Therefore, there is a need for an example protocol with guidelines for AGP alternative studies.

Most published studies evaluating AGP alternatives have been conducted in academic research settings, which typically house fewer pigs per pen than commercial production facilities. Because group size may impact pig performance, specifically in the nursery phase (Wolter et al., 2000), one may question whether the results of such studies could be different in a commercial setting. Furthermore, inherent environmental differences between academic research facilities and commercial pork production facilities create the need for more commercial-scale data.

The objective of this experiment was test the effects of two different group sizes and AGP alternative diets on nursery pig growth performance, in order to serve as a framework for future AGP alternative studies to ensure progress in assessing the scientific merit of said studies as rapidly as possible and to facilitate the comparison of experimental results across multiple studies.

Materials and methods

All experimental procedures were reviewed and approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC# 3-17-8465-S). The study was conducted in central Iowa in April and May 2017.

Animals, housing and management

One room of a commercial wean-to-finish research barn was populated with 1,300 barrows and gilts (6.14 ± 0.18 kg BW) derived from PIC 1050 females and 4 different sire lines (PIC Duroc, DNA Genetics Duroc, Genesus Duroc or PIC Pietrain) for a 42-d nursery study. The pigs were selected from a study evaluating sire lines, thus explaining the larger than normal

number of sires represented in the experiment. All pigs used in the experiment came from the same sow source and were weaned at 21 d of age. On d 1 after birth, all pigs were given iron and gentamicin injections. Before weaning, pigs were treated on an individual basis with injectable antibiotics (gentamicin, ceftiofur, or enrofloxacin) as needed. At weaning, pigs were vaccinated for porcine circovirus type 2 and *Mycoplasma hyopneumoniae* (Circumvent PCV-MG2, Merck Animal Health, Madison, NJ), and ileitis (Porcilis Ileitis, Merck Animal Health). Due to the flow schedule at the sow source, approximately half of the pigs were weaned 4 d prior to the start of the experiment and held at the sow farm while the other half was weaned 2 d prior to the start of the experiment. For the duration of the experiment, pigs were housed in a tunnel-ventilated barn. Each pen was equipped with a 4-space automatic dry self-feeder and nipple water drinker, fully slatted concrete floors, and metal rod penning and gates. Pigs were given *ad libitum* access to feed and water for the duration of the experiment. An automatic feeding system (Big Dutchman, Holland, MI) was used to deliver a specified amount of feed to each pen. Air temperature in the room averaged 28.5°C ± 1.4, 28.7°C ± 1.2, 27.1°C ± 0.5, 24.5°C ± 1.1, 25.8°C ± 0.9, 26.5°C ± 2.2, in weeks 1-6, respectively.

Experimental design

Experimental treatments were arranged in a split-plot design with 4 dietary treatments evaluated across 2 group sizes. The dietary treatments included a negative control (NC) with no AGP, a positive control (PC) consisting of the NC diet with either chlortetracycline hydrochloride (phase 1 and 3) or tiamulin hydrogen fumarate (phase 2) added at the expense of corn, alternative diet 1 (ZA) consisting of the NC diet with zinc oxide (ZnO) plus a dietary acidifier (blend of phosphoric, fumaric, citric, and lactic acids; Kem-Gest, Kemin, Des Moines,

IA) added at the expense of corn, and alternative diet 2 (DR) consisting of the NC diet with a *Bacillus*-based direct-fed microbial (DFM; BioPlus 2B, Chr. Hansen, Hoersholm, Denmark) plus resistant potato starch (MSP[RS], MSP Starch Products Inc., Carberry, Manitoba, Canada) added at the expense of corn. Combinations of AGP alternatives were used, rather than single products, to first help satisfy the objective of testing a study design, rather than focusing on evaluating specific products. The specific combinations were chosen based on results from Schweer et al. (2017a) which indicated that AGP alternatives in the categories of zinc/copper, organic acids, and probiotics were most effective. Furthermore, zinc with an acidifier and a probiotic with a prebiotic likely have modes of action which either compliment or do not antagonize each other. Diets were fed in three phases (Tables 1 and 2) based on a feed budgeting system. When a pen consumed its entire allowance for a phase, feed for the next phase was given to that pen. In this manner, all pens were allowed to consume their entire budget for each phase before moving to the next phase. In order to associate pig weights with phase changes, weigh days were scheduled as close as possible to the first pens finishing their feed budget from the previous phase. Phase 1 was fed from d 0-11, phase 2 from d 12-24, and phase 3 from d 25-41.The first 2 phases were delivered in pelleted form, and the third phase feed was delivered as a mash. Feed was manufactured at 2 different commercial feed mills (phase 1 and 2 at the same mill, and phase 3 at another mill). Prior to diet manufacturing, the acidifier, ZnO, DFM, and RS products were hand-weighed on an analytical scale to the proper inclusion level, packaged in individual bags, and delivered to the commercial mill. Mix sheets used during mixing from both feed mills were validated after mixing to ensure these bags were added to the proper batches. In all phases, the diet containing the DFM was mixed last in order to avoid contamination in the other 3 diets.

Pigs were housed in groups of 31 (large groups; Fig. 1) or 11 pigs each (small groups; Fig. 2). In the small groups, a gate was installed to block off approximately two thirds of the pen to reduce usable floor space; the 2 outer spaces of the feeders were blocked off to achieve approximately equal feeder space per pig. Not counting the space occupied by each feeder, large pens had 0.41 m^2 per pig, and small pens had 0.42 m^2 per pig. Sixty pens were utilized for a total of 15 replicates of each diet (8 large groups and 7 small groups each), 32 replicates of large groups and 28 replicates of small groups. Pigs were assigned to blocks based on weaning date, sire line, and sex. Pigs held for 4 d or 2 d post-weaning were balanced within block to account for the potential influence of days post-weaning. Since 4 different sire lines were used, sire line was balanced within block. Mixed sex pens were used, and sex was balanced within block. A total of 8 blocks were used, and pens were assigned to experimental treatments so that each combination of diet and group size was represented in each block. However, since there were only 60 pens, one block had only four large groups and no small groups.

Characterization of health status

The pigs originated from a sow source that was negative for porcine reproductive and respiratory syndrome virus (PRRSV; confirmed through negative oral fluid and serum PCR analysis). Oral fluids, serum samples, and necropsies of pigs that died were used to confirm or rule out exposure to specific pathogens (Table 3). All diagnostic tests, including necropsies, were conducted at the Veterinary Diagnostics Laboratory at Iowa State University in Ames, Iowa. If a sample was positive for a specific pathogen, the whole barn was considered to have exposure to that pathogen.

Under the direction of a veterinarian, pigs were individually treated throughout the study with injectable antibiotics (ceftiofur or enrofloxacin) for symptoms of lethargy, gauntness, severe diarrhea, coughing, or other signs of illness. Flunixamine was also given for a small number of cases of coughing and labored breathing. Individual medical treatments were recorded daily by pen to determine if diet and group size influenced the number of treatments required. Pigs were removed from the study and housed in a hospital pen if they were injured, extremely ill, or did not improve after treatment. The daily number of pigs removed was recorded by pen. Pigs found dead were also recorded and included in the daily removal records.

Oral fluid samples were collected via rope sampling from 2 pens per dietary treatment (8 pens total) on d 0, 21, and 40. Pens were chosen for oral fluid collection based on fixed special sampling, so that each area of the barn was equally represented (Rotolo et al., 2017). A cotton rope was hung in the pen for approximately 1 h, and fluid was extracted from the rope by placing the saturated end into a plastic bag and squeezing out the fluid (Prickett et al., 2008). The resulting fluid samples were transferred to a plastic tube and stored at -20 °C until analysis. Oral fluid samples were analyzed using polymerase chain reaction (PCR) for PRRSV, Influenza type-A virus of swine (IAV-S), Porcine epidemic diarrhea virus (PEDV), Porcine deltacoronovirus (PDCoV), and *Mycoplasma hyopneumoniae*. Oral fluid samples were also collected from 4 pens exhibiting clinical symptoms (coughing, sneezing, lethargy) and tested for PRRSV and IAV-S on d26. Blood samples were collected from 1 pig per pen in 2 pens per dietary treatment (using the same pens as oral fluid collections) for a total of 8 blood samples on d 1 and 28. At the end of the trial (d41), 8 pigs per dietary treatment (one from each of the large pens) were euthanized for a separate experiment and blood was collected from each. Ten milliliters of blood were collected by jugular venipuncture and centrifuged at 2,000 x *g* for 10 min at 4°C, and the resulting serum

was stored at -80 °C for later analysis. Serum samples from d 41 were tested for PEDV and *Mycoplasma hyopneumoniae* using PCR. The goal of this diagnostic testing was to establish a general knowledge of disease exposure and health status of the pigs used in the study.

Diet sample analysis

Feed samples were taken directly from the feeders of 8 pens per dietary treatment during the middle of each feeding phase. To obtain each sample, the feed in each feeder was stirred to assist in homogeneity, and an approximately 200 g sample was taken by hand. All 8 samples for each dietary treatment were then pooled and homogenized, and sub-samples were taken from this composite sample and stored at -20°C prior to analysis. Diet samples were analyzed for DM (method 930.15), CP (method 990.03), ether extract (method 945.16), and Zn, Ca, P, and Na (method 985.01) at a commercial laboratory (Midwest Laboratories, Omaha, Nebraska, AOAC, 2007). *Bacillus*-spore enumeration in diet samples was performed at Midwest Laboratories using the *Bacillus* heat shock method (Jackson, 2015). Diets were analyzed for resistant starch (RS) content using a commercially available kit (Megazyme, Wicklow, Ireland; method 2002.02, AOAC 2007). The goal of analyzing diet samples for *Bacillus*, Zn, and RS was to confirm the presence of the additives in the final mixed diets. Diets were not analyzed for the inclusion of the acid blend due to the current unavailability of an assay to quantify the specific acids included in the blend.

Growth performance data collection

Pigs were weighed by pen on a floor scale (validated with a standard check weight at each use) at the beginning and end of the experiment, and at the end of each feed phase (d11 and

d24) to determine average daily gain (ADG). Feed offered was weighed by the automatic feed delivery system, and remaining feed was weighed at the end of each phase to determine ADFI and G:F, measured as total BW gain:total feed intake. Pen, removal date, BW at removal, and reason for removal were recorded for each pig found dead or removed from the study. This information was used to calculate pig days for each phase and the overall experimental period.

Calculations and statistical analysis

The total number of medical treatments per pen was calculated as a proportion by dividing the total number of treatments given for the whole experimental period by the number of pigs placed in the pen (either 31 or 11). The proportion of total removals per pen was calculated by dividing the total number of pigs removed for the whole period in each pen by the number of pigs placed in the pen (either 31 or 11). Pig days were used to calculate ADG, ADFI, and G:F.

The UNIVARIATE procedure of SAS (SAS Inst. Inc. Cary, NC) was used to determine homogeneity of variances and to identify outliers. Observations were considered outliers if greater than 3 standard deviations from the mean. Residual plots were also used to verify equality of variances and normality of the residuals. It was determined that all variables analyzed met the assumptions for parametric tests, so the same model was used to analyze all the data. The MIXED procedure of SAS was used to analyze the data with pen as the experimental unit and initial BW as a covariate. The fixed effects were diet, group size, and diet × group size interaction. Block was considered a random effect. Differences were considered significant if $P < 0.05$ and tendencies if $0.05 \geq P < 0.10$.

Results

Diet analysis

Results of Zn and RS analysis confirmed their presence in the complete feed in their respective dietary treatments (Table 1 and 2). With respect to the DFM product, after the experiment was completed, it was discovered that a separate *Bacillus*-based DFM was included in the vitamin-mineral premix used at the commercial feed mill that manufactured the phase 1 and 2 diets; thus, a DFM product had been added to all phase 1 and 2 diets. Consequently, *Bacillus* spore counts were much higher than expected, although they were also quite variable (data not shown). In the phase 1 and 2 diets, *Bacillus* counts in the DR diet were not as high as expected; recovery varied from 20-60% of expected when taking into account both the DFM in the premix and the added DFM in the DR diet. Additionally, there was a low recovery of *Bacillus* in the DR diet from phase 3, but spore counts were elevated in this diet compared to the NC, PC, and ZA diets. The *Bacillus* product was tested and confirmed to contain viable spores very close to the level specified on the product label (91% recovery). All test products were pre-weighed and the correct amounts per batch were delivered to the feed mills to ensure they were added at the correct quantity. Evaluation of the mix sheets confirmed that these pre-weighed bags were indeed added. We cannot explain why the assayed spore counts fell short of expected, other than perhaps the difficulty of assaying low concentrations in complete feed, as compared to a premix.

Growth performance

Due to naturally occurring health challenges reported below, overall pig performance was below that expected for this facility (Table 4).

For the overall period (d0-41), there were impacts of both dietary treatment and group size, and their interaction, on piglet growth performance. There was an interaction between diet and group size for ADG ($P = 0.012$) and ADFI ($P = 0.015$). Pigs fed the PC had higher ADG and ADFI than the NC for both group sizes ($P < 0.05$), and pigs fed the ZA diet only had a higher ADG and ADFI than the NC in the large groups ($P < 0.05$). Small groups fed the NC and DR diets had higher ADG compared to large groups fed these diets ($P < 0.05$). However, small and large groups had similar ADG for the PC and ZA diets ($P > 0.05$). The mean ADG for large groups was 0.280 kg and was 0.293 kg for small groups (main effect $P = 0.006$). Small groups had similar ADFI to large groups except for the NC control diet where small groups had higher ADFI ($P < 0.05$). There was no interaction between diet and group size for G:F; pigs fed the PC diet were more efficient than pigs fed the NC, ZA, and DR diets (diet $P < 0.001$), and small groups were more feed efficient than large groups (group size $P = 0.004$). There was no impact of the DR diet on growth performance ($P > 0.05$).

Within the individual feeding phases, performance responses for diet and group size treatments showed similar patterns to the overall treatment data (data not shown). In phase 1 and 3, no interactions between diet and group size were observed ($P > 0.05$). The main effect of group size was not significant for ADG, ADFI, or G:F in phase 1 and 2 ($P > 0.10$) but was significant in phase 3 where small groups had greater ADG and G:F than large groups ($P < 0.01$). The main effect of diet was present in all phases in a similar pattern to the overall results. In phase 3, ADG and G:F were similar to phase 2, which likely reflects depressions in growth performance due to PRRSV.

Animal health and morbidity

The pigs experienced acute diarrhea and septicemia in the first week of the experiment and a PRRSV challenge in the fourth week of the experiment (confirmed by PCR analysis of oral fluids on d 26; Table 3). Mortality was 1.8%, and morbidity (pigs removed from the study for illness or injury) was 6.1%. Mortality was not statistically analyzed due to the low numbers in each treatment. The number of mortalities per treatment were as follows: NC diet, 8; PC diet, 3; ZA diet, 7; DR diet, 6; large groups: 18; small groups: 6.

On d 5, all pigs were given gentamicin through the drinking water for 6 d to treat the diarrhea. Culture of liver and lung tissue from pigs that died during this time confirmed exposure to *Salmonella (S. infantitis)*, *Actinobacillus suis*, and *Streptococcus suis*. Several deaths due to mulberry heart disease prompted water treatment with vitamin E and selenium for 5 d (d 15 to 19). A PRRSV challenge was confirmed on d 26 of the study after observations of lethargy, heavy breathing, coughing, sneezing, and decreased feed intake. Pigs were individually treated as described in the materials and methods section for symptoms for the remainder of the study. A timeline and results of all necropsies are listed in Table 5; results of all diagnostic testing are listed in Table 3.

There were no interactions between diet and group size for medical treatments or removals, so only main effects are presented (Table 6). Pigs fed the PC diet required fewer medical treatments than pigs fed the NC or DR diet, and the ZA diet was intermediate between NC and PC ($P = 0.024$). There was no effect of group size on number of medical treatments ($P = 0.706$). The number of pigs removed from the study, including mortality and morbidity, was not influenced by dietary treatment. However, the number of removals was lower in small groups than in large groups ($P = 0.049$).

Discussion

The swine industry is seeking effective alternatives to AGPs, and inconsistent results from AGP alternative studies has led to the need for evaluating AGP alternative testing protocols and study designs. The objective of this experiment was to evaluate the effects of AGP alternative diets and test group size on nursery pig performance. This data can then be used to provide a better framework of standards that can be used as a model for future studies testing the efficacy of AGP alternatives that will aid in comparing and interpreting results across those studies. The majority of published studies evaluating alternatives to AGPs have been conducted in academic research settings, and consequently, most studies have used relatively small groups of pigs (Schweer et al., 2017a). The literature review conducted by Schweer et al. (2017a) showed that experiments with a positive response to an AGP alternative had, on average, more pigs per pen than studies that did not show a positive response. The observed interactions between diet and group size indicate that consideration of group size may be necessary in studies evaluating AGP alternatives. Improvements in performance due to the ZA diet were only detected when pigs were housed in large groups. Higher removal rates were observed when pigs were housed in large groups, possibly indicating a higher-stress environment. These results may suggest a greater potential for this combination of additives to be effective under higher-stress situations, which may occur in larger group sizes. Furthermore, the benefit of AGPs seemed smaller when pigs were housed in small groups. Small groups fed the NC and DR diets had increased ADG compared to large groups. The PC and ZA diets seemed to somewhat compensate for slower gain in large groups as small and large groups had similar ADG when fed these diets. Improved growth performance when pigs are housed in smaller groups is in agreement with previous reports of this trend in nursery pigs (Wolter et al., 2000; Wolter et al.,

2001). McGlone and Newby (1994) also reported higher morbidity rates in pens of 40 pigs compared to pens of 10 or 20. These results indicate that group size may impact the outcomes of AGP alternative studies, and perhaps positive responses to specific AGP alternatives are less pronounced in studies where pigs are housed in smaller groups.

The growth-promoting effects of sub-therapeutic levels of antibiotics in swine diets are well documented (Cromwell, 2002). Improvements in ADG, ADFI, and G:F observed in this study due to AGP inclusion are similar in magnitude to previous reports (Cromwell, 2002). The current improvements are higher than the values reported by Dritz et al. (2002) which could be due to the poor performance of the NC treatment, perhaps due to health status. It should also be noted that the chlortetracycline inclusion level in the present diets is higher than some previous studies have used, but the levels of antibiotics used in this study were compliant with the 2017 VFD for this particular farm. Separately, ZnO and acidifiers have shown beneficial effects, yet results have been inconsistent; few studies have looked at these in combination. Pharmacological levels of Zn have also proven effective in improving growth performance of nursery pigs, in addition to decreasing diarrhea (Pettigrew, 2006; Heo et al., 2010; Pérez et al., 2011; Pluske, 2012). Walsh et al. (2007) and Li et al. (2008) both reported improvements in growth performance of nursery pigs due to acidifiers, though Boas et al. (2016) reported no improvements. Schweer et al. (2017a) reported that acidifiers resulted in ADG improvements in 33.8% of studies.

Inclusion of DFM's has also given inconsistent responses. Kyriakis et al., (1999) Papatsiros et al. (2011), and Hu et al., (2014) reported improved growth performance, but many studies have also reported no improvements (Bhandari et al., 2008; Liao and Nyachoti, 2017). Resistant potato starch as a prebiotic has been shown to reduce diarrhea (Bhandari et al., 2009),

and in combination with a DFM has also improved ADG (Krause et al., 2010). However, studies evaluating resistant potato starch are uncommon.

It is clear that the PRRSV challenge impacted the performance of this group of pigs. Based on the standard feed budget used at this farm, expected feed intake during phase 3 would be 1.0-1.2 kg/pig/day. Pigs consumed, on average, only 0.52 kg/pig/day during this period. Compared to estimates from NRC (2012) for 11-25 kg pigs, the pigs gained 46% less and ate 45% less per day. However, in phases 1 and 2, prior to the PRRSV outbreak, pigs performed as expected (0.21 kg/day compared to the 0.21 kg/day estimate for 5-7 kg pigs, and 0.31 kg/day compared to the 0.34 kg/day estimate for 7-11 kg pigs; NRC, 2012). Severely reduced feed intake and low growth rate demonstrates the impact of the PRRSV challenge on growth performance, which is typical for pigs challenged with this virus (Schweer et al., 2017b). The present results were likely influenced by this health challenge, especially in phase 3 when pigs were consuming far less feed than expected and therefore were not receiving the desired amount of the AGP alternatives, potentially decreasing their effect.

Pigs fed the PC diet required almost 40% fewer medical treatments, suggesting that AGPs were beneficial to pig health and welfare during a disease challenge. The number of medical treatments required when pigs were fed the ZA diet was intermediate between the NC and PC diets, indicating that this diet may have also benefited pig health. Few studies report medical treatments, but Pérez et al. (2011), as an example, reported a decrease in the number of required medical treatments for pigs fed ZnO during a pathogenic *E. coli* challenge.

Considerations for future studies

There are likely many factors responsible for the inconsistent responses observed in studies evaluating AGP alternatives (Allen et al., 2013; Thacker, 2013). To increase the value of future studies, it will be highly beneficial to provide more information on study conditions than has previously been the case. When such information is provided, the context of the study will be more apparent, and it will be much easier to compare studies conducted in different locations and in different environments. Figure 3 outlines proposed necessary components that should be included and reported in AGP alternative studies. The remainder of this discussion will elaborate on a few specific components.

Health status is an important consideration when alternatives are being evaluated, as products may have greater or less efficacy under certain health conditions. Some evidence exists to suggest that AGPs are more effective on commercial farms than in academic-type research settings (Cromwell, 2002; Dritz et al., 2002), and this has been hypothesized to be partially due to lower pathogen load and incidences of "sub-clinical" disease (Zimmerman, 1986). If health status can affect the response to AGPs, then it is logical to propose that it could also influence the effects of AGP alternatives as well.

Indeed, health status has been discussed repeatedly as a potential reason for inconsistencies in response to AGP alternatives (Allen et al., 2013; Boas et al., 2016). Some studies have shown the potential for AGP alternatives to mitigate a health challenge (Bhandari et al., 2008; Gebru et al., 2010; Heo et al., 2010); benefits of AGP alternatives to animal health during a disease challenge would be of great interest to the swine industry. Thus far, the impact of specific AGP alternatives in the presence of particular pathogens is not well understood, and information about health status is mostly absent in published AGP alternative studies (Schweer

et al., 2017a). Documentation of the pathogens present in a group of pigs that may influence the outcome of a study will help to build an understanding of how AGP alternatives may perform under varying health conditions. In this study, the collection of oral fluid and serum samples as well as necropsies of pigs that died allowed for the identification or exclusion of critical pathogens, including PRRSV, as influential factors in this group of pigs. Collection and testing of diagnostic samples, especially at the beginning and end of a study, can be used to assess and document pathogen exposure. If clinical signs of illness are observed, additional samples should be collected, based on the symptoms, to characterize the illness. Major changes in health status throughout a trial should be reported. Table 7 outlines examples of potential pathogens of interest and methods of testing for them.

Determining pathogen presence, or the presence of agents/active infections, will involve identifying genetic material of a pathogen (generally through PCR), detecting an antigen (through ELISA or immunohistochemistry), or detecting a viable pathogen through isolation (Christopher-Hennings et al., 2012). Pathogen exposure is determined by measuring seroconversion, which confirms a prior infection or presence of a maternal antibody and is done by detecting antibody in the serum (Christopher-Hennings et al., 2012). The specific procedure for defining health status via pathogen presence or exposure will likely depend on the nature of a study and the pathogens involved, and a strategy may need to be adapted for each study and pathogen of interest. It is also important to report the medical treatment regimen used if pigs need to be treated for illness.

While it is important to confirm the presence of feed additives through diet analysis, it may not be possible to be fully quantitative in this respect, due perhaps to limitations of the assay, or due to transformation of the additive during the feed manufacturing process. In this

study, analysis of feed samples for *Bacillus* spore counts revealed that a *Bacillus* product was included in the vitamin-mineral premix that was used in the phase 1 and 2 diets. Thus, the phase 1 and 2 diets all had greater spore counts than expected. Additionally, the spore counts in all the phase 1 and 2 diets were unexpectedly variable, and overall recovery was low (ranging from 20-50% in phase 1 and 2 diets; DR diets had an average recovery of 23% in phase 1 and 2). This made it difficult to determine if the DFM product was correctly added to the DR diets. It was clearer that the DFM product was added correctly in the phase 3 diets, although recovery of the product was not as high as expected (roughly 30%) and may also point to variability or low recovery of the *Bacillus* assay in general.

To the authors' knowledge, there is currently no assay readily available to analyze for the specific acids contained in the acid blend that was used in this experiment. Zinc levels in the ZA diets were slightly lower than expected but were much higher in the ZA as intended (Table 1). When considering the RS content of the potato starch product (approximately 78%, DM basis), the DR diet in phase 1 and 2 showed only 62% recovery of expected values of RS. Since these were pelleted, the low recovery could be due to heat and water application during the pelleting process, which can cause starch to gelatinize and increase its susceptibility to degradation by alpha-amylase (Svihus and Zimonja, 2011). When possible, it is crucial to analyze diets for the AGP alternatives being tested to confirm their presence as intended, as these outcomes can influence the interpretation of study results.

With future study design in mind, sample size calculations were conducted (Table 8) using the standard deviations generated in the overall data to predict the sample size that would be needed to detect differences of practical significance and to determine if required sample size would differ according to pig group size. Though group size may be an important consideration

in AGP alternative studies as previously discussed, it does not appear that a larger sample size would necessarily be needed for one pig group size over the other.

In conclusion, the methodology used in this study resulted in the ability to compare the impact of dietary treatments on growth performance, morbidity, and medical treatments to establish a description of population health status. This was facilitated by careful planning and execution of the experimental protocol as well as strict record keeping and observation. The results suggest that group size is an important factor to consider when designing and interpreting AGP alternative studies. As research on AGP alternatives continues, the credibility and impact of future studies will be improved with proper design, protocol implementation, and consistent reporting of pertinent study information and results. Careful consideration of group size, sample size, the study components mentioned above and how these factors may influence study outcomes will be advantageous to the swine industry's rate of progress in identifying effective alternatives to growth-promoting antibiotics.

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Table 3.1. Ingredient and nutrient composition of experimental diets (as fed basis): phase 1 and 2[1]

	Phase 1				Phase 2			
	Dietary treatment[2]							
	NC	PC	ZA	DR	NC	PC	ZA	DR
Ingredient, %								
Corn	34.24	33.94	33.64	29.19	52.80	52.62	52.40	47.75
Soybean meal 47% CP	17.50	17.50	17.50	17.50	27.50	27.50	27.50	27.50
Whey permeate	20.73	20.73	20.73	20.73	4.88	4.88	4.88	4.88
Dried yeast	11.12	11.12	11.12	11.12	3.56	3.56	3.56	3.56
Rolled oat groats	7.50	7.50	7.50	7.50	5.00	5.00	5.00	5.00
Choice white grease	3.48	3.48	3.48	3.48	3.48	3.48	3.48	3.48
Spray-dried plasma	3.00	3.00	3.00	3.00	-	-	-	-
Limestone	0.70	0.70	0.70	0.70	0.69	0.69	0.69	0.69
L-lysine HCl	0.51	0.51	0.51	0.51	0.48	0.48	0.48	0.48
MHA methionine	0.35	0.35	0.34	0.34	0.35	0.35	0.35	0.35
Monocalcium phosphate	0.31	0.31	0.31	0.31	0.54	0.54	0.54	0.54
VTM premix[3]	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Salt	0.15	0.15	0.15	0.15	0.28	0.28	0.28	0.28
Choline	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
L-Threonine	0.08	0.08	0.08	0.08	0.16	0.16	0.16	0.16
L-Tryptophan	0.03	0.03	0.03	0.03	-	-	-	-
CTC[4]	-	0.30	-	-	-	-	-	-
Tiamulin[4]	-	-	-	-	-	0.18	-	-
Zinc oxide	-	-	0.30	-	-	-	0.20	-
Acidifier[5]	-	-	0.30	-	-	-	0.20	-
DFM[6]	-	-	-	0.05	-	-	-	0.05
Potato starch[7]	-	-	-	5.00	-	-	-	5.00
Analyzed values								
Resistant starch[8], %	-	-	-	1.89	-	-	-	1.82
DM%	89.0	88.8	89.0	88.8	87.6	87.7	87.3	87.3
Ether extract, %	5.60	5.96	6.04	5.76	6.06	6.45	6.13	6.14
Ca, %	0.68	0.72	0.64	0.72	0.59	0.61	0.57	0.61
P, %	0.61	0.62	0.63	0.60	0.51	0.53	0.51	0.52
Na, %	0.30	0.31	0.30	0.31	0.15	0.15	0.16	0.16
CP, %	21.00	21.60	20.40	21.00	19.60	19.80	19.10	19.00
Zinc, ppm	461	347	1900	459	432	357	1160	406

[1]Phase 1 was fed from d0-11, phase 2 was fed from d12-24. Feed budget was 2.2 kg/pig for phase 1 and 5.4 kg/pig for phase 2.

[2]NC = negative control, PC = positive control: NC + dietary antibiotics, ZA = NC+ ZnO + dietary acidifier, DR = NC + *bacillus*-based direct-fed microbial + 5% resistant starch.

[3]VTM premix provided per kg of complete diet: 0.21 ppm Cr as Cr_2O_3, 10 ppm Cu as $CuSO_4$, and Cu-MHA chelate, 0.31 ppm I as calcium iodate, 82 ppm Fe as $FeSO_4$, 21 ppm Mn as MnO and Mn-MHA chelate, 0.31 ppm Se as selenium yeast, 170 ppm Zn as ZnO and Zn-MHA chelate, 1,701 IU vitamin D3, 11,337 IU vitamin A, 45.3 IU vitamin E, 4.53 mg menadione, 0.23 mg biotin, 1.7 mg folic acid, 51 mg niacin, 15.6 mg pyridoxine, 28.3 mg pantothenic acid, 8.5 mg riboflavin, 39.7 mg vitamin B12, 514.4 FTU phytase (AxtraPhy, Danisco Animal Nutrition, Marlborough, UK). Premix also contained per kg of complete diet 0.06 g of *bacillus*-based direct-fed-microbial ($1.6x10^3$ CFU/g).

[4]CTC= Chlortetracycline hydrochloride (Auromycin-100, Zoetis, Parsippany, NJ); Tiamulin hydrogen fumarate (Denagard 10, Elanco, Greenfield, IN).

[5]Blend of phosphoric, fumaric, citric, and lactic acids (Kem-Gest, Kemin, Des Moines, IA).

[6]*Bacillus* spp. based direct-fed-microbial, provided $1.1x10^6$ CFU/g of complete diet (BioPlus 2B, Chr. Hansen, Hoersholm, Denmark).

[7] Resistant potato starch (MSP[RS], MSP Starch Products Inc., Carberry, Manitoba, Canada).

[8] Diets with no value did not have high enough resistant starch content to be accurately measured by this assay.

Table 3.2. Ingredient and nutrient composition of experimental diets (as fed basis): phase 3[1]

	Dietary Treatment[2]			
	NC	PC	ZA	DR
Ingredient, %				
Corn	47.59	47.19	47.29	42.54
Soybean meal 46.5% CP	35.95	35.95	35.95	35.95
Corn DDGS	10.00	10.00	10.00	10.00
Choice white grease	3.20	3.20	3.20	3.20
Limestone	1.03	1.03	1.03	1.03
Lysine sulfate, 54.6%	0.67	0.67	0.67	0.67
Monocalcium phosphate	0.54	0.54	0.54	0.54
Salt	0.46	0.46	0.46	0.46
DL-Methionine	0.19	0.19	0.19	0.19
VTM premix[3]	0.15	0.15	0.15	0.15
L-Threonine	0.13	0.13	0.13	0.13
Vitamin E	0.05	0.05	0.05	0.05
L-Tryptophan	0.04	0.04	0.04	0.04
Phytase[4]	0.01	0.01	0.01	0.01
CTC[5]	-	0.40	-	-
Zinc oxide	-	-	0.10	-
Acidifier[6]	-	-	0.20	-
DFM[7]	-	-	-	0.05
Potato starch[8]	-	-	-	5.00
Analyzed values				
Resistant starch[9], %	-	-	-	3.90
DM, %	88.4	88.5	88.3	87.9
Ether extract, %	6.73	6.04	5.95	5.69
Ca, %	0.67	0.75	0.71	0.68
P, %	0.60	0.61	0.60	0.58
Na, %	0.21	0.26	0.22	0.21
CP, %	24.2	24.3	24.2	23.9
Zinc, ppm	138	196	701	240

[1]Phase 3 was fed from d25-41

[2]NC = negative control, PC= positive control: NC + dietary antibiotics, ZA = NC+ ZnO + dietary acidifier, DR = NC + *bacillus*-based direct-fed microbial + 5% resistant starch.

[3]Vitamin-trace mineral premix provided per kg of complete diet: 11,013 IU of vitamin A, 1,651 IU of vitamin D, 33 IU of vitamin E (dl-alpha tocopheryl acetate), 11 IU of vitamin E (d-alpha tocopheryl acetate), 4.4 mg of vitamin K, 0.029 mg of vitamin B_{12}, 5.51 mg of riboflavin, 38.55 mg of niacin, 22.03 mg of pantothenic acid, 0.22 mg of biotin, 1.10 mg of folic acid, 0.88 mg of pyridoxine, 0.396 mg of Co as $CoCO_3$, 0.015 g of Cu as CuO or $CuSO_4$, 0.22 mg of I as ethylenediamine dihydroiodide (EDDI) or CaI_2, 0.15 g of Fe as $FeSO_4$, 0.031 g of Mn as MnO or $MnSO_4$, 0.31 mg of organic Se as selenium yeast, and 0.15 g of Zn as ZnO or $ZnSO_4$.

[4] OptiPhos 2000 (Huvepharma Inc., Peachtree City, GA).

[5] Chloratetracycline hydrochloride (Chlormax 50, Alpharma, Bridgewater Township, NJ).

[6] Blend of lactic, citric, fumaric, and phosphoric acids (Kem-Gest, Kemin, Des Moines, IA).

[7] *Bacillus* spp. based direct-fed-microbial product, provided $1.1x10^6$ CFU/g of complete diet (BioPlus 2B, Chr. Hansen, Hoersholm, Denmark).

[8] Resistant potato starch (MSP[RS], MSP Starch Products Inc., Carberry, Manitoba, Canada).

[9] Diets with no value did not have high enough resistant starch content to be accurately measured by this assay.

Table 3.3. Results of diagnostic testing throughout experiment (d 0-41)

Day[1]	Pathogen[2]	Result[3]	Testing method[4]
3	*Salmonella (S. infantitis*)	Positive	Liver culture
3	*Actinobacillus suis*	Positive	Lung culture
3	*Streptococcus suis*	Positive	Lung culture
11	*Mycoplasma hyorhinis*	Positive	Fibrin swab PCR
26	PRRSV	Positive	Oral fluid PCR
26	IAV	Negative	Oral fluid PCR
26	*Streptococcus suis*	Positive	Lung culture
26	*Haemophilus parasuis*	Positive	Lung culture
40	PEDV	Negative	Oral fluid PCR and serology
40	PDCoV	Negative	Oral fluid PCR
40	*Mycoplasma hyopneumoniae*	Negative	Oral fluid PCR and serology

[1]Day of sample collection

[2]PRRSV=porcine reproductive and respiratory syndrome virus, IAV=influenza A virus, PEDV=porcine epidemic diarrhea virus, PDCoV=porcine deltacoronovirus

[3]Samples were collected at necropsy from pigs that died as determined necessary by the diagnostic veterinarian. On d 26, oral fluid samples from 4 symptomatic pens were collected and tested. On d 40, oral fluid and serum samples from 8 pens, equidistantly spaced throughout the barn, were collected and tested. If a sample was positive for a specific pathogen, the whole barn was considered to have exposure to that pathogen

[4]PCR=polymerase chain reaction

Table 3.4. Effects of dietary treatment and group size, and their interaction, on nursery pig growth performance, d0-41

	Treatment[1]									*P*-value		
	Large group				Small group							
Item, kg	NC	PC	ZA	DR	NC	PC	ZA	DR	SEM	Diet	Group size	Diet×group size
Start BW	6.12	6.11	6.11	6.12	6.09	6.09	6.09	6.08	0.089	0.997	0.013	0.958
End BW	17.32	20.13	18.25	17.16	18.46	20.01	17.94	17.69	0.361	<0.001	0.154	0.080
ADG	0.26	0.33	0.28	0.25	0.29	0.33	0.27	0.28	0.009	<0.001	0.006	0.012
ADFI	0.40	0.47	0.43	0.40	0.43	0.47	0.42	0.42	0.011	< 0.001	0.144	0.015
G:F	0.64	0.69	0.65	0.62	0.67	0.69	0.66	0.66	0.010	< 0.001	0.004	0.203

[1]NC=negative control; PC= positive control: NC + dietary antibiotics; ZA= NC+ ZnO + dietary acidifier; DR= NC + *bacillus*-based direct-fed microbial + 5% resistant starch. Group size treatments: pigs were housed in groups of either 31 (large group) or 11 (small group) pigs per pen.

Table 3.5. Timeline of necropsies and diagnostic results

Day	Treatment[1]	Diagnosis	Pathogens confirmed present[2]
3	DR, large group	Pneumonia, septicemia	*Salmonella, actinobacillus suis, streptococcus suis*
4	ZA, small group	Pneumonia, septicemia	-
5	ZA, large group	Mulberry heart disease	-
5	PC, large group	Pneumonia, septicemia	-
11	ZA, large group	Pneumonia, septicemia	*Mycoplasma hyorhinis*
11	DR, large group	Pneumonia, meningitis	-
13	NC, large group	Mulberry heart disease	-
17	ZA, large group	Pneumonia, septicemia	-
17	ZA, large group	Mulberry heart disease	-
26	NC, large group	PRRSV, interstitial pneumonia	*Streptococcus suis*, PRRSV
26	DR, large group	PRRSV, interstitial pneumonia	*Streptococcus suis*, PRRSV
38	PC, small group	Intestinal torsion	-

[1] NC=negative control; PC= positive control: NC + dietary antibiotics; ZA= NC+ ZnO + dietary acidifier; DR= NC + *bacillus*-based direct-fed microbial + 5% resistant starch.
Group size treatments: pigs were housed in groups of either 31 (large group) or 11 pigs per pen (small group).
[2]Further testing for specific pathogens at necropsy was done at the discretion of the veterinarian.
[3]PRRSV=porcine reproductive and respiratory syndrome virus.

Table 3.6. Effects of dietary treatment and group size on medical treatments and removals, d 0-41

	Diet[1]					Group Size[2]				
Item	NC	PC	ZA	DR	SEM	*P*-value	Large	Small	SEM	*P*-value
Medical treatments, proportion[3,5]	0.814[a]	0.506[b]	0.719[ab]	0.923[a]	0.152	0.024	0.759	0.722	0.136	0.706
Removals, proportion[4,5]	0.086	0.062	0.073	0.059	0.017	0.666	0.087	0.053	0.0121	0.0486

[1]NC=negative control; PC= positive control: NC + dietary antibiotics; ZA= NC+ ZnO + dietary acidifier; DR= NC + *bacillus*-based direct-fed microbial + 5% resistant starch.

[2]Group size treatments: pigs were housed in groups of either 31 (large group) or 11 (small group) pigs per pen.

[3]Medical treatments calculated as total number of medical treatments administered per pen divided by number of pigs allotted to pen (31 or 11).

[4]Removals calculated as total number of pigs removed from study (found dead or removed for illness or injury) divided by number of pigs allotted to pen (31 or 11).

[5]Means within a row without a common superscript differ significantly ($P < 0.05$). Interaction *P*-value for medical treatments and removals not significant. ($P > 0.10$).

Table 3.7. Examples of methods for determining pathogen exposure in studies

Pathogen[1]	Sample to test	Testing method[2]
PRRSV	Oral fluids, or serum	PCR, abELISA (or both)
PEDV	Oral fluids	PCR
	Serum	abELISA
PDCoV	Oral fluids	PCR
IAV	Oral fluids	PCR
	Serum	abELISA
Mycoplasma hyopneumoniae	Deep swab	PCR
	Oral fluids	PCR
	Serum	abELISA
Porcine circovirus	Oral fluids	PCR
	Serum	abELISA, PCR
Mycoplasma hyorhinis	Oral fluids	PCR
Haemophilus parasuis	Oral fluids	PCR
Rotavirus	Oral fluids	PCR
TGEV/ PRCV	Oral fluids	PCR
Lawsonia intracellularis	Oral fluids, feces	PCR
Actinobacillus pleuropneumoniae	Serum	Serology
	Tonsil scrape	PCR
Salmonella	Serum,	Serology
	Feces, rectal swab	Culture, PCR
E. Coli	Rectal swab	Culture
Brachyspira	Rectal swab	Culture, PCR
Actinobacillus suis	Nasal swab	Culture, PCR
Streptococcus suis	Lung	Culture

[1]PRRSV=porcine reproductive and respiratory syndrome virus, PEDV=porcine epidemic diarrhea virus, PDCoV=porcine deltacoronovirus, IAV=influenza A virus, TGEV/PRCV= transmissible gastroenteritis virus/porcine respiratory coronavirus

[2]PCR=polymerase chain reaction, abELISA= ELISA for antibody detection

Table 3.8. Sample size calculations[1]

Variable	Group size	Standard deviation[2]	Effect size	Sample size (n/trt)
ADG, kg	31 pigs/pen	0.036	0.05	9
	11 pigs/pen	0.032	0.05	7
ADFI, kg	31 pigs/pen	0.042	0.07	6
	11 pigs/pen	0.032	0.07	4
G:F	31 pigs/pen	0.036	0.05	9
	11 pigs/pen	0.038	0.05	10

[1]$\alpha = 0.05$; power = 0.80

[2]Estimates of standard deviations associated with each group size (31 or 11 pigs/pen) obtained from current experiment (d 0-41 data was used)

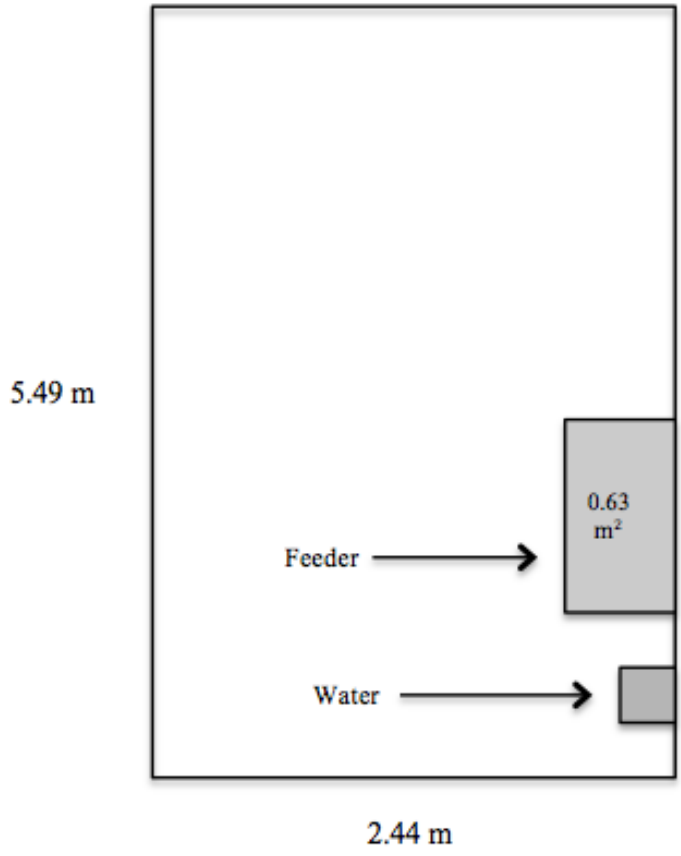

Figure 3.1. Large pen configuration. Pens were stocked with 31 pigs (0.41 m^2 per pig)

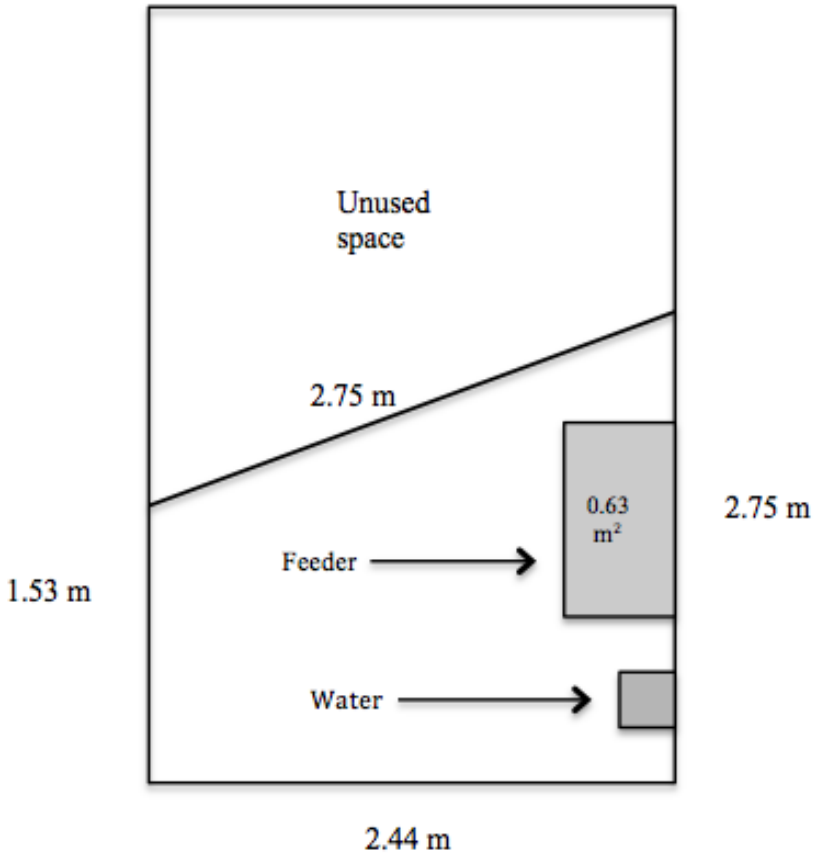

Figure 3.2. Small pen configuration. Pens were stocked with 31 pigs (0.42 m^2 per pig)

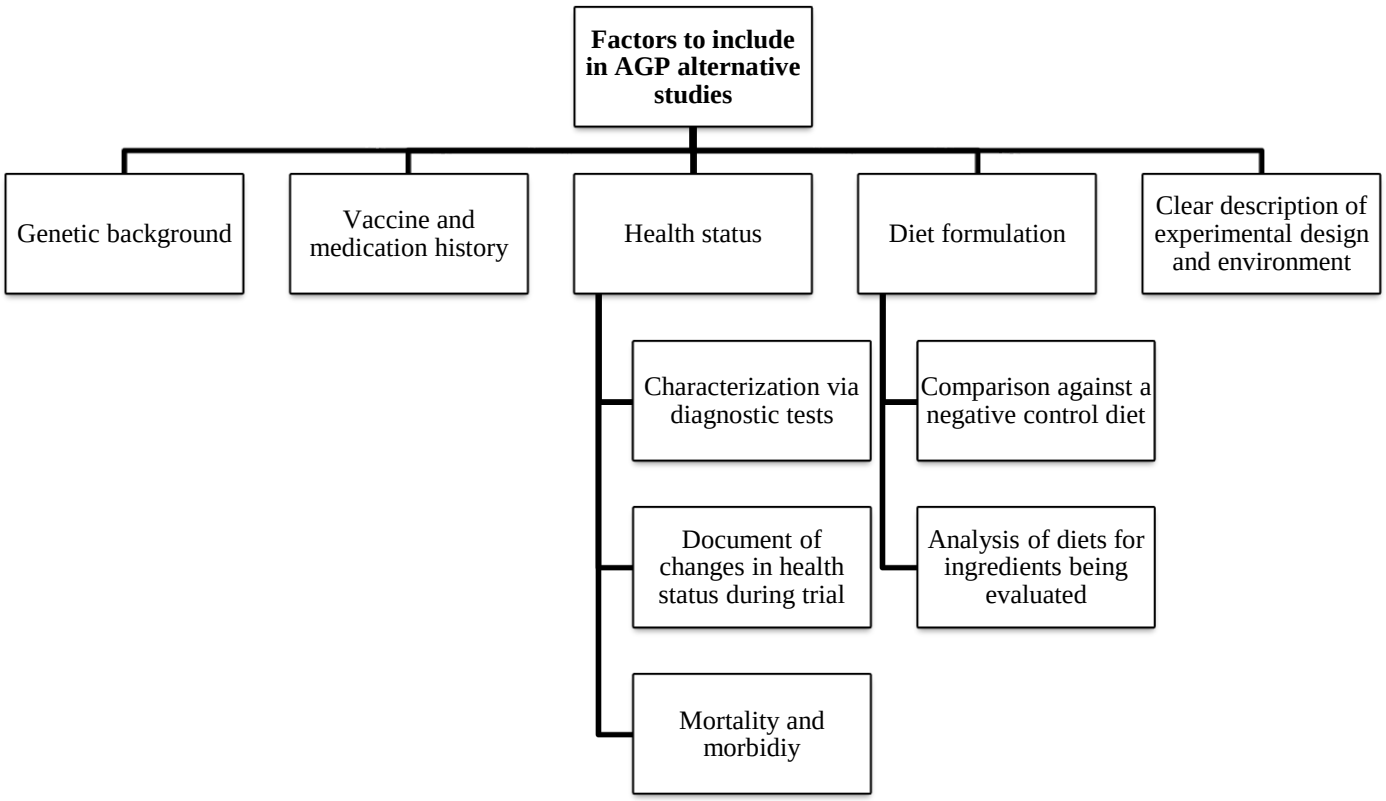

Figure 3.3. Proposed necessary study components to be included and reported in studies evaluating alternatives to antibiotic growth promoters (AGP) for pigs. When critical information is included in reports of AGP alternative studies, the context of the study is better understood. This will aid in making comparisons across multiple studies and will lead to faster and more valuable conclusions about the effectiveness of AGP alternative products.

CHAPTER 4

INTEGRATIVE SUMMARY

General Discussion

Feed additives are commonly used in swine diets for purposes such as increased nutrient digestibility, improved growth performance, or disease prevention. Additives can include exogenous enzymes, acidifiers, antibiotics, plasma products, phytogenic compounds, and more. Ideally, these add value to a diet and improve productivity. However, proper evaluation of feed additives is critical for effective use. The objective of this thesis was to investigate the methods with which swine feed additives, specifically phytase and alternatives to growth promoting antibiotics, are evaluated and to address issues that may impede their proper evaluation.

Exogenous phytase is undoubtedly useful in swine diets as it makes dietary P more available to the pig. When diets are formulated using phytase, some value for P contribution is assigned to phytase to account for its P releasing capabilities. This results in removal of some supplemental P from the diet and reduces the P content of manure. Adequate quantities of dietary P are crucial for optimal bone development and growth performance of pigs (Cromwell, 2005). Thus, to effectively formulate diets using phytase, accurate estimates of P-releasing capabilities of phytase are necessary. Traditionally phytase has been evaluated in studies using P-deficient diets; increases in characteristics such as bone mineral ash or ADG are compared to a standard curve created with inorganic P (Selle and Ravindran, 2008). This approach has two potential flaws. First, it may create a welfare concern for experimental animals due to impaired bone development and potential lameness (Cromwell, 2005; Vigors et al., 2014). Second, feeding P-deficient diets alters P-status (Cromwell, 2005; Berndt and Kumar, 2009) and therefore may

modify the pig's response to increased dietary P availability achieved through the use of phytase. This may occur do to the upregulation of P absorption in the intestine, or increased mobilization of P from bone (Cromwell, 2005) Therefore, evaluation of phytase and P release in models that use P-adequate diets may be more representative of normal physiological conditions, and the circumstances under which it is used in commercial practice.

Chapter 2 investigated the effects of adding phytase to a P-adequate diet on P and Ca digestibility and metabolism. These effects were compared when phytase was added to a P-deficient diet. The usefulness of urinary P as a predictor of P release by phytase was also evaluated. It was found that phytase improved digestibility of P when added to a P-adequate diet; however, the increase in digestible P was not as great as expected based on manufacturer's recommendations, possibly due to previous values being estimated using P-deficient diets. Though urinary P increased linearly, indicating the diets were in fact above P-requirement, pigs were able to elevate P retention as phytase levels increased. Therefore, urinary P alone was shown to be unsuitable as an indicator of P release by phytase.

With a different study design, it is possible that urine P could be used as a predictor for P release by phytase. However, this would likely require feeding diets very low in Ca such that any excess absorbed P would not be retained in the body and could be quantified in the urine. This may be useful because it would assure that P released by phytase had indeed been absorbed, but it would also present difficulties due to feeding impractical Ca to P ratios.

It was also found in chapter 2 that the improvement in digestibility due to phytase was slightly larger when 200 FTU/kg was added to the P-deficient diet than to the P-adequate diet. This may indicate a difference in phytase's P-releasing capabilities when P-adequate diets are fed, though the exact reason for this is still unknown. Further research should be conducted

evaluating phytase sources in P-adequate models, since different types and sources of phytase differ in their P-releasing efficacy (Dersjant-Li et al., 2015). The study would have benefited from the comparison of more levels of phytase added to the P-deficient diet to see if this discrepancy was present at multiple phytase inclusion levels. Due to statistical constraints, this study was not able to accommodate more dietary treatments. Evaluation of multiple levels of phytase added to a P-adequate diet in comparison to multiple levels in a P-deficient diet would be useful in further elucidating the impact of dietary P level on phytase efficacy.

In 2017, the Veterinary Feed Directive (VFD) went into effect in the United States. The VFD vastly changed the way livestock producers can use antibiotics. Antibiotics that were previously used as antibiotic growth promoters (AGPs) can no longer be used for growth promotion. Effective alternatives to AGPs in swine diets will be important for the successful reduction of antibiotic use in the swine industry in the coming years. Currently, there are many ingredients available with the potential to be alternatives to AGPs, but thus far their effectiveness in commercial production has not been well established. Results from studies where AGP alternatives are evaluated are inconsistent; for example, AGP alternatives improved ADG in only 29.3% of the 1,698 experiments reviewed by Schweer et al. (2017). To make conclusions about which AGP alternatives are effective in which situations, factors that influence the outcomes of AGP alternative studies need to be investigated, and essential study components need to be defined.

In chapter 3, interactions between the effects of dietary treatment and group size on nursery pig growth performance indicated that group size may be an important factor in AGP alternative studies. When pigs were housed in groups of 31, but not in smaller groups of 11, a diet containing zinc oxide and a blend of organic and inorganic acids improved growth

performance. This demonstrated that group size may impact results of AGP alternative studies. There may be an increased potential for AGP alternatives to improve performance when pigs are housed in larger groups, as would likely be the case in commercial production. It was also found that when pigs were housed in groups of 11, pig removals (including mortalities and pigs that were removed for illness or injury) were fewer than when pigs were housed in groups of 31. This may point to decreased stress and improved pig welfare in the smaller groups. One limitation of these data is that pigs removed from the study for illness or injury were not followed by treatment to the end of the study. So, to fully quantify morbidity and mortality, it would be valuable in future studies to keep track of removed pigs and record final outcomes. The outcomes of this study were likely influenced by the health status of this group of pigs, including a naturally occurring challenge with porcine reproductive and respiratory syndrome virus (PRRSV). The characterization of health status in future AGP alternative studies will assist in interpretation of results and will also help build knowledge about the effects of AGP alternatives in varying situations of health.

It is apparent that research on AGP alternatives will continue in the coming years as the swine industry seeks to reduce their use. As discussed in chapter 3, careful consideration of study design, group size, and inclusion of components such as characterization of health status and detailed background information will enhance be beneficial. Additionally, the majority of current published AGP alternative studies have been conducted in small-pen research environments (Schweer et al., 2017), so there remains a need for more commercial-scale research in general. In addition, proper negative and positive controls should be carefully considered. Because the use of antibiotics for growth promotion is no longer allowed under the 2017 VFD, the levels of in-feed antibiotics now used (as prescribed by a veterinarian in compliance with the VFD) may be

at higher, therapeutic, rather than sub-therapeutic, doses. In some situations, the comparison of an alternative ingredients to a positive control in this manner may not be useful, as some pork production systems use no antibiotics at all; the industry is likely to continue to reduce use of in-feed antibiotics. Therefore, the use of traditional AGP's as a positive control may no longer be practical, and studies should carefully consider the positive control used to compare "alternative" ingredients against.

This thesis highlights the importance of not only evaluating feed additives to verify their efficacy in swine diets, but also of considering the methods with which additives are evaluated. Where phytase is concerned, it may be more appropriate to evaluate P release in diets that are not deficient in phosphorus. Future studies should consider using P-adequate diets rather than P-deficient diets or should validate P release values generated with P-deficient diets in studies using P-adequate diets. Comprehensive collection and reporting of important information in AGP alternative studies and validation in commercial settings may be necessary to determine their true effectiveness. Improvements in methodology will ultimately increase the practicality of using certain feed additives and their benefits for pigs and will contribute towards greater confidence in accurate diet formulation and improved feeding practices.

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