Effect of Diet Complexity, Multi-enzyme Complexes, Essential Oils, and Benzoic Acid on Weanling Pigs

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of Master of Science

Auburn, Alabama
May 7, 2016

Keywords: weanling pig, diet complexity, enzymes, essential oils, benzoic acid

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ABSTRACT

The objective of this study was to investigate the effect of supplementing a simple corn-soybean meal (SBM) diet with multi-enzyme complexes, essential oils, and benzoic acid on growth performance, serum metabolite profile, serum cytokines, and gut microbiota in weanling pigs. Forty-eight gilts and 48 castrated males (initial body weight, 7.96 ± 0.89 kg) weaned at 3 to 4 wk of age were randomly assigned to 4 dietary treatments with 6 replicate pens (4 gilts or 4 castrated males/pen) per treatment. A typical complex diet containing various special ingredients was formulated and used as a positive control (POS) diet. A simple corn-SBM, negative control (NEG) diet was formulated to be iso-lysinic to the POS diet, and the NEG diet was supplemented with multi-enzyme complexes (ENZ) or multi-enzyme complexes, essential oils, and benzoic acid (ALL). Feed additives were included in the diet by replacing the part of corn. All 4 diets were formulated to meet or exceed the 2012 NRC nutrient requirements. During the fourth week of the study, approximately 5 mL of blood was collected via vena cava puncture using a sterile needle and evacuated tube. An aliquot was stored at -20ºC, and samples were pooled by pen and analyzed for serum metabolites and cytokines. Similarly, fecal samples for bacterial enumeration were collected from, at least, 3 pigs from each pen by rectal stimulation during the fourth week of the study. Fecal samples were immediately chilled with ice, pooled by pen, and an aliquot was immediately used for bacterial enumerations. Pigs had ad libitum access to feed and water throughout the study. From d 0 to 7, pigs fed the POS diet consumed more ($P < 0.05$) feed, Lys, and digestible energy (DE) and gained more weight ($P < 0.05$) than those fed the NEG and ALL

diets. Pigs fed the POS diet also consumed more ($P < 0.05$) DE than those fed the ENZ diet. Similarly, from d 7 to 14, pigs fed the POS diet consumed more ($P < 0.05$) feed, Lys, and DE and gained more weight ($P < 0.05$) than those fed the NEG and ALL diets. Pigs fed the POS diet also consumed more ($P < 0.05$) DE than those fed the ENZ diet. Pigs fed the ENZ diet gained more weight ($P < 0.05$) than those fed the NEG diet. From d 14 to 21, pigs fed the POS diet consumed more ($P < 0.05$) feed, Lys, and DE, and gained more weight ($P < 0.05$) than those fed the other 3 diets. Pigs fed the POS diet consumed more ($P < 0.05$) DE than those fed the other 3 diets from d 21 to 28. Overall (d 0 to 28), pigs fed the POS diet consumed more ($P < 0.05$) feed, Lys, and DE, and gained more weight ($P < 0.05$) than those fed the other 3 diets. Serum total protein concentration in pigs fed the ENZ and ALL diets was greater ($P < 0.05$) than those fed the POS and NEG diets. Serum albumin concentration in pigs fed the POS diet was greater ($P < 0.05$) than those fed the NEG and ALL diets, and it was greater ($P < 0.05$) in pigs fed the ENZ diet than those fed the NEG diet. Serum globulin and urea nitrogen (SUN) concentrations in piglets fed the POS diet were less ($P < 0.05$) than those fed the other 3 diets. The albumin to globulin ratio, glucose and cholesterol concentrations in pigs fed the POS diet were greater ($P < 0.05$) than those fed the NEG, ENZ, and ALL diets. Dietary treatments had no effect on any of the serum cytokines and fecal microbiota. Because weaning is the most crucial period in the pig's life, it is important to feed weanling pigs with a highly palatable and digestible diet to satisfy the pigs' nutritional requirement. For that reason, over the years, a complex diet has been extensively used by the pig industry, even though it is costly. The results indicated that weanling pigs' performance can be affected by dietary treatments. Pigs fed the complex diet performed better and had different serum metabolite profile compared with those fed the simple corn-SBM diet. Supplementation of the simply corn-SBM diet with multi-enzyme complexes, essential oils, and

benzoic acid had no effect on growth performance. Similarly, serum metabolism profile, cytokine concentrations, and fecal microbiota concentrations in weanling pigs were generally not affected by supplementation of the NEG diet with various feed additives. Further research is needed to explore the possibility of using a simple corn-SBM diet for weanling pigs by supplementing with various feed additives.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude and appreciation to my major professor, Dr. Lee I. Chiba, for his teaching, supervision, and guidance throughout the graduate program. I would like to express my gratitude to Dr. Werner G. Bergen and Dr. Nar K. Gurung for their willingness to serve on the advisory committee and their support, encouragement, and constructive advice. I would like to thank all the faculty and staff of the Department of Animal Sciences and Swine Research and Education Center for their support and inputs throughout the program. I would also like to expresses deep appreciation to the fellow graduate students. I would like to thank my family for their continued support and encouragement throughout my graduate program.

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I. INTRODUCTION

Weaning is the most critical period in the pig's life, and the trend in swine industry has been to wean pigs early. Many swine producers have been weaning their pigs at 3 to 4 weeks of age. At the time of weaning, pigs are exposed to several stressors, which limit the growth performance of weaned pigs. Pigs at weaning are suddenly removed from the sow and moved into a new environment where they have to consume dry feed from feeder and drink water from a drinker. Additionally, they are mixed with pigs from other litters and fight to establish new hierarchies. As a result, pigs go through a period of so called "growth check," and they may lose about 100 to 250 g body weight (BW) during the first day after weaning, which may not be recovered until 4 d post-weaning. The metabolizable energy (ME) intake can be only about 60 to 70% of the pre-weaning ME intake. In addition, although newborn pigs can produce sufficient enzyme to digest the fats and proteins in sow milk, activities of other enzymes necessary to utilize a corn-soybean meal diets are not sufficient, especially during the first week after weaning. For this reason, diets provided for weaning pigs should be highly palatable and digestible, and compatible with their enzyme secretion pattern.

A corn-soybean meal diet is traditionally used as a pig feed. Because pigs face challenges during weaning and the digestive system of those pigs is immature, it is essential to provide a diet that can ease the transition from sow's milk to a solid feed that can support maximum growth rate. Obviously, a simple corn-SBM diet cannot meet the weanling pig's nutritional requirements. Weaning pigs need a more "complex" diet that contains so called special

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ingredients such as dried whey, plasma protein, fish meal, which are highly palatable and digestible. Researchers have investigated the effect of diet complexity for weanling pigs over the years and concluded that complex diets can improve nursery pig growth performance during the early post-weaning period. However, providing such complex diets with highly palatable and digestible ingredients to weanling pigs can be rather costly.

In addition, pigs are born with, essentially, no protection against various challenges from disease organisms. For that reason, it's been a routine practice to include an antibiotic/antibiotics in weanling pig diets. Dietary antibiotics have been used as animal growth and health promoter for a long time. At the same time, the issue of "antibiotic resistance" has been a subject of concern for researchers, producers, and general public since, perhaps in the 1960s in the States and many other countries. With the complete ban of the use of antibiotics in feed in European countries and others, the current trend is to look for some alternatives to protect young pigs from various disease organisms.

Over the years, various exogenous enzymes or enzyme complexes have been developed to increase the digestibility by extracting more energy and nutrients from feedstuffs/diets. Similarly, it's been demonstrated in recent years that essential oils or benzoic acid or both can be used as an alternative to antibiotics because of their antibacterial and anti-oxidative effects. Previous studies indicated that essential oils may improve pig performance, nutrient digestibility, and intestinal ecosystem, and benzoic acid may reduce growth of some undesirable microbiota in the intestine of pigs.

It is possible that a simply, corn-SBM diet supplemented with multi-enzyme complexes can be used as an alternative to a complex diet containing a multitude of special ingredients to satisfy the energy and nutrient needs of weanling pigs. Similarly, essential oils and benzoic acid

can be a viable alternative in protecting weanling pigs from disease organisms. A study was conducted to test the hypothesis that a simple corn-SBM diet supplemented with a combination of multi-enzyme complexes, essential oils, and benzoic acid can be used as a replacement for a typical complex diet for weanling pigs. Specific objectives were to assess the effect of those feed additives on growth performance, serum metabolite profile and cytokines, and fecal microbiota in weanling pigs.

II. LITERATURE REVIEW

Introduction

A corn-soy diet is a gold standard for feeding pigs. However, such a diet may not be appropriate for weanling pigs because, in addition to being subjected to various stressors at weaning, their digestive system is immature or the secretion of various enzymes is not sufficient to utilize a corn-soy diet. Weanling pigs cannot digest such a diet efficiently and subsequently may cause diarrhea. For those reasons, traditionally, weanling pigs have been provided highly palatable and digestible "complex diets", including many "special ingredients" such as dried whey, fish meal, plasma protein, and others. However, providing such complex diets could be costly.

In addition, pigs are born with, essentially, no protection against various challenges from disease organisms. For that reason, it's been a routine practice to include an antibiotic/antibiotics in weanling pig diets. Dietary antibiotics have been used as animal growth and health promoter for a long time. However, the use of antibiotics may be limited in the future because of the public concerns on the antibiotic residue and antibiotic resistance issues. It is necessary to find alternative ways to enhance the health status of weanling pigs. With the development of various enzymes or enzyme complexes to extract more energy and nutrients from feedstuffs and essential oils and benzoic acid to provide some antimicrobial activities, it may be possible to use simple corn-soy diets more efficiently and successfully to feed weanling pigs.

Diet Complexity

Introduction

Weaning is one of the most critical period in the pig's life, and most pigs experience "growth check" after weaning, which affects future pig performance. Simple corn-soybean meal (SBM) diets cannot meet the weanling pig's requirements, and complex diets with some "special ingredient" must be provided. Those complex diets are, however, costly. For successful swine production, it is important to use corn-SBM meal as much as possible in producing pigs. It might be possible to improve the efficiency of utilization of simple corn-SBM diets by supplementing with feed additives, such as enzymes, essential oils, and benzoic acid.

Early Weaning of Pigs

The trend in the swine industry is to wean pigs early. Many swine producers have been weaning their pigs at 3 to 4 weeks of age, and they have been weaning much earlier in recent years. This trend is driven by economic factors, such as improving the number of pigs per sow per year and also to decrease death losses (Charles et al., 2000). For example, King et al. (1998) conducted study based on breeding-herd performance data from hundreds of farms and suggested that shorter lactation length were associated with more pigs weaned per sow per year. Furthermore, early weaning and removal of pigs to a second isolated site can reduce the potential for disease transfer from the sow to piglets. An additional benefit to off-site production is that it can satisfy the nutritional requirement of piglets optimally and have heavier and uniform pigs at 8 weeks of age (Charles et al., 2000).

Factors Limit the Performance of Weaned Pigs

At the time of weaning, pigs were exposed to serious stressors, which limit the performance of weanling pigs. For instance, with the sudden change to a solid diet at weaning,

feed intake is usually reduced during the first few days. Based on the review of Le Dividich and Sève (2000), the metabolizable energy (ME) intake account for only 60 to 70% of the pre-weaning milk ME intake, and it takes about 2 weeks to recover. Growth performance is also reduced with low feed intake. Pigs lose about 100 to 250 g body weight (BW) during the first day after weaning and recover this BW loss in about 4 d post-weaning (Le Dividich and Sève, 2000). Newborn pigs can produce sufficient enzymes to digest fats and proteins in sow milk. However, activities of other enzymes, such as amylase, lipase, trypsin, and chymotrypsin, are decreased during weaning (Jensen et al., 1997). Lindemann et al. (1986) noted that there was a depression in the activities of pancreatic enzymes during the first week after weaning. For this reason, diets provided for weaned pigs should be highly digestible and compatible with their enzyme secretion patterns.

Comparison of Simple and Complex Diet

A corn-SBM diet is traditionally used as a pig feed. Because pigs are facing challenges during the weaning and the digestive system of those pigs is immature, it is essential to formulate a diet that will ease the transition from sow's milk to a nursery diet to maintain maximum growth rates. Obviously, simple corn-SBM diet cannot meet the weanling pig's nutritional requirements. Weanling pigs need a more "complex" diet. Typically, diets containing dried whey, plasma protein, fish meal, and others are called complex diets. Researchers have investigated diet complexity for weanling pigs and concluded that complex diets can improve nursery pig performance during the early post-weaning period (Whang et al., 2000; Wolter et al., 2003). For instance, Mahan et al. (2004) conducted a study with 135 post-weaning pigs and assigned to three treatments: a complex diet using several protein sources, a semi-complex diet with fewer

protein, and a simple corn-SBM diet. The result showed that feed intake and feed efficiency improved as diet complexity increased.

Antibiotics

Antibiotics have been widely used in food animal production. They are used for many purposes, including the therapeutic treatment of clinically sick animals, disease prophylaxis during periods of high risk of infection, promotion of growth, and improvement of feed efficiency (McEwen and Fedorka-Cray, 2002). Food animals are raised in groups or herds, and are often in confined conditions that promote the spread of infectious diseases. However, the majority of antibiotics aren't given to animals that are sick. Instead, it's a routine practice in the animal industry to mix these drugs with livestock feed and water as a substitute for healthier living conditions and to make chickens, pigs, and cows grow faster (Butaye et al., 2003).The problem with feeding antibiotics to animals that are not sick is that it kills off weak bacteria and creates the perfect environment for antibiotic-resistant bacteria to multiply and thrive.

Antimicrobial resistance may threatens public health because these antibiotic-resistant bacteria are likely to be transferred through the food chain to humans (Levy et al., 1976), or resistance genes in these bacteria can be transferred to the zoonotic bacteria and make pathogenic bacteria withstand the effect of antimicrobial agents (Low et al., 1997). As a result, some drugs may no longer work to treat infections. In January of 2006, the European Union took actions against the overuse of antibiotics on livestock by banning the use of antibiotics on livestock for the purpose of growth promotion. And the FDA began to seriously regulate the use of antibiotics on livestock, and prohibited the phrase "growth promotion" from the use of any antibiotics in April of 2012 (Gilbert, 2012). Concerns about the use of antibiotic growth promoters and the development of antibiotic-resistant bacteria have led researchers to investigate various methods

of maintaining and improving the performance of animals in the absence of antibiotic growth promoters (Turner et al., 2001).

Feed Additives

With the development of enzymes or enzyme complexes, essential oils, and benzoic acid, it might be possible to use a simple corn-SBM diet more efficiently, which may reduce the cost of diets. Enzymes have been demonstrated to increase the digestibility of diets to a greater extent by extracting nutrients from feed ingredients. For example, Zhang et al. (2014) noted that the feed intake and gain to feed intake ratio tended to be greater with the supplemented enzymes. Frølich (1990) indicated that application of enzymes could break down phytate and release phosphorus and other minerals from plant base feedstuffs. Essential oils and benzoic acid are used as feed additives because of their antimicrobial effect. Previous studies indicated that essential oil may improve pig performance (Cho et al., 2005; Kroismayr et al., 2008), nutrient digestibility (Yan et al., 2010), and intestinal ecosystem (Manzanilla et al., 2009). Knarreborg et al. (2002) noted that benzoic acid could reduce growth of microbiota in the intestine of piglets.

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Enzymes and Enzyme Complexes

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Introduction

Enzymes are needed to break down protein, carbohydrates, and lipids so that the body can utilize them. Enzymes are also involved in activating and hastening chemical reaction in the body, which are essential to life. Food producing animals must be supplied with nutritionally adequate diets, but the adequate content of energy and nutrients in feed is not sufficient to meet the animal's needs because of the antinutritive factors in some feedstuffs. Exogenous enzymes can be used to target the different antinutritive components in plant feedstuffs. Phytase is the best-known exogenous enzyme and has been used for a long time, while carbohydrases became

more popular in recent years (Olukosi, 2013). Supplementation of an animal diet with phytase and carbohydrases may alleviate the anti-nutritional effects that are associated with phytate and non-starch polysaccherides (NSP). A great portion of phosphorus in plant based ingredients is bound to phytate, and phytase is used to break down the phytate molecule and release phosphorus and other mineral elements (Selle and Ravindran, 2007). The NSP are complex carbohydrates that non-ruminant animals cannot digest, and carbohydrases are used for the breakdown of NSP and increase the availability of plant based ingredients (Bedford, 2000).

Phytate

Phytate is found in most plant seeds as the principal storage form of P at concentrations from 5 to 25 g/kg, contributing between 1.4 and 7 g phytate-P/kg (Maenz, 2001). Phosphorus and inositol in phytate forms are not, in general, bioavailable to nonruminant animals because these animals lack the enzymes required to remove phosphate from the phytate molecule (Kornegay, 2001). As a result, a large amount of P in phytate-P is wasted and expensive inorganic P must be supplemented in the diet to meet the animal's requirement (Knowlton et al., 2004). In addition, phytate has a strong binding affinity to some divalent minerals, such as Ca, Fe, and Zn which in turn reduces the availability and utilization of those minerals (Ockenden et al., 2004). The obvious solution to these antinutritional effects of phytate is to releasing P from phytate in the proximal gastrointestinal tract into lower molecular weight of monophosphate. This approach will release P from phytate, and reduce anti-nutritive effect of phytate as the lower molecular weight of monophosphate esters are less antinutritional (Persson et al., 1998).

Phytase

Phytase dephosphorylates insoluble phytic acid in grains and oilseeds into orthophosphate and inositol phosphates (Selle and Ravindran, 2007). In the broader term,

phytases are classified as 3- and 6-phytase on the basis of its effect on the site of the initial dephosphorylation of the phytic acid molecule. Generally, 3-phytase is of microbial origin and hydrolyzing P at the carbon 3 atom of the inositol ring, whereas 6-phytas is of plant origin and cleaving P at the carbon 6 atom of the inositol ring (Kornegay, 2001). Phytase catalyzes the hydrolysis of phytate to a series of lower myo-inosital phosphates esters and inorganic phosphate, which followed by a step-wise dephosphortlation reaction (Vats and Banerjee, 2004).

Effect of Phytase on Animals

In Poultry. Various studies on the effect of microbial phytase on growth performance have been conducted. In the study of Simons et al. (1990), different dietary phytase concentrations [250, 375, 500, 750, 1,000, 1,500, and 2,000 phytase unit (FTU)/kg] were provided to broiler chickens. The results indicated that addition of 1,500 FTU phytase/kg increased weight gain and improved feed conversion ratio in broiler chickens from 0 to 24 d of age. Ravindran et al. (2000) fed broiler chickens with 3 concentrations of phytic acid (10.4, 13.2, and 15.7 g/kg), 2 concentrations of of non-phytate P (2.3 and 4.5 g/kg), and 3 concentrations of phytase (0, 400, and 800 FTU phytase/kg) from 7 to 25 d of age. Supplementation of diets with phytase improved weight gain and feed conversion ratio in broiler chickens, but the response was greater in broiler chickens fed 2.3 g non-phytate P/kg, resulting in a significant interaction between non-phytate P and phytase (Ravindran et al., 2000). On the other hand, Ravindran et al. (2001) supplemented a P-adequate diet with phytase and found that phytase have no effect on feed intake of broiler chickens.

In Swine. Several studies have been conducted to evaluate the effect of phytase on swine growth performance. In the study of Young et al. (1993), a corn-SBM weanling pigs diet was supplemented with 0, 500, and 1,000 FTU phytase/kg and the addition of the phytase increased

feed intake and weight gain. Kornegay and Qian (1996) supplemented piglet diets containing 2 available P levels (0.7 and 1.6 g/kg) with 5 phytase levels (0, 350, 700, 1,050, and 1,400 FTU phytase/kg) and also used 2 extra diets that supplied NRC recommended available P level (3.2 g/kg) and supplemented with 0 or 1,400 FTU phytase/kg. The addition of graded levels of phytase resulted in a linear increase in average daily feed intake, average daily gain, and gain to feed ratio in pigs consuming the lower available P (0.7 g/kg) concentration diet. Piglets fed the diet supplemented with 1,400 FTU phytase/kg with 3.2 g/kg available P further increased average daily feed intake and average daily gain. In the study of Jongbloed et al. (2000), growing pigs were fed the basal diet and the diet supplemented with 410 FTU phytase/kg. Microbial phytase improved average daily gain and feed conversion ratio. However, in the study of Omogbenigun et al. (2003), early-weaned pigs were fed the diet supplemented with 500 FTU phytase/kg, and there were no differences in average daily feed intake, average daily gain, or gain to feed ratio in the pigs fed the phytase compared to the control groups.

Non-starch Polysaccharides

Non-starch polysaccharides (NSP) include all the plant polysaccharides other than starch. They are the key components of the cell walls of various grains and cover a great variety of biological functions and chemical structures. The major polysaccharides of NSP are cellulose, hemicellulose, pentosans, and β-glucans, which cannot be hydrolyzed by the endogenous enzymes of non-ruminant animals (Bedford, 2000). Plant ingredients generally contain a mixture of both soluble and insoluble NSP in a ratio that varies according to the type and stage of maturity of grains. Soluble NSP form dispersions when mixed with water and have the ability to increase the viscosity of digesta, which slows down the activities of digestive enzymes and the

absorption of nutrients. These consequences lead to anti-nutritive effects of NSP for non-ruminant animals (Bedford and Schulze, 1998).

Carbohydrases

Exogenous carbohydrases are enzymes that can reduce inherent inefficiency of nutrient utilization and combat the possible ill-effects associated with the feeding diets that contain high concentrations of NSP (Bedford, 2000). Because NSP comprises of many compounds with very different structures, several exogenous enzymes have been developed to target this diverse group of carbohydrates.

Carbohydrases include many enzymes that can catalyze the reduction of carbohydrates to smaller carbohydrate polymers. The cell walls of different plants are made up of different structural carbohydrates, and specific carbohydrases are required to break those down efficiently and release more energy. Xylanase and glucanase account for 80% of the global carbohydrases market.

Xylanases can degrade the linear polysaccharide, *β*-1,4-xylan, into xylose, thus, breaking down part of hemicellulose, one of the major components of plant cell walls (Beg et al., 2001). Because of the diversity in the chemical structures of xylans derived from the cell walls of wood, cereal, or other plant materials, a large variety of xylanases with various hydrolytic activities, physicochemical properties, and structures are known. Glucanases are enzymes that break down glucans. Glucans are polysaccharide components of the cell walls of the higher plant family, particularly abundant in the endosperm cell walls of cereals with a great commercial value such as barley, rye, sorghum, rice, and wheat (Stone and Clarke, 1992). In addition to the xylanases and glucanases, other commercially available carbohydrases include: α-amylase, β-mannanase, α-galactosidase, and pectinase.

Effect of Carbohydrases on Animals

In poultry. Mathlouthi et al. (2002) evaluated the effect of rye- or corn-based diets containing xylanase and β-glucanase. Broiler chickens consuming the rye-based diet decreased weight gain, feed intake, and feed efficiency compared with those fed the corn-based diet. The addition of xylanase and β-glucanase to the rye-based diet improved growth performance to the level comparable with the corn-based diet. Tahir et al. (2005) provided broiler chickens a corn-SBM diet with the enzyme additives: 0.33 unit/g cellulase, and 2 units/g hemicellulase or cellulase and hemicellulase mixture. The researchers found that average daily gain and carcass weight were increased in broiler chickens fed the enzyme and enzyme mixture. But, feed intake of broiler chickens remained the same for all treatment groups. Cowieson and Ravindran (2008) observed improved weight gain and feed conversion ratio in broiler chickens fed a maize-based diet supplemented with a mixture of xylanase, amylase, and protease. Similarly, Olukosi et al. (2007a) indicated a dose-related increase in final weight, weight gain, and gain to feed ratio in broiler chickens that consumed the diet composed of rye, wheat, and SBM and supplemented with xylanase. On the other hand, there were no responses to supplementation of carbohydrases in other studies. For instance, Olukosi and Adeola (2008) fed broiler chickens with 4,000 unit xylanase/kg and they observed no effect on broiler chicken growth performance.

In Swine. There have been some reports that indicated the positive response to carbohydrases supplementation, especially to diets with a high-NSP concentration. Cadogan et al. (2003) supplemented the wheat diet with xylanase and found the increased average daily gain and average daily feed intake in pigs. Barrera et al. (2004) provided 5,500, 11,000, and 16,500 xylanase units/kg of wheat diet. The results showed an improvement in average daily gain and feed conversion ratio in growing pigs, especially those supplemented with 11,000 xylanase

units/kg. On the other hand, other studies showed no improvement in growth performance to carbohydrases supplementation. Kiarie et al. (2007) conducted a study to evaluate the effect of multi-carbohydrase enzymes on piglet performance. The enzyme supplement provide 500 units of pectinase, 50 units of cellulase, 400 units of mannanase, 1,200 units of xylanase, 450 units glucanase, and 45 units of galactanase per kilogram of diet. The results showed that multi-carbohydrase did not affect piglet growth performance during the entire experiment. Olukosi et al. (2007a) provided piglets with the basal diet composed of corn, rye, wheat, and SBM. Dietary treatments consisted of the basal diet supplemented with 400, 800, 1,600, 3,200, and 32,000 xylanase units/kg. The researchers found no effect of xylanase on growth performance. Similarly, some other researchers found that supplementing grower-finisher pig diets with xylanase had no effect on growth performance (Olukosi et al., 2007b; Woyengo et al., 2008).

Essential Oils and Benzoic Acid

Introduction

There is no doubt that dietary antibiotics have played an important role in animal production as a growth and health promoter. Dietary antibiotics presumably act on the intestinal microflora, leading to improved animal growth performance. However, the current trend is to looking for some alternatives to antibiotics because of public concerns on their residues and antibiotic-resistant bacteria. It is clear that controlling the microflora could positively influence animals' performance and some other feed additives with antimicrobial activities can be a potential alternative to in-feed antibiotics (Taylor, 2001).

Several researchers have reviewed and compared various compounds that can be considered as an alternative to antibiotics in animal production (Langhout, 2000; Taylor, 2001; Wenk, 2000). Essential oils and benzoic acid would be the two possible antibiotic alternatives.

An essential oil is a concentrated hydrophobic liquid containing volatile aromatic compounds. The term essential used here does not mean indispensable as with the term used in essential amino acids or essential fatty acid, which are nutritionally required by animals (Reeds, 2000). Essential oils are generally extracted from plants by distillation. Other process include expression, solvent extraction, and cold pressing (Simon, 1990; Greathead, 2003). Factors such as species, ecological and climatic conditions, harvest time, and part of the plant used can all affect the chemical composition of essential oils (Màthé, 2009). Essential oils are used in the feed industry because of their antimicrobial property. It has been demonstrated that essential oils may have antimicrobial activity against a wide range of microorganisms, including bacteria, protozoa, and fungi (Chao et al., 2000; Deans and Ritchie, 1987; Sivropoulou et al., 1996).

Benzoic acid ($C_7H_6O_2$) is a colorless crystalline solid and a simple aromatic carboxylic acid. Benzoic acid occurs naturally in many plants and it serves as an intermediate in the biosynthesis of many secondary metabolites (CICA, 2000). Salts of benzoic acid are used as food preservatives because it could inhibit the growth of mold, yeast, and some bacteria (Warth, 1991). In the European Union, benzoic acid is approved as food additives with an inclusion rate of 0.5 to 1.0% (Hansen et al., 2007).

Cytokine

Cytokines are small proteins that are primarily involved in the host response to diseases or infections (Dinarello, 2000). Cytokines include chemokines, interferons, interleukins, lymphokines, and tumor necrosis factor (TNF). Some cytokines promote cellular immune response and are called pro-inflammatory cytokines, which include interleukin-1 (IL-1), TNF, and chemokines. Other cytokines suppress the activities of pro-inflammatory cytokines and are called anti-inflammatory cytokines, which include IL-1Ra, IL-4, and IL-10 (Lucey et al., 1996).

Pro-inflammatory cytokines are primarily produced by the macrophages. These cytokines induce fever, and activate B and T lymphocytes and endothelial cells (Dinarello, 1991). Elevated plasma concentration of pro-inflammatory cytokines have been observed in patients with infections, trauma, or ischemia (Dinarello, 2000). Anti-inflammatory cytokines inhibit the production of IL-1, TNF, and other pro-inflammatory cytokines (Opal and DePalo, 2000). Under physiologic conditions, anti-inflammatory cytokines limit injurious effect of sustained or excess inflammatory reactions. Under pathologic conditions, these anti-inflammatory cytokines may downregulate cytokine production during severe infections (Munoz et al., 1991). Therefore, an interaction between these pro-inflammatory and anti-inflammatory cytokines determine the ultimate effect of disease.

Intestinal Microbiota

The intestinal tract is colonized by a large and diverse community of microbiota (Leser et al., 2002). The microbial community of the gut has useful functions on the host (Guarner and Malagelada, 2003). For example, gut microflora help the fermentation of non-digestible fiber. Non-ruminant animals lack enzymes to break down certain polysaccharides, while gut flora, especially anaerobic microbes, could turn these polysaccharides into short chain fatty acids (SCFA). Those SCFA can be used as source of energy and provide some health benefit for the host (Gibson, 2004). In addition, gut microorganisms prevent the host from pathogenic microbiota. Helpful bacteria prevent the growth of harmful bacterial species by competing for nutrients and attachment sites on the epithelium (Guarner and Malagelada, 2003).

Effect of Essential Oils on Inflammation

There have been some studies shown that essential oils have anti-inflammatory effect on animals. Juergens et al. (2004) conducted a study to investigate the role of essential oil in

inhibiting cytokine production in stimulated human monocytes and lymphocytes *in vitro*. It was demonstrated that essential oil is a strong inhibitor of TNF-α and IL-1β in both cell types. It also had an inhibitory effect on the production of the chemotactic cytokines IL-8 and IL-5. Some research has showed that essential oils may suppress cytokine production in animals. In humans, the anti-inflammatory effect of the essential oil was evaluated by Hart et al. (2000), and they found that essential oil inhibited the production of inflammatory mediators such as TNF-a, IL-1β, IL-8, and IL-10 by activated human blood monocytes. Li et al. (2012) supplemented weaning pigs with essential oil products, and they reported that IL-6 concentration was lower and TNF-α concentration was greater in the plasma of pigs fed essential oil products. In addition, Kim et al. (2013) fed essential oil to *E. acervalina*-infected chickens, and they found that IL-6, IL-8, and IL-10 concentrations were increased in chicks fed the essential oil diet.

Effect of Benzoic Acid on Inflammation

There are also some evidences that benzoic acid can modulate animal inflammatory immune response. For example, Abdalla et al. (2013) divided 90 broiler chickens into 3 dietary treatments: control diet, and the control diet supplemented with 0.1 or 0.2% benzoic acid. Their results indicated an increase in the concentration of IL-10 and a decrease in IL-6 and TNF-α in the chickens fed 0.2% benzoic acid. Similarly, Oh et al. (2012) conducted a study to evaluate the effect of benzoic acid on immune response of weaning pigs, and they found that supplemented with 0.5% benzoic acid decreased the concentration of IL-1β, IL-6, and TNF in the small intestine. However, in the study of Walsh et al. (2012), no differences in serum and mucosal TNF concentrations in weanling pigs supplemented with benzoic acid were observed.

Effect of Essential Oils on Regulation of Gut Microflora

In vitro. Antibacterial activity of essential oils and phytogenic products have been well documented *in vitro* experiments (Dorman and Deans, 2000; Hammer et al., 1999; Si et al., 2006). For example, Lee and Ahn (1998) found that cinnamon essential oil inhibited growth of *Bifidobacterium bifidum* isolated from human feces. Sokmen et al. (2004) conducted a study to evaluate the *in vitro* antimicrobial properties of essential oil extracted from endemic plant, and the results showed that essential oil inhibited the growth of microorganisms. Other studies also reported *in vitro* essential oil antimicrobial activities. Michiels et al. (2009) characterized essential oil against pig gut flora during *in vitro* incubation, and their results indicated that essential oil has the potential to modulate gut microflora. Henn et al. (2010) investigated oregano essential oil on antimicrobial activity in weanling pigs, and the results showed that it had *in vitro* antimicrobial action against tested microorganisms.

In vivo. It would be expected that intake of essential oil affects the gastrointestinal microflora population, but the results were not consistent. For example, in ruminant species, McIntosh et al. (2003) feed a commercial blend of essential oils to dairy cattle. They found that essential oils inhibited the growth of bacteria in ruminal fluid. While, Benchaar et al. (2007) reported that supplementing cow diets with mixture of essential oils did not changes rumen microbial counts. In poultry, Mitsch et al. (2004) investigated two different blends of essential oils on *Clostridium perfringens* of broiler chickens. Their results indicated that essential oils reduced *Clostridium perfringens* concentrations in the jejunum, cecum, and feces. Jamroz et al. (2005) supplied broiler chickens with 100 mg/kg plant extract and observed the increase in *Lactobacilli* and reductions in *E. coli and Clostridium perfringens* populations. In swine, Michiels et al. (2009) investigated essential oils on pig gut microflora, and they reported that the pigs consumed essential oils decreased gastrointestinal *E. coli* count but did not affect

Lactobacilli. Ahmed et al. (2013) evaluated the effect of essential oils on weaned piglets. They indicated increased fecal *Lactobacilli* count and decreased *Salmonella* and *E. coli* counts in the piglets fed essential oils. However, Cross et al. (2007) and Muhl and Liebert (2007) reported that essential oils had no effect on the intestinal microflora populations in broiler chickens and weaned piglets, respectively.

Effect of Benzoic Acid on Regulation of Gut Microflora

In vitro. To screen benzoic acid for its anti-microbial effects in gastrointestinal content, a batch culture system was established. Benzoic acid markedly decreased coliform and lactic acid bacteria counts in the small intestine content from piglets (Knarreborg et al., 2002). In another experiment, Piva and Biagi (2010) evaluated the effect of benzoic acid on bacterial growth in an *in vitro* fermentation system. Different concentrations (7.5, 15, 30, 60, 120, and 240 mmol/L) of benzoic acid were used. The results showed that benzoic acid inhibited bacterial activity at any given concentrations, even at the low concentrations (7.5, 15, or 30 mmol/L).

In vivo. To determine the efficiency of benzoic acid on intestinal microflora, several feeding experiments were conducted. Józefiak et al. (2007) supplied 2.5, 5, and 7.5 g/kg of benzoic acid to broiler chicken diets. Lactic acid bacteria population increased in the group fed the diet with 7.5 g benzoic acid. *Coliform* tended to decrease with the supplementation of benzoic acid in the crop and ileal contents, but no difference on the cecal content. The same researchers conducted another study and supplied broiler chickens with 0.1 or 0.2% benzoic acid. The results showed that lactic acid bacteria population were the lowest in the group fed 0.1% benzoic acid. *Coliform* bacteria population decreased following the increased benzoic acid supplementation (Józefiak et al., 2010). In contrast, Abdalla et al. (2013) found 0.2% benzoic acid in the broiler chicken diet increased the total bacterial and *coliform* counts with decreased in

the *Lactobacilli* count. And, in swine, Kluge et al. (2006) supplied 5 or 10 g/kg of benzoic acid to piglets. In the stomach, the number of total aerobic, total anaerobic, lactic acid bacteria, and gram-negative bacteria was reduced, whereas the number of gram-negative bacteria in the duodenum and the number of total aerobic bacteria in the ileum were reduced in the piglets supplemented with benzoic acid. Oh et al. (2012) supplied 0.5% benzoic acid to weaning pigs, and they found a greater concentration of *Lactobacillus*, *L. plantarum*, and *B. subtillis* in the cecum compared with the control group. However, Torrallardona et al. (2007) supplied 0.5% benzoic acid to piglets and found that benzoic acid had no effect on intestinal microflora.

Effect of Essential Oils on Animal Performance

In recent years, many researchers have documented the effect of essential oils on the growth performance of animals, but the results have not been consistent. In ruminant species, no changes in dry matter intake, milk production, and milk components were observed when dairy cows were fed 750 mg or 2 g of essential oils per day (Benchaar et al., 2006b, 2007). Similarly, supplementation of dairy cows with another essential oil (peppermint) at 20 g/kg dry matter had no effect on milk yield and milk composition (Hosoda et al., 2005). Benchaar et al. (2006a) also evaluated growth performance of beef cattle fed a silage based-diet supplemented with 2 or 4 g/day of a commercial mixture of essential oil compounds. Results showed that dry matter intake and average daily gain were not affected by the addition of the essential oil mixture. But, the gain to dry matter intake ratio was improved. In poultry, Halle et al. (2005) supplemented 2 to 20 g/kg of oregano essential oils to broilers and found the reduced daily feed intake and improved feed efficiency. Lee et al. (2003) fed commercial essential oils to broiler chickens. Feed intake, weight gain, and feed to gain ratio were not different among treatments in their experiment. Amad et al. (2011) conducted a study to examine the effect of a phytogenic feed additives on

broiler chickens. The treatments were the basal diet supplemented with 0, 150, 750, or 1,500

mg/kg of phytogenic feed additive. Body weight, weight gain, and feed intake were not

influenced by the feed additive, but feed conversion ratio improved in phytogenic feed additive

treatment groups. Bampidis et al. (2005) conducted a study to test the effect of oregano essential

oil on turkeys. Body weight, feed intake and feed conversion efficiency were not affected by

essential oil treatment. In contrast, Cornelison et al. (2006) conducted a study to evaluated the

use of hops in broiler chicken diets. Addition of hops at 0 to 2.27 kg per ton resulted in an

improvement in bodyweight and feed conversion efficiency when compared with the control

group. In swine, Li et al. (2012) evaluated the effect of adding essential oils to the diets of

weaned pigs. Over the entire experiment, average daily gain was improved for the pigs fed the

essential oils. On the other hand, other researchers found contradicting results. For instance,

Muhl and Liebert (2007) fed a commercial phytogenic feed additive to weaned pigs. During

experiment, no effect of phytogenic feed additive on average daily gain, feed intake, and feed

conversion ratio were observed. Maenner et al. (2011) assessed the effect of two different

phytogenic products on weaned piglets. Results showed that the feed additives had no effect on

feed intake or body weight gain in the piglets. But, the improvement in gain to feed ratio was

observed.

Effect of Benzoic Acid on Growth Performance

Many studies have illustrated the effect of benzoic acid on the growth performance of

poultry and swine. In poultry, Józefiak et al. (2007) reported dietary inclusion of 5 and 7.5 g/kg

benzoic acid depressed body weight gain and increased feed conversion ratio in broiler chickens.

The same group of researchers provide broiler chickens with 0.1 or 0.2% benzoic acid, and

reported similar results (Józefiak et al., 2010). Amaechi and Anueyiagu (2012) provided broiler

chickens with 0, 0.6, 1.2, 1.8, and 2.4% benzoic acid. Results showed that the body weight gain and feed conversion ratio of broiler chickens fed 0.6 and 1.2% benzoic acid were greater than those fed 1.8 and 2.4% benzoic acid. The broiler chickens consumed 1.8 and 2.4% benzoic acid decreased total feed intake. However, Abdalla et al. (2013) indicated that supplying 0.2% benzoic acid to broiler chickens decreased the final body weight, feed intake, and body weight gain. Olukosi and Dono (2014) reported that supplying 0.2% benzoic acid to broiler chickens have no effect on feed intake, body weight gain, and gain to feed ratio. In swine, Kluge et al. (2006) supplied piglets with 5 and 10 g/kg of benzoic acid. Supplementation of the diet with benzoic acid resulted in a dose-dependent increase in feed intake, body weight gain, and feed conversion ratio. And those results are consistent with the findings by Torrallardona et al. (2007). In contrary, Nemechek et al. (2013) fed 0.5% benzoic acid to weanling pigs, and they found no differences in average daily gain, average feed intake, or feed conversion ratio. It was consistent with Devi et al. (2015) who found that feeding 0.5% benzoic acid to growing-finishing pigs had no effect on feed intake and weight gain.

Summary

Early-weaned pigs are facing serious stressors. It is important to provide a highly palatable and digestible diets to alleviate depressed growth performance during the weaning period. Although a corn-SBM diet is traditionally used as a pig feed, such diet may not be appropriate for weanling pigs because of many digestive and metabolic challenges. For this reason, weanling pigs have been fed a complex diet that containing many "special ingredients," such as dried whey, plasma protein, and fish meal.

Obviously, providing such a complex diet to weanling pigs is costly. With the development of many suitable feed additives, such as enzymes, essential oils, and benzoic acid, it might be possible that a simple corn-SBM diet can be utilized by weanling pigs more

efficiently, which may reduce the cost of diets. Enzymes can be used to extract more energy and nutrients from a diet, and essential oils and benzoic acid can protect piglets from various potential pathogens. The public concerns about the residues and antibiotics resistance have led to the ban of antibiotics as feed additive in, e.g., European Union. Therefore, evaluating fully the potential antimicrobial effects of essential oils and benzoic acid might be even more important in today's pig production.

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III. EFFECT OF DIET COMPLEXITY, MULTI-ENZYME COMPLEXES, ESSENTIAL OILS, AND BENZOIC ACID ON WEANLING PIGS

Effect of Diet Complexity, Multi-enzyme Complexes, Essential Oils, and Benzoic Acid on Weanling Pigs[1]

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A B S T R A C T

The objective of this study was to investigate the effect of supplementing a simple corn-soybean meal (SBM) diet with multi-enzyme complexes, essential oils, and benzoic acid on growth performance, serum metabolite profile, serum cytokines, and gut microbiota. Forty-eight gilts and 48 castrated males (initial body weight, 7.96 ± 0.89 kg) weaned at 3 to 4 wk of age were randomly assigned to 4 dietary treatments with 6 replicate pens (4 gilts or 4 castrated males/pen) per treatment. A typical complex diet containing various special ingredients was formulated and used as a positive control (POS) diet. A simple corn-SBM, negative control (NEG) diet was formulated to be iso-lysinic to the POS diet, and the NEG diet was supplemented with multi-enzyme complexes (ENZ) or multi-enzyme complexes, essential oils, and benzoic acid (ALL). Feed additives were included in the diet by replacing the part of corn. All 4 diets were formulated to meet or exceed the 2012 NRC nutrient requirements. During the fourth week of the study, blood samples were collected to determine serum metabolite profile and cytokines. Similarly, fecal samples were collected for bacterial cultivations during the fourth week of the study. Pigs had ad libitum access to feed and water throughout the study. From d 0 to 7, pigs fed the POS diet consumed more ($P < 0.05$) feed, Lys, and digestible energy (DE) and gained more weight ($P < 0.05$) than those fed the NEG and ALL diets. Pigs fed the POS diet also consumed more ($P < 0.05$) DE than those fed the ENZ diet. Similarly, from d 7 to 14, pigs fed the POS diet consumed more ($P < 0.05$) feed, Lys, and DE and gained more weight ($P < 0.05$) than those fed the NEG and ALL diets. Pigs fed the POS diet also consumed more ($P < 0.05$) DE than those fed the ENZ diet. Pigs fed the ENZ diet gained more weight ($P < 0.05$) than those fed the NEG diet. From d 14 to 21, pigs fed the POS diet consumed more ($P < 0.05$) feed, Lys, and DE, and gained more weight ($P < 0.05$) than those fed the other 3 diets. Pigs fed the POS diet consumed more ($P <$

0.05) DE than those fed the other 3 diets from d 21 to 28. Overall (d 0 to 28), pigs fed the POS diet consumed more ($P < 0.05$) feed, Lys, and DE, and gained more weight ($P < 0.05$) than those fed the other 3 diets. Serum total protein concentration in pigs fed the ENZ and ALL diets was greater ($P < 0.05$) than those fed the POS and NEG diets. Serum albumin concentration in pigs fed the POS diet was greater ($P < 0.05$) than those fed the NEG and ALL diets, and it was greater ($P < 0.05$) in pigs fed the ENZ diet than those fed the NEG diet. Serum globulin and SUN concentrations in pigs fed the POS diet were less ($P < 0.05$) than those fed the other 3 diets. The albumin to globulin ratio, and glucose and cholesterol concentrations in pigs fed the POS diet were greater ($P < 0.05$) than those fed the NEG, ENZ, and ALL diets. Dietary treatments had no effect on any of the serum cytokines and fecal microbiota. The results indicated that weanling pigs' performance can be affected by dietary treatments. Pigs fed the complex diet performed better and had different serum metabolite profile compared with those fed the simple corn-SBM diets. Supplementation of the simply corn-SBM diet with multi-enzyme complexes, essential oils, and benzoic acid had no effect on growth performance. Similarly, serum metabolism profile, cytokine concentrations, and fecal microbiota concentrations in weanling pigs were generally not affected by supplementation of the NEG diet with various feed additives. Further research is needed to explore the possibility of using a simple corn-SBM diet for weanling pigs by supplementing with various feed additives.

Keywords: Weanling pig, Diet complexity, Enzymes, Essential oils, Benzoic acid

1. Introduction

Weaning is the most critical period in the pig's life. At the time of weaning, pigs are exposed to serious stressors, which limit the performance of weanling pigs (Le Dividich and Sève, 2000; van Beers-Schreurs et al., 1998). For this reason, diets provided for weanling pigs should be highly palatable and digestible. Although corn and soybean meal (SBM) have been traditionally used as a pig diet, it does not meet the weanling pig's energy and nutritional requirements because of many digestive, metabolic, and immunological challenges (Jensen et al., 1997; Lindemann et al., 1986). Weanling pigs need a more "complex" diet. A diet containing, e.g., dried whey, plasma protein, and fish meal, which are highly digestible or palatable or both, are called a complex diet. Researchers have investigated the diet complexity for weanling pigs over the years and concluded that a complex diet can improve nursery pig performance (Whang et al., 2000; Wolter et al., 2003). However, providing such a complex diet to weanling pigs can be rather costly.

In addition, dietary antibiotics have been used as animal growth and health promoter for a long time. However, the public concern over the routine use of antibiotics has led to the ban of antibiotics as a feed additive in Sweden in 1986 and Switzerland in 1999 (Wenk, 2003) and the restricted use in 1999 and the complete ban in 2006 in the European Union (Janczyk et al., 2009; Windisch et al., 2008). Considering the ban in those countries and also ongoing discussions on the use of antibiotics in other countries, it is important to explore new ways to improve and protect the health status of farm animals (Wenk, 2003; Windisch et al., 2008).

With a great development of various enzymes or enzyme complexes in fairly recent years, it may be possible for weanling pigs to extract more energy and nutrients from feed ingredients (Zhang et al., 2014) such as corn and SBM. Similarly, some phytogenic feed

additives (Windisch et al., 2008), such as essential oils, and benzoic acid can be incorporated into diets to improve the productivity of weanling pigs because of their antimicrobial and anti-oxidative activities. Previous studies indicated that essential oils may improve pigs' performance (Cho et al., 2005; Kroismayr et al., 2008), nutrient digestibility (Yan et al., 2010), and intestinal ecosystem (Manzanilla et al., 2009). Likewise Knarreborg et al. (2002) reported that benzoic acid can reduce growth of microbiota in the intestine of piglets.

The present study was conducted to assess the possibility of replacing a typical complex diet for weanling pigs with a simple corn-SBM diet supplemented with various feed additives. Specific objective was to investigate the effect of supplementation of a simple corn-SBM diet with multi-enzyme complexes, essential oils, and benzoic acid on growth performance, serum metabolite profile, serum cytokines, and fecal microbiota.

2. Materials and methods

2.1. Animals and facilities

The protocol for animal care was approved by the Institutional Animal Care and Use Committee of Auburn University. A total of 96 piglets weaned at 4 wk of age (initial body weight, 7.96 ± 0.89 kg) were randomly assigned to 4 dietary treatments with 6 replicate pens per treatment (Cochran and Cox, 1957). Because of the number of pigs available at one time, the study was conducted in 3 trials, and each trial used 16 females and 16 castrated males. Each pen (1.5 m^2) contained 4 gilts or 4 castrated males with similar body weight. Pigs were housed in an environmentally controlled nursery with slotted floors and allowed ad libitum access to feed and water throughout the 4-wk study. Pig weights and feed consumption data were collected weekly.

2.2. Dietary treatments

Instead of using the phase feeding program, the decision was made to use only 1 diet during the starter phase. A typical complex, positive control (POS) diet was formulated to contain 13.0 g/kg standard ileal digestible (SID) Lys (NRC, 2012), and the corn-SBM diet included spray dried whey (Honeyville, Brigham City, UT), fish meal (Seven Springs Farm, Check, VA), poultry fat, plasma protein (Appetein, APC Inc, Ankey, IA), as well as antibiotic (Tylan-10 Sulfa-G, Livestock Concepts, Hawarden, IA). A simple, corn-SBM negative control (NEG) diet was formulated to be iso-lysinic to the POS diet, and the NEG was supplemented with multi-enzyme complexes (DSM Nutritional Products, Parsippany, NJ; ENZ) and multi-enzyme complexes, essential oils (CRENA, DSM Nutritional Products), and benzoic acid (Vevovitall, DSM Nutritional Products; ALL). Feed additives were included in the diets by replacing the part of corn. Minerals and vitamins for all diets were provided in amounts calculated to meet or exceed the NRC (2012) recommendations. Feed samples were collected from each batch of feed mixed, and pooled sub-samples were analyzed for crude protein and minerals (AOAC, 2000).

2.3. Collection of blood and fecal samples

During the fourth week of the study, approximately 5 mL of blood was collected via vena cava puncture using a sterile needle and evacuated tube. Blood samples were allowed to clot and serum samples were separated by centrifugation at $1,500 \times g$ for 15 min at room temperature to obtain clean serum samples. An aliquot was stored at -20ºC, and samples were pooled by pen and analyzed for serum metabolites and cytokines. Similarly, fecal samples for bacterial enumeration were collected from, at least, 3 pigs from each pen by rectal stimulation during the fourth week of the study. Fecal samples were immediately chilled with ice, pooled by pen, and an aliquot was immediately used for the assay.

2.4. Analysis of blood and fecal samples

Pooled serum samples were analyzed for total protein, albumin, globulin, serum urea nitrogen (SUN), glucose, cholesterol, and triglyceride using an automatic analyzer at the Auburn University Clinical Pathology Laboratory (Mule et al., 2006). Likewise, pooled serum samples were used for the multiplex cytokine assay (Discovery Assay; Eve Technologies Corp, Calgary, AB, Canada). The multiplex assay consisted of granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFNγ), interleukin (IL)-1α, IL-1Ra, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, and tumor necrosis factor-α (TNFα).

Bacterial counts in the pooled fecal samples were determined by the plate-count technique (Shen et al., 2009). A 25-g sample of feces was homogenized in 225 mL of buffered peptone water and serially diluted from 10^{-1} to 10^{-7} for the analysis. *Coliform* concentrations were determined by plating this homogenate onto violet red bile agar (Difco, Detroit, MI) and incubated aerobically at 37ºC for 24 h. For *Lactobacillus*, samples plated onto MRS agar (Difco) and incubated anaerobically at 37ºC for 48 h. For total anaerobes and aerobes, samples were plated onto plate-count agar (Difco) and incubated anaerobically or aerobically at 37ºC for 48 h. The number of bacteria was quantified based on colony forming units (cfu) on the culturing plate.

2.5. Statistical analysis

Data were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The pen was considered as the experimental unit. Trial and treatment, along with appropriate body weight as a covariate, were included in the statistical model. Covariates considered for the analysis were initial body weight for growth performance data and third week body weight for cytokines, microbiota, and serum metabolites data. The results of the initial statistical analyses

indicated that trial and trial × treatment interactions were not important source of variation, thus, the data for the 3 trials were combined and analyzed accordingly. The PDIFF option was used to assess the effect of treatments. The alpha level used in the determination of significance for all the analysis was $P < 0.05$ with trends at $P < 0.10$.

3. Results

3.1. Growth performance

From d 0 to 7, pigs fed the POS diet consumed more ($P < 0.05$) feed, Lys, and digestible energy (DE) and gained more weight ($P < 0.05$) than those fed the NEG and ALL diets (Table 2). Pigs fed the POS diet also consumed more ($P < 0.05$) DE than those fed the ENZ diet. Gain to feed and gain:Lys intake tended to be greater for pigs fed the POS diet than those fed the other 3 diets (treatments, $P = 0.073$). Similarly, from d 7 to 14, pigs fed the POS diet consumed more ($P < 0.05$) feed, Lys, and DE and gained more weight ($P < 0.05$) than those fed the NEG and ALL diets. Pigs fed the POS diet also consumed more ($P < 0.05$) DE than those fed the ENZ diet. Pigs fed the ENZ diet gained more weight ($P < 0.05$) than those fed the NEG diet. Gain to feed, gain:Lys intake, or gain:DE intake was not affected by dietary treatments.

From d 14 to 21, pigs fed the POS diet consumed more ($P < 0.05$) feed, Lys, DE, and gained more weight ($P < 0.05$) than those fed the other 3 diets. Gain to feed, gain:Lys intake, or gain:DE intake was not affected by dietary treatments. Pigs fed the POS diet consumed more ($P < 0.05$) DE than those fed the other 3 diets from d 21 to 28. Pigs fed the POS diet seemed to consume more feed and Lys than those fed the other 3 three diets, but the differences were not statistically significant. Overall (d 0 to 28), pigs fed the POS diet consumed more ($P < 0.05$)

feed, Lys, DE, and gained more weight ($P < 0.05$) than those fed the other 3 diets. There was no effect of dietary treatments on the efficiency of utilization of feed, Lys, or DE.

3.2. Serum metabolites

The effect of feed additives on metabolites concentrations in the serum of weanling pigs during the fourth week of the study is presented in Table 3. Total protein concentration in piglets fed the ENZ and ALL diets was greater ($P < 0.05$) than those fed the POS and NEG diets. Albumin concentration in pigs fed the POS diet was greater ($P < 0.05$) than those fed the NEG and ALL diets, and it was greater ($P < 0.05$) in pigs fed the ENZ diet than those fed the NEG diet. Globulin and SUN concentrations in pigs fed the POS diet were less ($P < 0.05$) than those fed the other 3 diets. The albumin to globulin ratio, glucose and cholesterol concentrations in piglets fed the POS diet were greater ($P < 0.05$) than those fed the NEG, ENZ, and ALL diets. Triglyceride concentration in pigs fed the POS diet seemed to be less than those fed the other 3 diets, but there was no statistically significant difference.

3.3. Serum cytokines and fecal microbiota

The effect of feed additives on serum cytokine concentrations and fecal microbiota in weanling pigs is presented in Tables 4 and 5, respectively. The concentrations of GM-CSF, IFNγ, or IL-6 was either not detected or detected inconsistently, thus, the data were not presented (Table 4). Weanling pigs fed the ENZ diet seemed to have greater IL-2 concentration than those fed the ALL diet. The IL-8 concentration seemed to be greater in pigs fed the ENZ diet. And, the concentration of IL-18 seemed to be lower in pigs fed the NEG diet. However, those differences in cytokines among dietary treatments were not statistically significant. Similarly, although there seemed to be some differences among treatments on some microbiota, dietary treatments had no effect on any of the fecal microbiota.

4. Discussion

A corn-soy diet is the gold standard for feeding pigs, however, such diet may not be appropriate for weanling pigs. Weanling pigs are subjected to various stressors at weaning, and their digestive system is immature or the secretion of digestive enzymes is not sufficient. For that reason, traditionally, weanling pigs have been provided complex diets containing many special ingredients such as dried whey, fish meal, and others, which are highly palatable and digestible. However, using such diets can be rather costly. With the development of, e.g., complex enzymes, it might be possible to use corn-SBM diets more successfully for weanling pigs.

In addition, it's been a common practice to include antibiotics as an animal growth and health promoter in, e.g., weanling pig diets. However, because of the public concerns over antibiotic resistance and the possible residue problems, the dietary use of antibiotics for such purpose has been banned in many countries (Janczyk et al., 2009; Wenk, 2003; Windisch et al., 2008). Considering such bans and ongoing discussions on the dietary use of antibiotics, it's crucial to find alternatives (Wenk, 2003; Windisch et al., 2008) for successful and sustainable pig production. Although various approaches, such as maintaining good housing or climatic conditions (Wenk, 2003) or including non-antibiotic growth promoters (e.g., organic acids, prebiotics, and probiotics) in weanling pig diets (Windisch et al., 2008) to improve and protect the health status and promote their growth, the use of essential oils and benzoic acid can be a viable alternative.

The effect of supplementing weanling pig diets with enzymes, essential oils, and benzoic acid individually have been reported over the years (Knarreborg et al., 2002; Kroismayr et al., 2008; Zhang et al., 2014). However, the combination effect of dietary enzymes, essential oils,

and benzoic acid on weanling pigs has not been explored fully. Therefore, in the present study, the effect of multi-enzyme complexes, essential oils, and benzoic acid on growth performance, metabolic profile, and health status was investigated.

In the present study, pigs fed the NEG diet consumed less feed, Lys, and DE, and had reduced ADG and gain to feed ratio compared with those fed the complex POS diet. Previous research has shown that feeding piglets a simple diet can result in reduced body weight gain (Whang et al., 2000), which was probably due to reduced feed intake as observed in the present study (Dritz et al., 1996). Moreover, Dritz et al. (1996) found that the effect of the diet complexity on feed intake was most pronounced in the early period after weaning. In the current study, the POS diet had effect on pigs' growth performance during the first 2 wk, but the impact of diet complexity on growth performance on last 2 wk was less.

The results of the present study indicated that supplementing the NEG diet with multi-enzyme complexes had no effect on feed intake, Lys intake, DE intake, body weight gain, and gain to feed, Lys, or DE intake. The lack of improvement in pigs growth performance as a result of multi-enzyme supplementation has been reported in several studies (Kiarie et al., 2007; Omogbenigun et al., 2003). Based on some research, this result could be attributed to the age of pigs. For example, phytase supplementation was found to improve performance on grower-finisher pigs more than on early-weaned pigs (Harper et al., 1997). Furthermore, carbohydrases seemed to be most effective in swine diets formulated with higher fiber ingredients, such as wheat distillers dried grains with soluble, barley, or rye (Emiola, 2009; Omogbenigun et al., 2004). The carbohydrases seemed less effective when included in diets based on corn and SBM. Jones et al. (2010) found that galactosidase, galactomannanase, β-glucanase, or xylanase supplementation did not improve animal performance when added to corn–SBM based diets

containing 30% corn distillers dried grains with solubles. The efficacy of carbohydrases seems to be dependent on the amount of available substrates for the enzymes to break down. Fiber substrate composition and concentration likely play a role in the efficacy of carbohydrase enzymes (Bedford, 2000). Because corn is highly digestible and low in fiber, carbohydrases may not consistently improve growth performance (Partridge, 2000).

In previous studies, the effect of essential oils and benzoic acid on animal growth performance has been rather inconsistent. For example, Zhang et al. (2014) showed that weanling pigs fed diet supplemented with 0.5% benzoic acid and 0.01% essential oils improved ADG and gain to feed ratio. On the other hand, other researchers (Maenner et al., 2011; Muhl and Liebert, 2007; Nemechek et al., 2013) indicated that the diet supplemented with essential oils or benzoic acid had no effect on feed intake, body weight gain, or feed conversion ratio. Similarly, in present study, no difference in growth performance was observed in piglets fed the ALL diet compared with those fed the ENZ diet.

Serum metabolite data may provide information about animal metabolic activities. Lowrey et al. (1962) indicated that the total protein concentration can be used as an indicator of efficiency of utilization of dietary protein. In the current study, pigs fed the POS diet had a greater growth rate than those fed the other 3 diets, thus pigs may utilized crude protein more efficiently for growth than those fed the other 3 diets, and that might be the possible reason that pigs fed the POS diet had lower serum total protein concentration.

In the pigs, the liver increases albumin synthesis in response to the increase of available amino acids (Busher, 1990). In the present study, pigs consumed the POS diet had greater albumin concentration than those consumed other diets. It may be explained by the fact that the POS diet included spray dried whey and fish meal, which are highly digestible, and may have

provided more available amino acids than other diets. Serum total protein consists of albumin and globulin. Generally, total serum protein and albumin are determined directly, and total serum globulin is determined by the difference. Because pigs fed the POS diet had greater albumin concentration than those fed other diets, it may have resulted in a relatively lower globulin concentration and greater albumin to globulin ratio.

Serum urea nitrogen is another important indicator of protein and amino acid adequacy and efficiency of amino acid utilization (e.g., Coma et al., 1995). Eggum (1970) found that the serum urea content increased with the crude protein content in the diet. In the present study, piglets fed the NEG, ENZ, and ALL diets had a greater concentration of SUN compared with those fed the POS diet. That was likely due to the relatively greater protein content of the NEG, ENZ, and ALL diets. The increased glucose concentration in pigs fed the POS diet may be explained by a relatively greater carbohydrate content of the POS diet compared with the NEG, ENZ, and ALL diets, which had greater crude protein content than the POS diet (Fabian et al., 2004).

In the current study, pigs fed the POS diet have greater serum cholesterol concentration than those fed the other 3 diets. Pigs fed the POS diet grown faster than those fed the other 3 diets, which may resulted in greater accumulation of body fat and higher cholesterol concentration. Pigs consumed the POS diet had numerically lower serum triglyceride concentration than those consumed the other 3 diets. Pigs fed the POS diet had greater growth rate than those fed the other 3 diets, and it is possible that pigs on the POS treatment used the potential energy for growth, thus, reducing serum triglyceride concentration.

Cytokines play an important role in mediation of immune response to infection. The IL-1, IL-6, and TNF-α are typical pro-inflammatory cytokines produced by macrophages of the innate

immune system as a rapid response against disease and infection (Dinarello, 1991, 2000). Pié et al. (2004) reported early and transient response in the gene expression of inflammatory cytokines in the gastrointestinal tract is associated with weaning. There have been some studies that showed the anti-inflammatory effect of essential oils and benzoic acid. Li et al. (2012) reported that the IL-6 concentration was lower and TNF-α concentration was greater in the plasma of weanling pigs supplemented with essential oils. Oh et al. (2012) found dietary supplementation with 0.5% benzoic acid decreased the concentration of IL-1β, IL-6 and TNF-α in the small intestine of weanling pigs. However, Walsh et al. (2012) did not found any differences in the serum and mucosal TNF-α concentration of weanling pigs supplemented with benzoic acid. In the present study, feeding pigs the diet supplemented with essential oils and benzoic acid had no effect on serum cytokine concentrations.

Several studies have reported the effect on intestinal microflora when essential oils and benzoic acid have been included in animal diets (Józefiak et al., 2007; Kluge et al., 2006; Mitsch et al., 2004; Michiels et al., 2009). On the other hand, Muhl and Liebert (2007) fed weaned pigs with a commercial phytogenic feed additives and found no effect on microbial count in feces. Furthermore, Torrallardona et al. (2007) reported that supplementation of the piglet diet with 0.5% benzoic acid had no effect on intestinal microflora. In the current study, feeding the diet containing essential oils and benzoic acid had no effect on the fecal microbiota.

It is known that healthy pigs do not respond to antimicrobial agents when they are housed under clean and disinfected conditions (Lee et al., 2003). In the present study, weanling pigs were kept in a clean, sanitary environment, which may have diminished the efficacy of the possible antimicrobial and anti-oxidative dietary additives. In addition, although it has been demonstrated in the *in vitro* studies that essential oils and benzoic acid have antimicrobial

activity (Dorman and Deans, 2000; Knarreborg et al., 2002; Si et al., 2006), such an effect may not be observed under varying *in vivo* conditions (Wenk, 2003). The microbiota in the digestive tract may depend on many factors such as feed composition, digestive tract pH, nutrient absorption rate, and others (Wenk, 2003). Those factors may influence the effect of essential oils and benzoic acid on intestinal microbiota. Besides, the minimum inhibitory concentration of essential oils or benzoic acid, which is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of test microorganism (Andrews, 2001), may not have exceeded because of the dilution effects during the passage of digesta.

5. Conclusion

The results indicated that weanling pigs' performance can be affected by dietary treatments. Pigs fed the complex diet performed better and had different serum metabolite profile compared with those fed the simple corn-SBM diets. Supplementation of the simply corn-SBM diet with multi-enzyme complexes, essential oils, and benzoic acid had no effect on growth performance. Similarly, serum metabolism profile, cytokine concentrations, and fecal microbiota concentration in weanling pigs were generally not affected by supplementation of the NEG diet with various feed additives. Further research is needed to explore the possibility of using a simple corn-SBM diet for weanling pigs by supplementing with various feed additives.

Conflict of interest statement

The authors certify that there is no financial and (or) personal relationships with other individuals or organizations that can affect the current research project improperly, or no

professional or personal interest of any nature or kind in any product, service, and (or)

organization that could to construed as influencing the present article.

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Table 1
Composition of weanling pig diets (as-fed basis) [1,2,3].

Item	POS	NEG	ENZ	ALL
Ingredient (g/kg)				
Corn	413.6	514.9	514.2	511
Soybean meal (48% CP)	331.7	457.4	457.4	457.4
Spray dried whey[4]	150	-	-	-
Fish meal[5]	40	-	-	-
Poultry fat	30	-	-	-
Plasma protein[6]	10	-	-	-
Dicalcium phosphate	6.6	12.8	12.8	12.8
Limestone	7.1	8.9	8.9	8.9
Salt	3.5	3.5	3.5	3.5
Enzyme mixture[7]	-	-	0.75	0.75
Essential oil[8]	-	-	-	0.1
Benzoic acid[9]	-	-	-	3
Antibiotics[10]	5	-	-	-
Vitamin-trace mineral premix[11]	2.5	2.5	2.5	2.5
Calculated composition				
DE (Mcal/kg)	3.61	3.43	3.43	3.42
CP (g/kg)	242.8	260.7	260.7	260.4
Ca (g/kg)	8	8	8	8
P (g/kg)	7	7	7	7
Ca:P	1.14	1.14	1.14	1.14
Lys (g/kg)	13	13	13	13
Lys:DE (g/Mcal)	3.6	3.79	3.79	3.8
Analyzed composition (g/kg)				
CP	242.3	246.3	273.8	269.5
Ca	7.6	8.9	6.7	9.3
P	6.1	6.2	5.8	6.2

[1] POS = positive complex diet; NEG = simple corn-soybean meal, negative diet; ENZ = NEG diet supplemented with multi-enzyme complexes; and ALL = NEG diet supplemented with multi-enzyme complexes, essential oils, and benzoic acid.
[2] Weanling pig diets were fed from 7.96 ± 1.78 to 20.70 ± 3.76 kg.
[3] CP = crude protein; and DE = digestible energy.
[4] Spray dried whey: Honeyville (Brigham City, UT).
[5] Fish meal: Seven Springs Farm (Check, VA).
[6] Plasma protein: Appetein (APC Inc, Ankeny, IA).
[7] Enzyme mixtures (RONOZYME; DSM Nutritional Products, LLC, Parsippany, NJ): 0.11 g/kg MultiGrain (2,700 fungal xylanase unit xylanase, 700 fungal beta-glucanase unit β-glucanase, and 800 cellulase unit cellulase/g); 0.09 g/kg VP (50 fungal beta-glucanase unit β-glucanase and 5,000 pectinase unit pectinase/g); 0.09 g/kg WX (1,000 fungal xylanase unit

xylanase/g); 0.11 g/kg Rumistar (600 kilo novo unit amylase/g); 0.35 g/kg HiPhos2700 (10,000 phytase unit phytase/g).

[8] Essential oil: CRINA (DSM Nutritional Products, LLC).

[9] Benzoic acid: Vevovitall (DSM Nutritional Products, LLC).

[10] Antibiotics: Tylan-10 Sulfa-G (Livestock Concepts, Hawarden, IA).

[11] Provided the following (unit/kg diet; Nutra Blend, Neosho, MO): Fe (ferrous sulfate), 150 mg; Zn (zinc oxide), 150 mg; Mn (manganous oxide), 37.5 mg; Cu (copper sulfate), 150 ppm; I (ethylenediamine dihydroiodide), 5 ppm; Se (sodium selenite), 3 ppm; vitamin A, 6,614 IU; vitamin D_3, 1,102 IU; vitamin E, 26 IU; vitamin B_{12}, 0.03 mg; menadione (menadione Na bisulfite complex), 1 mg; riboflavin, 6 mg; D-pantothenic acid (D-Ca pantothenate), 45 mg; niacin, 28 mg; and choline (choline chloride), 110 mg.

Table 2
Effect of feed additives on growth performance of weanling pigs [1,2,3].

Item	Diet				SEM	*P*-value
	POS	NEG	ENZ	ALL		
d 0 to 7						
ADFI (g)	298[a]	232[b]	264[ab]	261[b]	8.30	0.013
Lys intake (g/d)	3.88[a]	3.01[b]	3.44[ab]	3.39[b]	0.11	0.013
DE intake (Mcal/d)	1.08[a]	0.79[b]	0.91[b]	0.89[b]	0.03	0.002
ADG (g)	256[a]	182[b]	211[ab]	170[b]	11.7	0.01
G:F (g/kg)	849	782	789	656	30.5	0.073
Gain:Lys intake (g/g)	65.3	60.2	60.7	50.4	2.35	0.073
Gain:DE intake (g/Mcal)	235	228	230	192	8.44	0.148
d 7 to 14						
ADFI (g)	647[a]	552[b]	587[ab]	570[b]	12.3	0.041
Lys intake (g/d)	8.41[a]	7.18[b]	7.62[ab]	7.41[b]	0.16	0.041
DE intake (Mcal/d)	2.33[a]	1.90[b]	2.01[b]	1.95[b]	0.05	0.004
ADG (g)	537[a]	416[b]	486[ac]	456[bc]	15.1	0.004
G:F (g/kg)	833	752	830	797	17.4	0.111
Gain:Lys intake (g/g)	64.1	57.8	63.8	61.3	1.34	0.111
Gain:DE intake (g/Mcal)	231	219	242	233	4.91	0.173
d 14 to 21						
ADFI (g)	874[a]	737[b]	789[b]	745[b]	16.6	0.005
Lys intake (g/d)	11.35[a]	9.58[b]	10.26[b]	9.68[b]	0.22	0.005
DE intake (Mcal/d)	3.15[a]	2.53[b]	2.71[b]	2.55[b]	0.07	< 0.001
ADG (g)	648[a]	528[b]	542[b]	529[b]	15.2	0.015
G:F (g/kg)	745	722	685	709	13.7	0.504
Gain:Lys intake (g/g)	57.3	55.6	52.7	54.5	1.06	0.5
Gain:DE intake (g/Mcal)	207	210	200	207	3.79	0.825
d 21 to 28						
ADFI (g)	982	849	871	853	24.6	0.113
Lys intake (g/d)	12.77	11.04	11.32	11.08	0.32	0.113
DE intake (Mcal/d)	3.54[a]	2.91[b]	2.99[b]	2.91[b]	0.09	0.017
ADG (g)	624	555	561	576	23.6	0.594
G:F (g/kg)	633	653	637	671	16.5	0.801
Gain:Lys intake (g/g)	48.7	50.2	49	51.6	1.27	0.802
Gain:DE intake (g/Mcal)	175	190	186	196	4.92	0.404
d 0 to 28						
ADFI (g)	700[a]	592[b]	628[b]	607[b]	13.6	0.007
Lys intake (g/d)	9.10[a]	7.70[b]	8.16[b]	7.89[b]	0.18	0.007
DE intake (Mcal/d)	2.52[a]	2.03[b]	2.15[b]	2.08[b]	0.05	< 0.001
ADG (g)	516[a]	420[b]	450[b]	433[b]	10.7	< 0.001
G:F (g/kg)	738	711	715	715	7.75	0.588
Gain:Lys intake (g/g)	56.8	54.7	55	55	0.6	0.589

Gain:DE intake (g/Mcal)	205	207	208	209	2.07	0.889

a-c Within a row, means without a common superscript differ ($P < 0.05$).

[1] POS = positive complex diet; NEG = simple corn-soybean meal, negative diet; ENZ = NEG diet supplemented with multi-enzyme complexes; and ALL = NEG diet supplemented with multi-enzyme complexes, essential oils, and benzoic acid.

[2] Stater diets were fed from 7.96 ± 1.78 to 20.70 ± 3.76 kg.

[3] ADFI = average daily feed intake; Lys = lysine; DE = digestible energy; ADG = average daily gain; G:F = gain to feed ratio; and SEM = pooled SEM.

Table 3
Effect of feed additives on plasma metabolite concentrations in weanling pigs [1,2,3].

Item	Diet				SEM	*P*-value
	POS	NEG	ENZ	ALL		
Total protein (g/dL)	4.63[b]	4.72[b]	4.88[a]	4.87[a]	0.03	< 0.01
Albumin (g/dL)	3.78[a]	3.49[b]	3.65[ac]	3.58[bc]	0.05	0.02
Globulin (g/dL)	0.85[b]	1.22[a]	1.22[a]	1.29[a]	0.05	< 0.01
A:G ratio	4.56[a]	2.94[b]	3.02[b]	2.79[b]	0.20	< 0.01
SUN (mg/dL)	14.32[b]	17.61[a]	18.37[a]	17.78[a]	0.49	< 0.01
Glucose (mg/dL)	134[a]	116[b]	115[b]	120[b]	2	< 0.01
Cholesterol (mg/dL)	81.77[a]	70.41[b]	71.18[b]	75.13[b]	1.39	< 0.01
Triglyceride (mg/dL)	41.67	50.80	50.56	51.30	2.11	0.23

[a-c] Within a row, means without a common superscript differ ($P < 0.05$).

[1] POS = positive complex diet; NEG = simple corn-soybean meal, negative diet; ENZ = NEG diet supplemented with multi-enzyme complexes; and ALL = NEG diet supplemented with multi-enzyme complexes, essential oils, and benzoic acid.

[2] A:G Ratio = albumin to globulin ratio; SUN = serum urea nitrogen.

[3] SEM = pooled SEM.

Table 4
Effect of feed additives on plasma cytokine concentrations (pg/mL) in weanling pigs [1,2,3].

Item	Diet				SEM	*P*-value
	POS	NEG	ENZ	ALL		
IL-1α	4.78	2.19	7.51	2.74	1.29	0.44
IL-1β	260	479	307	253	50	0.40
IL-1Ra	242	238	351	308	28	0.54
IL-2	21	22	59	7	10	0.33
IL-4	57	111	114	162	22	0.62
IL-8	275	285	696	361	73	0.13
IL-10	197	154	317	120	43	0.40
IL-12	1401	1267	1523	1339	62	0.61
IL-18	1150	668	1269	1070	102	0.24
TNFα	8.18	7.99	10.95	6.39	2.22	0.94

[1] POS = positive complex diet; NEG = simple corn-soybean meal, negative diet; ENZ = NEG diet supplemented with multi-enzyme complexes; and ALL = NEG diet supplemented with multi-enzyme complexes, essential oils, and benzoic acid.

[2] IL-1 α = interleukin-1 α; IL-1 β = interleukin-1 β; IL-1 Ra = interleukin-1 Ra; IL-2 = interleukin-2; IL-4 = interleukin-4; IL-8 = interleukin-8; IL-10 = interleukin-10; IL-12 = interleukin-12; IL-18 = interleukin-18; TNF α = tumor necrosis factors α.

[3] SEM = pooled SEM.

Table 5

Effect of feed additives on microbiota content ($\log_{10}$cfu/g) in feces of weanling pigs [1,2].

Item	Diet				SEM	*P*-value
	POS	NEG	ENZ	ALL		
Total aerobes	10.71	10.73	10.55	10.51	0.07	0.60
Total anaerobes	9.90	10.17	10.20	10.17	0.08	0.29
Coliforms	7.39	6.65	7.35	6.92	0.24	0.57
Lactobacillus	10.85	10.79	10.87	10.70	0.05	0.69

[1] POS = positive complex diet; NEG = simple corn-soybean meal, negative diet; ENZ = NEG diet supplemented with multi-enzyme complexes; and ALL = NEG diet supplemented with multi-enzyme complexes, essential oils, and benzoic acid.

[2] SEM = pooled SEM.

IV. SUMMARY AND CONCLUSION

Weaning is the most critical period in the pig's life, and the trend in swine industry has been to wean pigs early. Many swine producers have been weaning their pigs at 3 to 4 weeks of age. At the time of weaning, pigs are exposed to several stressors, which limit the growth performance of weaned pigs. Pigs at weaning are suddenly removed from the sow and moved into a new environment where they have to consume dry feed from feeder and drink water from a drinker. Additionally, they are mixed with pigs from other litters and fight to establish new hierarchies. As a result, pigs go through a period of so called "growth check," and they may lose about 100 to 250 g body weight (BW) during the first day after weaning, which may not be recovered until 4 d post-weaning. The metabolizable energy (ME) intake can be only about 60 to 70% of the pre-weaning ME intake. In addition, although newborn pigs can produce sufficient enzyme to digest the fats and proteins in sow milk, activities of other enzymes necessary to utilize a corn-SBM diets are not sufficient, especially during the first week after weaning. For this reason, diets provided for weaning pigs should be highly palatable and digestible, and compatible with their enzyme secretion pattern.

A corn-SBM diet is traditionally used as a pig feed. Because pigs face challenges during weaning and the digestive system of those pigs is immature, it is essential to provide a diet that can ease the transition from sow's milk to a solid feed that can support maximum growth rate. Obviously, a simple corn-SBM diet cannot meet the weanling pig's nutritional requirements. Weaning pigs need a more "complex" diet that contains so called special ingredients such as

dried whey, plasma protein, fish meal, which are highly palatable and digestible. Researchers have investigated the effect of diet complexity for weanling pigs over the years and concluded that complex diets can improve nursery pig growth performance during the early post-weaning period. However, providing such complex diets with highly palatable and digestible ingredients to weanling pigs can be rather costly.

In addition, pigs are born with, essentially, no protection against various challenges from disease organisms. For that reason, it's been a routine practice to include an antibiotic/antibiotics in weanling pig diets. Dietary antibiotics have been used as animal growth and health promoter for a long time. At the same time, the issue of "antibiotic resistance" has been a subject of concern for researchers, producers, and general public since, perhaps in the 1960s in the States and many other countries. With the complete ban of the use of antibiotics in feed in European countries and others, the current trend is to look for some alternatives to protect young pigs from various disease organisms.

Over the years, various exogenous enzymes or enzyme complexes have been developed to increase the digestibility to extract more energy and nutrients from feedstuffs/diets. Similarly, it's been demonstrated in recent years that essential oils or benzoic acid or both can be used as an alternative to antibiotics because of their antibacterial and anti-oxidative effects. Previous studies indicated that essential oil may improve pig performance, nutrient digestibility, and intestinal ecosystem, and benzoic acid may reduce growth of some undesirable microbiota in the intestine of pigs.

The present study was conducted to assess the possibility of replacing a typical complex diet for weanling pigs with a simple corn-SBM diet supplemented with various feed additives. Specific objective was to investigate the effect of supplementation of a simple corn-SBM diet

with multi-enzyme complexes, essential oils, and benzoic acid on growth performance, serum metabolite profile, serum cytokines, and fecal microbiota.

Forty-eight gilts and 48 castrated males (initial body weight, 7.96 ± 0.89 kg) weaned at 3 to 4 wk of age were randomly assigned to 4 dietary treatments with 6 replicate pens (4 gilts or 4 castrated males/pen) per treatment. A typical complex diet containing various special ingredients was formulated and used as a positive control (POS) diet. A simple corn-SBM, negative control (NEG) diet was formulated to be iso-lysinic to the POS diet, and the NEG diet was supplemented with multi-enzyme complexes (ENZ) or multi-enzyme complexes, essential oils, and benzoic acid (ALL). Feed additives were included in the diet by replacing the part of corn. All 4 diets were formulated to meet or exceed the 2012 NRC nutrient requirements. During the fourth week of the study, blood samples were collected to determine serum metabolite profile and cytokines. Fecal samples were collected for bacterial cultivations. Pigs had ad libitum access to feed and water throughout the study.

From d 0 to 7, pigs fed the POS diet consumed more feed, Lys, and digestible energy (DE) and gained more weight than those fed the NEG and ALL diets. Pigs fed the POS diet also consumed more DE than those fed the ENZ diet. Similarly, from d 7 to 14, pigs fed the POS diet consumed more feed, Lys, and DE and gained more weight than those fed the NEG and ALL diets. Pigs fed the POS diet also consumed more DE than those fed the ENZ diet. Pigs fed the ENZ diet gained more weight than those fed the NEG diet. From d 14 to 21, pigs fed the POS diet consumed more feed, Lys, and DE, and gained more weight than those fed the other 3 diets. Pigs fed the POS diet consumed more DE than those fed the other 3 diets from d 21 to 28. Overall (d 0 to 28), pigs fed the POS diet consumed more feed, Lys, and DE, and gained more weight than those fed the other 3 diets. Serum total protein concentration in pigs fed the ENZ and

ALL diets was greater than those fed the POS and NEG diets. Serum albumin concentration in pigs fed the POS diet was greater than those fed the NEG and ALL diets, and it was greater in pigs fed the ENZ diet than those fed the NEG diet. Serum globulin and urea nitrogen (SUN) concentrations in pigs fed the POS diet were less than those fed the other 3 diets. The albumin to globulin ratio, glucose and cholesterol concentrations in pigs fed the POS diet were greater than those fed the NEG, ENZ, and ALL diets. Dietary treatments had no effect on any of the serum cytokines and fecal microbiota.

The results indicated that weanling pigs' performance can be affected by dietary treatments. Pigs fed the complex diet performed better and had different serum metabolite profile compared with those fed the simple corn-SBM diets. Supplementation of the simply corn-SBM diet with multi-enzyme complexes, essential oils, and benzoic acid had no effect on growth performance. Similarly, serum metabolism profile, cytokine concentrations, and fecal microbiota concentrations in weanling pigs were generally not affected by supplementation of the NEG diet with various feed additives. Further research is needed to explore the possibility of using a simple corn-SBM diet for weanling pigs by supplementing with various feed additives.

V. CUMULATIVE BIBLIOGRAPHY

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VI. APPENDICES

**Appendix A**: Principle of the Total protein Analysis (Roche Diagnostics, Indianapolis, IN)

Under alkaline conditions, divalent copper in the biuret reagent reacts with protein peptide bonds to form the characteristic purple-colored biuret complex:

$$\text{Protein} + \text{Cu}^{2+} \xrightarrow{\text{Alkaline solution}} \text{Cu-protein complex}$$
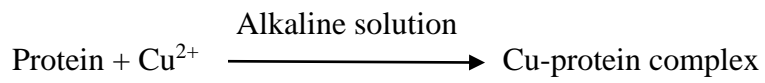
The color intensity is directly proportional to the protein concentration, which can be measured photometrically.

Appendix B: Principle of the Albumin Analysis (Roche Diagnostics, Indianapolis, IN)

It is a colorimetric assay with endpoint method. At a pH of 4.1, albumin displays a sufficiently cationic character to be able to bind with bromocresol green (BCG), an anionic dyestuff to form a blue-green complex:

$$\text{Albumin} + \text{BCG} \xrightarrow{\text{pH 4.1}} \text{Albumin BCG complex}$$
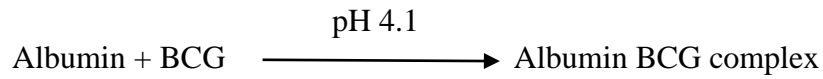
The color intensity of the blue-green color is directly proportional to the albumin concentration and can be measured photometrically.

Appendix C: Principle of the Urea nitrogen Analysis (Roche Diagnostics, Indianpolis, IN)

Urea is hydrolyzed by urease to form CO_2 and ammonia:

$$Urea + H_2O \xrightarrow{\text{Urease}} 2\ NH_4^+ + CO_2$$
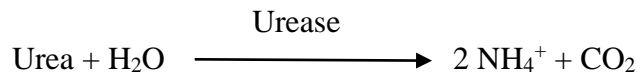
The ammonia formed then reacts with α-ketoglutarate and NADH in the presence of GLDH to yield glutamate and NAD^+:

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADH \xrightarrow{\text{GLDH}} L\text{-glutamate} + NAD^+ + H_2O$$
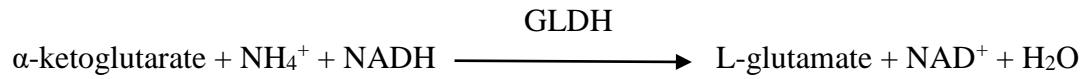
The decrease in absorbance due to consumption of NADH is measured kinetically.

Appendix D: Principle of Glucose Analysis (Diagnostic Chemicals Ltd)

Glucose is phosphorylated to hexokinase in the presence of adenosine triphosphate (ATP) and magnesium to form glucose-6-phosphate (G-6-P) and adenosine diphosphate (ADP):

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase, Mg}^{2+}} \text{Glucose-6-phosphate} + \text{ADP}$$
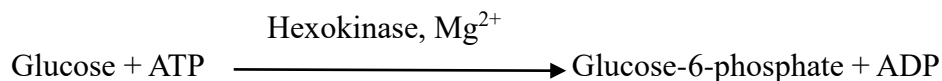
G-6-P is then oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) in the presence of nicotinamide adenine dinucleotide (NAD$^+$) producing 6-phosphogluconate and NADH:

$$\text{Glucose-6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADH} + \text{H}^+$$
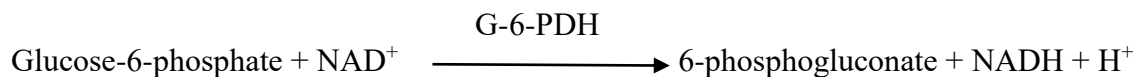
The formation of NADH causes an increase in absorbance at 340 nm which is directly proportional to the concentration of glucose in the sample.

Appendix E: Principle of Cholesterol Analysis (Roche Diagnostics, Indianpolis, IN)

Cholesterol is determined enzymatically using cholesterol esterase and cholesterol oxidase as follows. Cholesterol esters are cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids:

$$\text{Cholesterol esters} + H_2O_2 \xrightarrow{\text{Cholesterolesterase}} \text{Cholesterol} + RCOOH$$
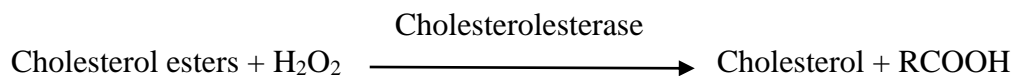
Cholesterol is converted by oxygen with the aid of cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$
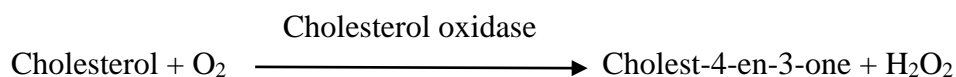
The hydrogen peroxide created forms a red dyestuff by reacting with 4-aminophenazone and phenol under the catalytic action of peroxidase:

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{4-(p-benzoquinone-monoimino)-phenazone} + 4H_2O$$
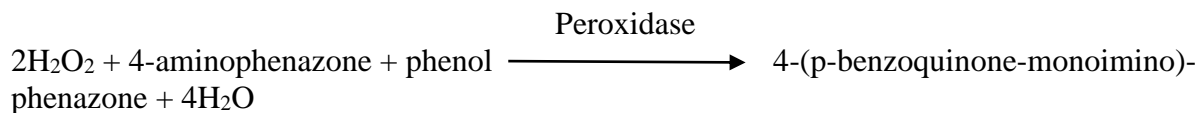
The color intensity is directly proportional to the concentration of cholesterol and can be determined photometrically.

Appendix F: Principle of the Triglyceride Analysis (Diagnostic chemicals Ltd., Oxford. CT)

Serum triglycerides are hydrolyzed to glycerol and free fatty acids by lipase:

$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{fatty acids}$$
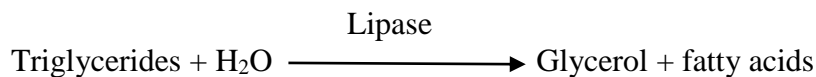
In the presence of ATP and glycerol kinase (GK), the glycerol is phosphorylated to glycerol-1-phosphate:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK, } Mg^{2+}} \text{Glycerol-1-phosphate} + \text{ADP}$$
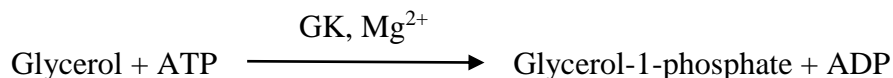
Glycerol-1-phosphate is then oxidized by glycerol phosphate oxidase (GPO) to yield hydrogen peroxide (H_2O_2):

$$\text{Glycerol-1-phosphate} + O_2 \xrightarrow{\text{GPO}} H_2O_2 + \text{dihydroxyacetone phosphate}$$
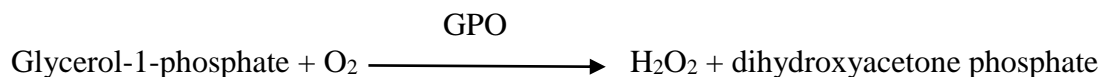
The hydrogen peroxide causes oxidative coupling of p-chlorophenol and 4-aminoantipyrine, producing a red colored quinoneimine dye complex:

$$H_2O_2 + \text{p-cholorophenol} + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine dye} + H_2O_2$$
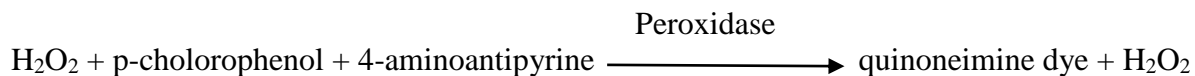
The increase in absorbance at 520 nm due to the formation of the quinoneimine dye is directly proportional to the concentration of triglycerides in the sample.

Appendix G: Microbiota Plate Count Procedures

1. Sample preparation

    Weight 25 g solid or semi-solid sample into a sterile stomacher bag containing 225 mL of peptone water diluent, beat in the blender for 1-2 min, and then formulate into 1:10 sample solution.

2. Dilution

    For each dilution use fresh 1 mL sterile pipette. Pipette 1 mL of 1:10 sample solution into a tube containing 9 mL of peptone water, shake up the tube and then formulate 1:100 sample solution. Repeat above operation procedures until the desired dilution is obtained.

3. Plating and cultivation

    For determination of total aerobic or anaerobic bacteria, according to the estimation of live bacteria count to be tested, select 2-3 proper continuous dilution degrees, for each dilution degree, 1 mL of sample solution is placed into 2 sterile plates. Pour 15-20 mL plate count agar (tempered to 46ºC) into each petri-dish and swirl plates to mix. Allow agar to solidify. Incubate the dishes, aerobically or anaerobically at 36ºC for 48 h.

    Similarly, according to the estimation of live bacteria count to be tested, select 2-3 proper continuous dilution degrees, for each dilution degree, 1 mL of sample solution is placed into 2 sterile plates. For determination of *coliform*, pour 10-12 mL of Violet Red Bile Agar (tempered to 48ºC) into each petri-dish and swirl plates to mix. Allow to solidify. Overlay with 3-5 mL VRBA and allow to solidify. Incubate the dishes, inverted at 36ºC for 18-24 h. For determination of *lactobacillus*, pour 15-20 mL MRS agar (tempered to 46ºC) into each petri-dish and swirl plates to mix. Allow agar to solidify. Anaerobically incubate the dishes, inverted at 36ºC for 48 h.

4. Counting the colonies

Select the plates for the number of colonies between 30 colony-forming units (CFU) and 300 CFU. Record the dilution times and corresponding plate count.

5. Calculation

Calculate the average plate count of both plate, multiply the average value by corresponding dilution times, to serve as the total plate count in one gram of sample.

Appendix H: The reference of enzyme activities

Multi-grain, VP, WX, Hiphos:

Http://nutricionanimal.info/wp-content/uploads/2014/11/tabla-comparativa-enzimas.pdf

RumiStar:

Vargas-Rodriguez C.F., Engstrom, M., Azem, E., Bradford, B.J., 2014. Effect of dietary

amylase and sucrose on productivity of cows fed low-starch diets. J. Dairy Sci., 97, 4464–4470.

Appendix I: Definition of enzyme units

Fungal Beta-Glucanase (FBG) unit is the amount of enzyme which degrades β-glucan to reducing carbohydrates with a reduction capacity equivalent to 1 μmol glucose per minute under pH 5.0 at 30ºC

Pectinase unit (PSU) corresponds to the amount of enzyme that generates 1 μmol of galacturonic acid from polygalacturonic acid per minute under pH 4.0 at 40ºC.

Fungal xylanase unit (FXU) was defined as the amount of enzyme that liberates 1 μmol of xylose from arabinoxylan per minute under pH 6.0 at 50ºC.

Kilo Novo Units (KNU) is the amount of enzyme which breaks down 5.26 g starch per hour at Novo's standard method for determination of α-amylase.