Effect of Feed Additives and Toxic Elements on Swine Growth Performance, Nutrient Digestibility, Immune Function and Reproductive Performance

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by

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Abstract

To evaluate the effects of peptide in combination of zinc oxide (Zno) or acidifiers (Exp 1), and gossypol (G) from cottonseed meal (CSM) (Exp 2&3) on growth performance, complete blood cell counts (Exp 1, 2&3), nutrient digestibility (Exp 1), plasma gossypol (Exp 2&3) and semen quality (Exp 3), weaned pigs (Exp 1), growing gilts (Exp 2) and growing boars (Exp 3) were randomly allotted to dietary treatments. Treatments for Exp 1 during phase 1&2 were: (1) Positive Control (PC), formulated to meet NRC (2012) nutrient requirements; (2) Negative control (NC), fish meal was reduced to achieve -0.13% SID lysine; (3) NC + 0.25% peptide plus high level of zinc (0.25PZ); (4) NC + 0.50% peptide plus high level of zinc (0.5PZ); (5) 0.25% peptide (0.25P); (6) 0.50% peptide (0.5P); (7) 0.25% peptide + 0.1% sodium butyrate and 0.5% benzoic acid (PSB). In phase 3, all pigs were fed a common diet. Treatments in Exp 2 during phase 1 to 3 consisted of: (1) control diet, formulated to meet NRC, (2012) nutrient requirements without CSM (0% G); (2) inclusion of 1.21% CSM (0.01% G); (3) 2.42 % CSM (0.02% G); and (4) 4.84 % CSM (0.04% G). In Exp 3, dietary treatments were the same as those in Exp 2, except the 1.21% CSM (0.01% G) was removed. During phase 4, pigs were fed a common diet devoid of CSM (Exp 2&3). Each phase consisted of 14 days. Data were analyzed using the Mixed procedures of SAS as a RCBD with treatment as fixed effect, and BW block as random effect. In overall phase 1&2 of Exp 1, PSB pigs had similar ADG and BW when compared to those fed 0.25PZ and both were greater than NC pigs ($P < 0.05$). In Exp 2&3, gilts had a linear reduction ($P < 0.05$) and boars a quadratic ADG response ($P < 0.05$) as level of CSM increased in the diet during phase 3. These studies demonstrate that feeding nursery pigs with peptide in combination with acidifiers improved growth performance similar to that observed in pigs fed high levels of zinc oxide, indicating that acidifiers may be an alternative to high levels of ZnO in weaned pigs

diets without affecting their growth performance. Feeding gossypol from CSM up to 0.02% impaired growth performance in gilts and boars but not affected semen quality.

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Introduction

Introduction

The animal production industry must continue to advance in its efficiency to fulfill the demand for meat products to an ever-increasing human population. This demand for meat products correlates with the need for animal feed (Tiwari, 2018). The feed costs in the swine industry represent approximately the 70 % of total production cost (Shike, 2011; Patience et al., 2015). However, physiological, environmental, and social changes at weaning disrupt intestinal and immune system functionality, resulting in reduced feed efficiency and growth performance (Brooks et al., 2001; Campbell et al., 2013; Linden, 2015).

In order to be healthy and efficient, piglets must acclimate as soon as they can to a wide number of weaning stressors, such as the abrupt separation from the sow, transportation and handling, establishing a social hierarchy, co-mingling with pigs from other litters, different environment conditions, and changes in diet. When these stressors are excessive for the pig to overcome, they become more susceptible to pathogens, resulting in low growth performance and high mortality rate (Campbell et al., 2013). The inclusion of biosynthetic or additive products, such as probiotics, prebiotics, emulsifiers, organic acids, and macro-micro mineral supplements are dietary components used in swine nutrition to reduce digestive dysfunctions associated with the transition of diet after weaning (Zheng, 2018).

The swine industry is incorporating protein hydrolysates in nursey diets due to their high protein quality and palatability (Zhao, 2014). Most of these protein sources are obtained from animal protein sources such as porcine intestines and fish viscera (Hou et al., 2017). Results have shown that pigs fed fish peptides have similar performance compared to those fed fish meal (Norgaard, 2012). Besides, pigs fed fish peptides in combination with fish meal had increased feed intake compared to those fed soybean meal (Norgaard, 2012). Notably, there is evidence

where nursery pigs fed high protein quality feedstuffs in combination with zinc sources showed considerable improvement in protein digestibility and absorption (Wang et al., 2019). However, there exists the problem of environmental contamination from pigs fed high zinc diets (Burton, 2017). Interestingly, acidifiers have been shown to exert benefits to mucosal morphology, and controlling alterations in the gut microflora through bacteriostatic or bactericidal actions, and enhancing endogenous enzyme activity in the pig as well as improving growth performance similar to that observed in pigs fed high zinc from ZnO (Partanen, 2011).

Feral hogs, a prolific species categorized as one of the top 100 destructive invasive species (Lowel et al., 2000), have been overpopulated in the US, generating extensive damages to agricultural crops, pastures, native vegetation and animal life (Brown et al., 2019). According to the United States Department of Agriculture (2020), approximately $1.5 billion are lost due to agricultural damage by feral hogs every year. Furthermore, another concern related to feral hogs is that they act as carrier of diseases which could endanger domestic livestock and humans (Meng, 2009). Because of the extensive agricultural and environmental damage caused by feral hogs plus the threat they pose as a vector of diseases, it is crucial to find new strategies to eradicate or counteract the growth of this invasive species.

Cotton seed is one of the large vegitable oil sources used worldwide (Thirumalaisamy et al., 2016). The end product of the oil extraction is cottonseed meal, which is considered a good nutritional source for livestock animals due to its high crude protein level (Stein et al., 2016). However, the inclusion of this feedstuff is generally restricted in monogastric animal diets because of a toxic component called gossypol (Tanksley, 1990).

In addition to the negative effects of gossypol on growth performance (Fombad and Bryant, 2004) and blood cells (Zbidah et al., 2012), the detrimental effects of gossypol are

reported in male reproductive performance, impairing spermatogenesis (EL-Sharaky et al, 2010) by reducing the sperm concentration, sperm motility, total sperm counts (Dodou et al., 2005; Baker, 2019), as well as the synthesis of hormones related to the reproduction (EL-Sharaky et al., 2010).

This chapter's aim is to review the function of peptide in nursery diets and the benefits of acidifiers such as sodium butyrate and benzoic acid on gut health, immunology status and growth performance in weaned pigs. The impact of gossypol on growth performance and reproduction will also be evaluated.

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Chapter 1: Literature Review

Part 1

Fish Meal

Fish meal is a nutritional compound that has been used in weaned piglet diets. This protein source is used in diet formulations for increasing feed intake due to its high palatability (Jones et al., 2018). Fish meal is widely available around the world because of its competitive price in comparison to other animal protein sources (milk and blood), and for its high nutritional quality (Cho and Kim, 2011). It has synergistic effects when combined with other protein sources, either vegetables or animal origin, enhancing growth and decreasing feed costs (Miles and Chapman, 2012). For example, fish meal has large amounts of energy per unit weight (Zinn et al., 2009), exceptional protein level with an excellent balance of amino acids, vitamins and minerals, and the presence of omega 3 fatty acids (Li et al., 2014; Cho and Kim, 2011; Jones et al., 2018).

Nutritional Composition of Fish Meal

Protein Content

In general, fish meal containing crude protein levels ranging from 60 to 72 % are considered of good quality (Cho and Kim, 2011), but also the quality will depend on its organoleptic characteristics such as taste, color and odor (Barzana and Garcia-Garibay, 1994). NRC (2012) and Ween et al. (2017) indicated that fish meal has a crude protein content of 63.8 %, and 62 % respectively. This discrepancy in the nutritional values depend on the species of fish from which the meals are obtained (Barlow, 1993). Fish meal is recommended as a protein supplement for monogastric animals because it offers an excellent amino acid profile; and rich in some essential amino acids such as lysine and methionine.

Lipid Content

Lipid content in fish meal is inconsistent. The concentration depends on the processing methodologies and objectives of the processing plant. On average, this ingredient contains between 6 to 10 % of lipid; however, it can be as higher as 20 % (Cho and Kim, 2011). Fish meal is used to ameliorate the imbalance of lipid content in animal diets because it offers high level of omega-3 fatty acids, which is low in plant-protein sources. Furthermore, it is a good source of docosahexaenoic acid, eicosapentaenoic acid, and linoleic acid. In addition, the fish oil is an excellent source of energy for pigs, chickens, shrimp and even ruminants due to its high digestibility of more than 90% (Miles and Chapman, 2012).

Mineral Content

The mineral content of fish meal is mainly known for its calcium and phosphorous concentration. It is considered that ash levels between 17 % to 25 % is a product of good quality. Contrary to the phosphorus content of plants, which is not highly digestible by monogastric animals, this mineral in fishmeal is in a highly available form for most animals (Miles and Chapman, 2012). According to NRC (2012), fish meal contains calcium and phosphorus levels of 4.28 and 2.29 % respectively; having higher levels than other protein sources such as soybean meal, which has 0.31 % and 0.75 % of calcium and phosphorus. In addition, the available phosphorous in fish meal has better standardized total tract digestibility (0.82%), than soybean meal (0.66 %).

Fish Protein Hydrolysates

According to the FAO (2018), the aquaculture sector registered more than 171 million tons of fish in 2016, increasing 36% in comparison to 2016, when only 110.2 million tons were produced. Base on the type of seafood and processing method, between 30- 60 % of fish

production is not used for human consumption, and thus is classified as waste products (Halim et al., 2016; Mahro and Timm, 2007). The high amount of waste products derived from commercial fish processing plants can be utilized in the production of feed sources for animals. Including such components in animal feed provides high levels of proteins, protein concentrates and fish protein hydrolysates thus promoting the growth of domestic animals leading to greater protein availability for human consumption (Barzana & Garcia-Garibay,1994). However, fish protein concentrates are not potentially as beneficial as fish protein hydrolysates because they are deficient of bioactive properties (Spinelli et al., 1972).

Fish protein hydrolysates are subproducts obtained from fish waste protein that is hydrolyzed into small peptides, containing mainly between 2 to 20 amino acids and free amino acids (Hou et al., 2017; Chalamaiah et al., 2012; Barzana and Garcia-Garibay,1994). The general nutritional contents of most fish protein hydrolysates are 81-91 % of protein, no more than 5 % fat, 3-8% ash, and 1-8 % moisture (Venugopal, 2016). The hydrolysis of protein sources can take between 4 to 48 hours, depending on the technology or methodology used (Hou et al., 2017). Specific peptides and peptide mixtures obtained from the hydrolysis of fish meat are investigated worldwide and are considered to be bioactive products due to their positive effects in the treatment of certain health problems (Gevaert et al., 2016). There are different methods of hydrolysis used by industry and the specific method utilized depends on the nature of the protein source (Hou et al., 2017).

Hydrolysis of Proteins

Acid and alkaline hydrolysis are the cheapest method to hydrolyze proteins but has the negative impact in high losses of amino acids (Mustatea et al., 2019; Dai et al., 2014). On the other hand, the enzymatic hydrolysis is the ideal method to hydrolyze fish tissues because it

maintains protein quality (Huang et al., 2015), thus preserving losses of nutritional value. This method can be controlled to such a degree that it can provide specific products based on consumer needs (Pasupuleti and Braun, 2010). This process can be achieved through the use of synthetic or natural enzymes (Petrova et al., 2018). The natural enzymes that are widely used in this method are obtained from animals (trypsin and pepsin), plants (papain and bromelain) and microbes (bacterial and fungal proteases: Pasupuleti and Braun, 2010; Pasupuleki et al., 2010; Petrova et al., 2018). In addition, protein hydrolysis can be achieved by microbial fermentation which is the earliest methodology used for food preservation. Bacteria have the capacity produce endogenous proteolytic enzymes, which act on proteins, releasing peptides with bioactive properties (Abuine et al., 2019). Bacterial hydrolysis can be achieved in two different ways, each based on the moisture content of the target. The process for a liquid protein source differs from a process acting upon a low moisture solid source (Hou et al., 2017).

Functional or Bioactive Activity of Peptides

Animal protein sources, such as hydrolyzed marine products, have been demonstrated to contain beneficial bioactive peptides (Gevaert et al., 2016), and thus are categorized as an ideal food ingredient (Harnedy and FitzGerald, 2012). Bioactive peptides are defined as small chains of amino acids which possess potential, beneficial functions within the host (Walther and Sieber, 2011; Murray and FitzGerald, 2007). Generally, peptides with bio-functional activity, with some exceptions, usually do not contain more than 20 amino acids in their chain (Hou et al., 2017). The specific function of different bioactive peptides depends upon their unique amino acid sequence, and the specific method used for hydrolysis (Pihlanto-Leppälä, 2000; Harnedy and FitzGerald, 2012).

According to Cipolari et al. (2020), fish bioactive peptides have different physiological functions, of which the most relevant are as agonists, antagonists, hormones, mediators, effectors, cofactors, activators, and stimulators. These functions, determined by peptide structure, are mainly associated with cell signaling, where they operate as translators and carry out biochemical messages, bringing about structural, molecular and cellular changes that create a biological effect (Cooper and Hausman, 2004).

Antioxidant Activity

Peptides from animal protein hydrolysates have the capacity to decrease reactive oxygen species (ROS), reactive nitrogen species (RNS), and lipid peroxidation. Also, they can reduce levels of antioxidants and pro-inflammatory cytokines in the small intestine, thus enhancing gut health, and improving nutrient digestibility and growth performance (López, Gutierrez & Serena, 2014). During cellular respiratory and metabolic processes, ROS and RNS are continually produced, releasing hydroxyl radical OH, superoxide anion radials O_2, and non-free radical species such as hydrogen peroxide and singlet oxygen. An elevated presence of ROS leads to deterioration of DNA, proteins, and lipid molecules (Abuine et al., 2019).

According to Elias et al. (2008), the position of the amino acid in the peptide bond as well as its type and hydrophobicity are characteristics of high relevance regarding antioxidant activity. These amino acid characteristics are believed to breakdown the tertiary structure of proteins, allowing the entrance solvents to enter the oxidative molecules, thus reducing the synthesis of oxidative stress (Sanchez and Vásquez, 2017). It is also considered that the low molecular weight of some bioactive peptides plays an important role in the antioxidative scavenge capacity of oxygen and nitrogen oxidative species (Dong et al, 2008).

Antimicrobial Activity

Fish possess a vigorous immune system because of constant exposure to high levels of bacterial, viral, and parasitical pathogens. Based on this, fish antimicrobial peptides are biochemical molecules linked to the immune system. (Rauta et al., 2012). At present, the mechanism of action of such peptides is not totally understood.

It is believed, once in the gastrointestinal tract, antimicrobial peptides (AMPs) have the capacity to damage bacterial cell membranes (Lima et al., 2015; Shabir et al., 2018). This damage is mainly caused by two mechanisms: 1); once AMPs couple to the bacterial cell membrane it results in the formation of transmembrane pores which allow leaking of intracellular contents, and 2); AMPs could also penetrate the cell membrane, deactivating enzymes and inhibiting the synthesis of proteins and nucleic acids (Shabir et al., 2018).

Bioactive peptides with antimicrobial activity have been the best substitute of antibiotics, having the capacity to control bacteria, viruses, fungi, and mycobacteria (Reddy et al., 2004). It has been shown that AMPs such as peptide A3, P5, colicin E1, cecropin AD, and cipB-lactoferricin-lactoferrampin (cipB-LFC-LFA) have the capacity to promote the health status of the gastrointestinal tract, enhance intestinal microflora, improve nutrient digestibility and growth performance in pigs and poultry (Tang et al., 2009; Xiao et al., 2015).

Immunomodulatory Activity

Bioactive peptides have been shown to stimulate the immune system (Yang et al., 2009). It has been reported that bioactive peptides derived from fish hydrolysates can improve immune status through the stimulation of different mechanisms, such as lymphocyte proliferation (He et al., 2015), natural killer (NK) cell activity (Hartman and Meisel, 2007), splenocyte proliferation (Kim et al., 2018), and antibody production (Moughan and Markwick, 2013). They also possess

anti-inflammatory activity (Moughan and Markwick, 2013) by suppressing the synthesis of nitric oxide and decreasing the proliferation of tumor necrosis factor -α and interleukin-6, which are proinflammatory cytokines (Ahn et al., 2012).

According to Chalamaiah et al. (2018), the bioactive peptides that most likely enhance an immunomodulatory activity are composed of aliphatic-hydrophobic non polar amino acids (glycine, valine, leucine and proline), aromatic amino acids (phenylalanine, tyrosine), and polar charged glutamic acid.

Effects on Growth Performance

Fish meal and derivatives from fish wastes such as protein hydrolysates were found to improve growth performance in nursery pigs (kim and Ster, 2001; Gottlob et al., 2006). The high concentration of short peptide chains in the hydrolysates are palatable and are more readily absorbed than intact protein, even without being digested by pancreatic proteases, then leading to improved growth performance (Gilbert et al., 2008).

Thuy and Ha (2016) found that fish protein hydrolysates improved ADG, ADFI, and reduced feed cost/gain and diarrhea incidence when replaced 100% of fish meal inclusion in pig diets over 5 weeks after weaning. However, their results were inconsistent with nursery pigs when fed salmon protein hydrolysates. There appears to be some discrepancies when fish bioactive peptides were fed together with other bioactive peptides in replacement of fish meal in nursery diets. Zhantian et al. (2009) found that pigs fed fish protein hydrolysates in combination with spray-dried plasma had similar response when compared to pigs fed fish hydrolysates with soybean meal. Turker (2011) and Norgard et al. (2012) did not find any differences in BW, ADG and ADFI in pigs fed a soybean vs. a fish meal diet.

As mentioned previously, the inclusion of fish protein hydrolysates to the diet of pigs has shown positive results. However, the result of studies evaluating inclusion of the same specific bioactive peptide in nursery diets, replacing fish meal or in combination with other bioactive peptides has been inconsistent. Based on this, further research is needed to define the mode of action of fish protein hydrolysates when combined with other feed additives, which may enhance physiological activities of bioactive peptides.

Zinc Oxide

To prevent commensal bacteria disorder from post weaning stress, zinc oxide (ZnO) became a focus of swine nutritionists because it has demonstrated the ability to decrease the incidence of diarrhea, thus improving health and growth performance of weaned piglets (Carlson et al., 1999; Hojberg et al., 2005; Walk et al., 2015).

Even though the minimum dietary requirement of zinc for weaned piglets is approximately 100 ppm (NRC, 2012), the addition of high levels of zinc in the form of ZnO (2000 - 3000 ppm) is recommended by the feed industry. Pharmaceutical levels of zinc have been shown to stimulate growth performance and decrease diarrhea incidence. However, pigs do not have the capacity to utilize and digest such high concentrations entirely. As a result, when pig wastes are used as fertilizer, zinc may pollute soil and water sources (Cang et al., 2004). As a consequence, the use of high levels of zinc after June 2022 will be drastically restricted in several developed countries (Byrne, 2019). Therefore, it is necessary to find new strategies to replace high levels of zinc in the swine diet without compromising profitable growth performance.

Organic Acids

In previous decades, organic acids have been used in animal nutrition because they

displayed positive effects on performance, feed efficiency, intestinal morphology, and gut health (Pettigre, 2006; Cortyl, 2009; Papatsiros and Billinis, 2012). The livestock industry prioritizes its production efficiency with new genetic lines and minimizes the use of pharmaceutical strategies (antibiotics and high levels of zinc) that lead to bacteria resistance and environmental pollution (Byrne, 2019; Tugnoli et al., 2020). Acidifiers are used as a nutritional strategy that can help improve or maintain overall growth performance in livestock animals without detrimental the environmental effects observed with excess zinc.

Effects on Stomach pH

At weaning, piglets do not have the capacity to produce enough stomach hydrochloric acid due to lack of dietary stimuli if the piglet diet is supplied totally from sow´s milk, whereas the lactose is digested relatively easily by lactase produced by bacteria in the stomach (Kim et al., 2005; Lawlor et al., 2005). During the early postweaning period when pigs start to consume feed grains, the buffering capacity of diets containing high levels of protein and minerals hinder the stimulation of hydrochloric acid resulting in a high stomach pH of 5 or greater (Makkink, 2001; Kim et al., 2005). As a consequence, stomach pH maintains relatively high, affecting pepsin activation, which needs pH 2-3.5 to function efficiently (Ravindran & Kornegay, 1993; Mayer, 1994; Cortyl, 2009). Organic acids have been shown to significantly decrease stomach pH, which increase pepsinogens to catalyze pepsins and increasing proteolytic enzyme activity in weaning piglets (Kim et al., 2005; Desai et al., 2007).

Effects on Gastrointestinal Microbiota

Acidifiers have shown to control microbial populations in the gastrointestinal tract. Organic acids have the capacity to kill pH-sensitive bacteria such as *Listeria* spp, *Escherichia coli*, *Salmonella*, *Clostridia*, and coliforms. Once the acids come in contact with the bacterial

surface, the undissociated biochemical structure these acids can penetrate the cell wall and disrupt intracellular homeostasis (Gauthier, 2002; Kim et al, 2005). The accumulation of anionic molecules from these acids decreases the intracellular pH and causes an imbalance between intracellular and extracellular; therefore, bacteria die as a result of activation of the H+ -ATPase pump mechanism, diverting energy sources to maintain homeostasis instead of growth (Cortyl, 2009). The control of pathogens helps the proliferation of desirable bacteria such as *Lactobacilli* and *Bifidobacterium* that contribute to low diarrhea incidence.

Effects on Growth Performance and Nutrient Digestibility

The inclusion of organic acids in swine diets has been shown to improve growth performance in piglets (Tsiloyiannis et al., 2001; Oh, 2004). With the inclusion of 0.5 % benzoic acid, Guggenbuhl et al. (2007), and Diao (2016) found improvement in feed conversion ratio, and ileal nitrogen and energy digestibility, and a 7-13% increase in body weight when compared to pigs fed the control diet. Besides, benzoic acid has been shown to improve mineral digestibility, especially Ca and P (Diao et al., 2013). In addition, 0.9% sodium formate added to the nursery diet has also shown good results by increasing ADG, feed conversion ratio and nutrient digestibility of piglets (Suryanarayana et al., 2010;). Ravindran & Kornegay, (1993), and Devi et al. (2015) mentioned that organic acids in grower pigs are not as effective as in weaned pigs. Fumaric and citric acid are considered the most beneficial and preferred organic acids for weaned pigs (Tsiloyiannis, 2001; Suiryanrayna and Ramana, 2015). It is believed that an acidifier blend is more beneficial than a single organic acid. However, Ahamed et al. (2014) found that citric acid demonstrated better growth performance than an acidifier blend in 28 day old pigs, and Radecki et al. (1988), and Walz & Pallauf (1997) did not observe any

improvements in growth performance and nutrient digestibility in weaned pigs when citric and formic acid were included.

It can be concluded that the addition of acidifiers in the swine diet is a beneficial nutritional strategy that enhances the overall health status and functionality of the gastrointestinal tract which leads to improvements on growth performance and nutrient digestibility for nursery pigs. However, more research is needed regarding organic acids as a diet supplement to understand their mode of action which could possibly explain the contradictory results expressed in some previous studies.

Scope of Research

Fish bioactive peptides as feed supplements have benefitted the livestock feed industry because of their immunomodulatory, antioxidant, and antimicrobial activity. In addition, acidifiers exhibited significant improvements to gastrointestinal tract health. Individually and in combination, these two nutritional sources are discussed in more detail in chapter 2. To investigate these feed supplements more thoroughly, an experiment was conducted to determine the growth performance, hematology profile, and nutrient digestibility in nursery pigs fed fish-microbial peptides separately and in combination with pharmaceutical zinc or fish-microbial peptides plus acidifiers, using diets with reduced levels of fish meal and standard dietary zinc.

Part 2

Gossypol

Gossypol, 2,2´-bis 8-formyl-1,6,7-trihydroxy5-isopropyl-3-methylnaphthalenel, is a lipid- soluble polyphenolic compound with a molecular weight of 518.55 Daltons. It is produced by different parts of the cotton plant, with the seed possessing the highest concentration (Borem et al., 2003). The concentration of this molecule within cottonseed can vary widely depending on

types of cotton plants (Jan et al., 2008), and environmental conditions. It is known to increase concentration during rainy conditions, and decrease in high temperatures (Pons et al, 1953). The biochemical structure of gossypol is found as (-) negative and (+) positive enantiomers, with the (-) enantiomer having the highest activity. The (-) enantiomer is more toxic than the (+) enantiomer due its lower rate of excretion or detoxification by the organism (Wu et al., 1986; Freedman et al., 2003). The level of gossypol in whole cottonseed (Kernels) can range from 7,000 to 14,000 mg/Kg. The concentration of gossypol in cottonseed meal is approximately 0.1 to 0.2 %. The extraction of oil, the combination of steam and heat during extraction, and further processing such as pelleting can greatly reduce the level of gossypol in cottonseed meal (Jones and King, 1996).

Gossypol Toxicity

Gossypol is known to cause several negative impacts in growth performance, health, and reproductive status (Garland, 2015). The clinical and subclinical symptoms of toxicity depend on the level of cottonseed in diets, and the symptomatology is usually the same across all animal species. Erythrocyte fragility is the most frequent symptom evident in different species (Risco et al., 1992; Jan et al., 2008). This hematologic abnormality of blood cells shows up when there is high toxicity level in different organs. Animals with low toxicity exhibit pulmonary edema, reduced feed intake and weight loss (Jan et al., 2008). However, it has been shown that increased levels of lysine and iron in a diet can decrease the toxic effects due to the aldehyde group of gossypol molecules linking with amines, and the elipson amino group of lysine, forming protein-gossypol complexes (Strøm-Hansen et al., 1989; Soto-Blanco, 2008). Furthermore, gossypol has the capacity to interact and build chains with iron molecules, called iron-gossypol conjugates, which are indigestible compounds, resulting in a reduction of toxic effects (Jan et al., 2008). In

addition, gossypol exhibits degenerative effects on the immune response, decreasing lymphocyte and neutrophil levels (Braga et al., 2012).

Effects on Reproductive Physiology

Even though the structure of gossypol has been known for over a century, the first evidence of gossypol toxicity on reproduction was in Chinese men during the 1930-1940´s. Citizens of Wang Village, located in Jiangsu China, replaced soybean oil with cottonseed oil for cooking. This resulted in an almost total sterile population with a very low birth rate for almost a decade (Liu, 1957).

The sterility effect of gossypol is more evident in monogastric animals than in ruminants. The severity of the toxic effects depends on the inclusion rate of gossypol and age at which animals are fed. Gossypol induces infertility by disrupting sperm cell mitochondria and germinal epithelium, resulting in decreased motility and sperm concentrations (Randel et al., 1992; Gadhela, 2014; Garland, 2015). Mature rats and hamsters fed 10 and 20 mg/kg body weight experienced low spermatocyte quality (Hahn et al., 1981; El-Sharaky et al., 2010). In addition, gossypol affects the functionality of Leydig and Sertoli cells (Jan et al., 2008). El-Sharaky et al. (2010) found that gossypol causes testicular pathologies such as Sertoli cell toxicity and degeneration of seminiferous tubules.

Baker (2019) collected semen for a 10 week period while pigs were fed a diet containing 60 % cottonseed meal (0.74% gossypol). The results demonstrated a 21.3% reduction in sperm motility, and a 16.02% increase in static cells when compared to the control pigs, culminating in a low sperm fertilization potential. Even low concentrations of gossypol have caused toxic side effects including death. Ling-Yun et al. (1984) fed boars at 8 and 20 % of cottonseed containing 0.069% gossypol in the diet and discovered that libido, semen quality and testosterone decreased

as gossypol levels increased. Also, the high inclusion (20%) resulted in the death of several boars. Clawson and Smith (1966) fed growing pigs (24 kg BW) diets containing 80, 244, and 400 mg/kg of gossypol. There results established that the 80 mg/kg gossypol in diet did not have any effect on growth performance, whereas 244 and 400 mg/kg diets caused death after day 37.

Hormones related to reproductive function are also affected by gossypol. El-Sharaky et al. (2010) found degenerative damage in spermatozoids and a reduction in levels of testosterone, LH, and FSH in rats fed 20 mg/kg BW of gossypol. Rats fed gossypol at 25mg/kg BW for 5 consecutive days had reduced estradiol-17-β levels (Lin et al., 1985). Also, rats fed low levels of gossypol at 5 mg/kg BW had low levels of cytochrome P450, and enzyme associated with reproductive hormone synthesis.

Scope of Research

Based on the problems that involve feral hogs for farmers and society, an experiment was conducted to determine the effects of gossypol on growth performance and blood cell count and semen quality in growing pigs. In chapter 3 are shown in more detail the possible applicability of gossypol from cottonseed as control method to the over population of this invasive species.

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Chapter 2: Effects of Adding Bioactive Peptide and Sodium Butyrate plus Benzoic Acid, in Replacing High Level of Zinc Oxide on Growth Performance, Hematology Profile, and Apparent Total Tract Digestibility of Nutrients in Nursery Pigs

Effects of Adding Bioactive Peptide and Sodium Butyrate plus Benzoic Acid, in Replacing High Level of Zinc Oxide on Growth Performance, Hematology Profile, and Apparent Total Tract Digestibility of Nutrients in Nursery Pigs

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Abstract:

To evaluate the effect of bioactive peptide (P) in combination with high level of zinc (HZ) or acidifiers on growth performance, complete blood cell counts (CBC) and nutrient digestibility in nursery pigs, a total of 288 weaned pigs (PIC1050xDNA600) were stratified by initial body weight (BW) within gender and allotted to 1of 7 treatments. Treatments for phase 1&2 were: 1) nutrient adequate positive control with HZ (PC), 2) nutrient deficient negative control with HZ (NC, -0.13% SID Lysine by reducing fish meal), 3) NC+0.25% peptide (0.25PZ), 4) NC+0.5% peptide (0.5PZ), 5) NC+0.25% peptide with standard zinc (0.25P), 6) NC+0.5% peptide with standard zinc (0.5P), 7) as 5 + 0.1% sodium butyrate and 0.5% benzoic acid (PSB). All pigs were fed a common low Zn diet (197 ppm) during phase 3. Individual body weight, and pen feed disappearance were collected on d 0 and at the end of each phase, while blood from 2 close-to-average pen-BW pigs were collected on d 0, and at the end of phase 2 and 3 to determine average daily gain (ADG), gain:feed ratio (G:F), complete blood cell count (CBC), and blood urea nitrogen (BUN). Fecal samples were collected at the end of phase 2 to determine volatile fatty acids (VFA) concentration, and nutrient digestibility, using titanium dioxide as indigestible marker. Data were analyzed using the Mixed procedures of SAS as a RCBD with treatment as fixed effect, and BW block as random effect. In overall phase 1&2, pigs fed 0.25PZ had similar

ADG and BW when compared to those fed PSB and both were greater than NC pigs (P< 0.05). Pigs fed PSB diet had the greatest G:F ratio and nitrogen digestibility among treatments (P< 0.05). Increasing peptide in high zinc diets gradually decreased Neutrophil-to-lymphocyte ratio (P< 0.05). There were statistic significant differences in BUN concentration among phases (P< 0.05), while VFA`s concentration did not differ among treatments (P >0.05). This study indicates that the improvement in growth performance from pigs fed peptide is pharmaceutical zinc dependent and acidifiers can be an alternative to replace ZnO without affecting growth performance.

Key words: fish-microbial-peptide, sodium butyrate, benzoic acid, zinc oxide, nursery pigs.

Introduction

Transitioning from lactation to nursery is the most stressful stage for piglets due to the abrupt environmental, social, and nutritional changes, caused by the sudden diet change from sow milk to dry feed. Dry feed is less palatable and digestible, leading to disturbances in gut morphology and functionality (Brooks et al., 2001, Lallès et al., 2007, Campbell et al., 2013). These disturbances are linked to weak barrier integrity and compromised immune functions that allow the proliferation of harmful bacteria to dominate the microflora. Such a change in the microbiota can result in post weaning diarrhea (PWD), causing a lag in growth performance and possibly death (Amezcua et al., 2002; Fairbrother et al., 2005; Domeneghini et al., 2006).

The swine industry has been using pharmaceutical compounds such as antibiotics in nursery diets to prevent the effects of PWD on growth performance (Verstegen and Williams, 2002; Vondruskova et al., 2010; Padaoan, 2018). ZnO was later implemented as a growth promoter and has been used in nursery diets to prevent intestinal disorders in the gastrointestinal

tract of piglets during the first days following weaning. However, measures have been established in several European countries to phase out the inclusion of antibiotics and high level of zinc in meat producing animal diets due to the high possibility of bacterial resistance or environmental pollution, respectively (Lusk et al., 2006; Heo et al., 2012; Sneeringer, 2015). Therefore, finding alternative additives is critical to counteract the negative impact on growth performance and mortality in antibiotics free and low zinc operations (Stein, 2002).

Fish protein hydrolysates have demonstrated a benefit to several bioactive or functional activities related to intercellular signaling, thus enhancing several biological functions (Cipolari et al., 2020). It has been shown that bioactive peptides can stimulate the immune system, reduce oxidative stress, and exert antimicrobial activity (Kim et al., 2009; Hou et al., 2017; Cipolari et al., 2020). Due to their various physiological properties, they may be a source for generating new drugs (Kim and Wijesekara, 2010; Cipolari et al., 2020), or a substitute for pharmacological growth promoters (Guo, 2020). Marine bioactive peptides have been shown to enhance feed consumption in nursery pigs (Norgaard et al., 2012), and ADG in growing pigs (Thuy et al., 2016) using diets formulated with a reduced level of fish meal. Interestingly, Wei et al. (2020), found that fish protein hydrolysate in combination with a high level of zinc, modulate the immune system, and gut microbiota which resulted in improved growth performance.

Organic acids have demonstrated beneficial effects to the gastrointestinal tract (GIT) of young pigs (Papatsiros et al., 2012). Because of their acidic properties, they enhance digestive enzyme activity (Castro, 2005), reduce the growth of harmful bacteria (Hansen et al., 2007), increase nutrient digestibility and promote growth performance in pigs (Diao et al., 2016). Interestingly, organic acids have shown to have accelerated effects when combined with other acidifiers (Walsh et al., 2007).

Due to the increasing concern regarding environmental pollution and bacterial resistance caused by dietary zinc oxide, the objective of this study was to determine if acidifiers (sodium butyrate and benzoic acid) exhibit beneficial effects on growth performance of nursery pigs similar to those observed with pharmaceutical levels of zinc oxide when combined with fish and microbial protein hydrolysates.

Materials and Methods

The institutional Animal Care and Use Committee at the University of Arkansas reviewed and approved the protocols for this experiment (Protocol number: 20034).

Animals and Experimental Design

A total of 288 weaned pigs (PIC1050xDNA600), 21 days of age (5.85 Kg), were stratified by initial BW within gender and allotted to 1 of 7 treatments. Treatment 1 had 6 replicates while treatment 2 to 7 had 7 replicates, with 6 pigs per pen. Pigs remained on the same dietary treatment during the first two-phase feeding program and were fed a common diet during the last phase. Pigs were housed in 1.49 m × 1.20 m pens at the University of Arkansas conventional nursery facility, with ad libitum access to feed and water during all 3 phases of the experiment. The microenvironment beginning temperature of the nursery facility was set at 30º C and reduced 2 degrees per week until reaching 23º C by the end of the last phase.

Experimental Diets

Phase 1 (14 days) and 2 (13 days) dietary treatments were: (1) Positive control (PC), where the crude protein was formulated to meet (NRC, 2012) nutrient requirements; (2) Negative control (NC), where fish meal was reduced to achieve -0.13% SID lysine; (3) NC plus 0.25 % peptide (Peptiva Ultra ®, Vitech Bio-Chem Corp) plus high level of zinc from ZnO; (4) NC plus

0.50% peptide plus high level of zinc from ZnO; (5) NC plus 0.25% peptide with standard zinc; (6) NC plus 0.50% peptide with standard zinc; and (7) as treatment 5 plus 0.1% of sodium butyrate (SB, Villimax®, DSM Nutritional Products, Parsippany, NJ) and 0.5% of benzoic acid (BA, Vevovitall®, DSM Nutritional Products, Parsippany, NJ); (PSB). Dietary treatments 1, 2, 3, and 4 had 2026 ppm of zinc oxide (ZnO) in phase 1 and 1600 ppm of ZnO in phase 2. All pigs were fed a common diet during phase 3 (14 days) with 197 ppm ZnO. Diets were formulated without antibiotic.

Sample Collection and Processing

Individual BW on d 0, and BW and pen feed intake at the end of each phase were recorded to calculate average feed intake, average daily gain, and gain to feed ratio per phase. At the end of phase 2, fecal samples were collected and stored at -20 ºC. Feed and fecal samples were analyzed to obtain Apparent Total Tract Digestibility (ATTD) of nutrients. Feed samples were collected from each batch and storage at – 20 ºC.

Fecal and feed samples were dried in a forced air oven (Shel Lab, Model: SMO28-2, Cornellus, OR) at 50 ºC for 3 days. Samples were processed with a grinder (Arthur H. Thomas, Philadelphia, PA) using a 2 mm screen, kept overnight in an oven (BWR Scientific Gravity oven, Model: 1370 GM, Radnor, PA) at 100 ºC to obtain total dry matter and heated in an ash oven (Thermolyne/ Sybron Ashing Oven, Model: FA1938) for 8 hr. at 500 ºC to ascertain ash content. Mineral content was determined using the method established by Jones et. al. (1990), where sample digestion was accomplished using an Environmental Express Hot Block (Charleston, SC). Mineral levels were analyzed using an Inductively Coupled Plasma Atomic Emission Spectrophotometer (Spectro Arcos 160 SOP, Model: FHS16, Kleve, Germany). Fiber analyzers (ANKOM Technology, Macedon, NY) were used to determine Neutral Detergent Fiber (NDF)

and Acid Detergent Fiber (ADF). A calorimeter (Parr 6200 Calorimeter, Moline, Illinois) was used to analyze Gross Energy (GE) via rapid combustion procedure. Nitrogen (N) content was measured using the Dumas Combustion Method with a CHN-analyzer (Na-2000 N-Protein, Fisons Instruments S.p.A., Rodano (MI), Italy).

Nutrient Digestibility

0.30 % of Titanium dioxide (TiO_2) was used as a feed marker. After determining the GE, crude protein, minerals, ADF-NDF, and ash of feed and fecal samples, TiO_2 was quantified via spectrometer (Synergy™ HTX Multi-Mode Microplate Reader, Biotek, Winooski, VT), by applying the method established by Short et al. (1996) from which we calculated (ATTD) of nutrients.

Complete Blood Cell Count and Blood Cell Characteristics

On d 0, and at the end of each phase (d 14, 27 and 42) a 10 mL K2-EDTA tube (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ) was used to collect blood from the average BW male per pen via jugular vena puncture. Complete blood cell count was determined using a hematology profile system (Hemavet 950 FS, Drew Scientific, Waterbury, CT) within 1-4 hr. after collection.

Volatile Fatty Acid Content

A 1:1 ratio was obtained using 1 g of fresh fecal sample plus 1ml of deionized water. Samples were then centrifuged at 2500 g for 15 min, after which 1 ml of supernatant was analyzed via gas chromatography (Hewlett Packard 5890 Series II Gas Chromatograph, Wilmington, DE) to determine absolute concentration (mM) of acetate, butyrate, propionate, iso-butyrate, valerate, and iso-valerate.

Blood Urea Nitrogen (BUN) Content

BUN content was determined via spectrometer (Synergy™ HTX Multi-Mode Microplate Reader, Biotek, Winooski, VT), using the Urea Nitrogen Reagent (Calorimetric) Method, (TECO Diagnostic kid, Anaheim, CA). Blood samples were centrifuged at 2500 g for 15 min to separate plasma. Plasma samples and standards were diluted 5-fold with saline solution. Using a 96 well plate, 5 µl of samples were mixed with 150 µl of reconstituted BUN Enzyme Reagent and incubated for 10 min at room temperature (RT). Next, 150 µl of BUN color developer was added to each well. After 10 min of incubation at RT, absorbance was read at 630 nm wavelength.

Statistical Analysis

Data were analyzed using the PROC Mixed of SAS (SAS Institute, Inc., Cary, NC) as a Randomized Complete Block Design with treatment as the fixed effect, and BW block as random effect. Pen was the experimental unit for ANOVA. Also, orthogonal contrasts were used to determine linear, quadratic, and cubic effects of increased level of peptide in diets that contained high level of zinc oxide.

Results

Growth Performance

Piglets used for the trial displayed a good health status with just 1.74 % of mortality (5 pigs). Of these 5 pigs, 2 were from the PC group, 2 from the NC group, and 1from the 0.25% peptide plus acidifiers group (PSB).

BW and ADG results are shown in Table 7, and ADFI and G:F ratios are presented in Table 8. In phase 1, pigs fed 0.25% peptide plus zinc (0.25PZ) and 0.5% peptide plus zinc (0.50PZ) had greater ADG when compared to other treatments ($P < 0.05$), and a linear response

(P < 0.001) was observed with increasing peptide in pigs fed diets containing high levels of ZnO (NC, 0.25PZ and 0.50PZ), while ADG was similar in pigs fed increasing levels of peptide without supplemental Zn. In phase 2, ADG in pigs fed 0.25 % peptide (0.25P) was numerically higher than observed in pigs fed the NC or 0.50PZ, and was similar to those fed the PC and 0.25PZ. Pigs fed PSB had the greatest ADG among all treatments (P < 0.05). In phase 1&2, ADG in pigs fed PSB, 0.25PZ, and 0.5PZ was higher than gain in pigs fed the NC or those fed increasing levels of peptide without supplemental Zn (0.25P and 0.50P; P < 0.05). In fact, pigs fed 0.25P or 0.50P diets had either a similar or reduced response compared to pigs fed NC diet, respectively.

As for phase 1 BW, pigs fed both 0.25PZ and 0.50PZ had a higher BW compared to all other treatments (P < 0.05). Body weight in pigs fed PSB was similar to that observed in pigs fed the NC. Furthermore, a linear response was observed in pigs fed increasing levels of peptide in diets containing high zinc from ZnO (NC, 0.25PZ, and 0.50PZ) in phase 1 (Linear = 0.002) and 2 (P = 0.037). In phase 2, pigs fed PSB also had a similar BW to pigs fed 0.25PZ and 0.50PZ and had a higher BW than pigs fed NC, 0.25P or 0.50P diets. Concerning to the final BW, there appears no statistically significant differences among treatments (P = 0.109); however, pigs fed 0.25PZ and PSB were 1.25 kg and 0.74 kg heavier than those pigs fed the NC, respectively.

According to the ADFI results, pigs fed 0.50PZ in phase 1 had increased ADFI among all treatments (P < 0.05) except those fed the 0.25PZ diet which was similar (Figure 3). A linear response was observed in pigs fed a high level of pharmaceutical zinc with increasing level of peptide (Linear P = 0.006). In phase 2, pigs fed PSB had similar intake to pigs fed the 0.25PZ, 0.50PZ, and PC was higher than pigs fed 0.50P diet (P < 0.05). In phase 1&2, pigs fed 0.50PZ had a higher ADFI compared to pigs fed either the PC or NC diets or any of the diets without a

high level of Zn supplementation (0.25P, 0.50P, PSB) ($P < 0.005$). A linear response ($P = 0.014$) in ADFI was observed in pigs fed increasing levels of peptide with a high level of zinc.

Regarding G:F ratio in phase 1, pigs fed NC had lower G:F ratio than PC ($P < 0.05$) but increasing the level of peptide in a high ZnO diet showed a linear response (Linear $P = 0.039$). In phase 2 and in phase 1&2, G:F ratio between pigs fed the PC and NC diets was similar ($P > 0.05$), and the efficiency was improved in pigs fed the PSB diet without high Zn in both phases ($P > 0.05$) compared to pigs fed any other diet. Pigs fed PSB had the highest G:F ratio among treatments in phase 2 ($P = 0.003$) and in phase 1&2 ($P = 0.0002$). Similarly, feeding high levels of ZnO did not appear beneficial to G:F ratio in NP 2 as pigs fed 0.25P diet had improved G:F ratio compared to those fed the 0.25PZ and 0.50PZ diet (Figure 4).

ATTD of Nutrients

The ATTD of all nutrients increased as the level of peptide increased in diets containing a high level of zinc oxide (Figure 5; Linear $P \leq 0.05$). ATTD of DM, ash, GE and nitrogen in pigs fed PSB was similar to pigs fed 0.25PZ, 0.50PZ, 0.25P, and PC but higher than those fed NC diet ($P < 0.05$). Similarly, Pigs fed PSB, 0.25PZ, 0.50PZ, 0.25P, and PC ha similar ATTD of P, K, and Mg , but they all were higher than observed in pigs fed NC diet ($P< 0.05$).

Complete Blood Cell Count and Blood Cell Characteristics

Leukocyte differential and complete blood cell characteristics are shown in Tables 10 and 11, respectively. The percentage of lymphocytes was higher and neutrophil to lymphocyte ratio was lower in pigs fed the PC diet compared to those fed the NC ($P < 0.05$). Pigs fed diets with high supplemental ZnO had a lower percentage of neutrophils compared to those fed diets without supplemental ZnO (PC, NC, 0.25PZ 0.50 PZ vs. 0.25P, 0.5P and PSB; $P < 0.05$). Peripheral lymphocyte percentage was higher in pigs fed the PC diet compared to all other

treatments. Similarly, pigs fed the 0.25PZ diet had a higher percentage of lymphocytes compared to those fed the 0.50P or PSB diet ($P< 0.05$). Percentage of monocytes increased linearly with increasing level of peptide ($P< 0.05$) and at the highest level of peptide, the percentage of monocytes was higher than that observed in pigs fed PSB ($P< 0.05$). Pigs fed the 0.50P and PSB diets had a higher NLR ratio compared to those fed the PC diet (Figure 9).

Pigs fed PSB and 0.50PZ had similar concentrations of hemoglobin, and both were higher than those observed in pigs fed the PC or 0.50P diets (Figure 6; $P < 0.05$). Pigs fed PSB had a similar percentage of hematocrit compared to those fed 0.50PZ and NC, and higher than those pigs fed PC, 0.25PZ, 0.25P and 0.50P diets ($P < 0.05$).

Volatile Fatty Acid Content

The results of the volatile fatty acid (VFA) analysis are presented in Table 12. No differences were observed in VFA's between pigs fed the PC or NC diets ($P < 0.05$). A linear decrease in acetate ($P < 0.05$) and a tendency for a linear decrease ($P < 0.10$) in propionate was observed with increasing level of peptide in pigs fed high ZnO diets. Pigs fed the 0.50P diet had higher percentage of iso-butyrate compared to pigs fed the NC, PC and 0.25P diets ($P < 0.05$). Similarly, pigs fed 0.50P had a higher iso-valerate percentage compared to pigs fed the PC, NC, and 0.25P diets ($P < 0.05$). No differences were found in acetate, propionate, butyrate, or valerate concentration ($P > 0.05$).

Blood Urea Nitrogen (BUN) Content

Results of BUN are found in Figure 7 and 8. There were no significant differences in BUN content among treatments ($P > 0.05$). Statistic difference was observed by day, where pigs on d 27 had a reduction of 26.6% in BUN content when compared to the initial point on d 0, and then BUN increased by 34% by the end of trial on d 42 (Figure 7, Day $P < 0.0001$).

Discussion

These results demonstrated that feeding nursery pigs with increasing levels of peptide in combination with high zinc, protein deficient diets, improved BW, ADG, ADFI. Furthermore, pigs fed the PSB diet obtained a similar response to those fed peptide with a high concentration of zinc oxide (ZnO). Previous studies have demonstrated that a high level of pharmaceutical zinc in diets enhanced the growth performance of nursery pigs (Case and Carlson, 2002; Han and Thacker, 2010; Goodband et al, 2017). Recently, Wei et al. (2020) found that inclusion of 0.50% peptide in combination with a high ZnO level improved growth performance. Our results are consistent with those of Wei et al. (2020), with the exception that in our study we detected a better response with a lower concentration of peptide (0.25%).

There is evidence that benzoic acid at the 0.50% inclusion level (Diao et al., 2016; Kiarie et al., 2018), low level of sodium butyrate (Piva et al., 2002; Lu et al., 2008), and high inclusion level (>10%) of marine protein hydrolysates fed to piglets (Norgaard et al., 2012; Thuy et al., 2018) and broilers (Wagner and Bregendahl., 2007; Opheim et al., 2016;) can improve growth performance; however, there is a lack of research regarding the effects of peptide used in combination with acidifiers on growth performance. Our results showed that PSB improved BW by 0.74 Kg and FE by 10% over NC on d 27 post weaning. Also, ATTD of DM, Ash, ADF, GE, N and several micro minerals such as P, K, and Mg were higher in pigs fed PSB compared to those fed NC diets. Acidifiers have been revealed to improve growth performance via improving gastric enzyme activity (Hansen et al., 2007; Kil et al., 2011), to control harmful bacterial proliferation in the GIT, (Castro, 2005; Lu et al., 2008); and to serve as energy sources which helps to reduce intestinal tissue degeneration after weaning (Bosi et al, 1999; Partanen & Mroz, 1999). In this study, a positive improvement was obtained when peptide was fed in combination

with acidifiers, showing a similar response those fed high ZnO level plus peptide and better response than peptide alone, suggesting that peptide in combination with acidifiers may be able to replace high level of dietary ZnO in nursery diets.

Interestingly, nitrogen (N) was the nutrient with the most notable change regarding digestibility. PSB pigs had similar N digestibility compared to those fed 0.50PZ, and both were improved by 14.3 % and 8.64 %, respectively over those fed the PC. Adibi (2003) mentioned that specific dietary substrates such as peptide added to feed can up or down-regulate PepT1 transporters by improving the mRNA stability or enhancing the gene transcriptional rate. Rats fed with a specific peptide (Gly-Phe) exhibited elevated PepT1 mRNA and protein expression, leading to an increase in peptide transport activity within the cells of the intestinal mucosa (Shiraga et al.,1999). Additionally, Shiraga et al. (1999) found that Phe stimulated the PepT1 gene expression, while Gly-Gln, Gly and Gln did not impact PepT1 mRNA and transport activity. Thus, there remains the possibility that specific amino acids and peptide can potentially stimulate PepT1 expression and improve protein digestibility.

Furthermore, peptides have shown to increase the absorption rate of free amino acids. Wenzel et al. (2001) demonstrated in vitro that incubation of Caco-2 cells of humans where neutral, mono or dicationic dipeptides not only stimulated peptide uptake, but also amino acid utilization via b(0, +) transporter. Keohane et al. (1985) tested 5 different partial peptide hydrolysates (egg, albumin, lactalbumin, casein-soy-lactalbumin, and meat-soy-lactalbumin) in humans, finding that amino acid residues such as threonine, phenylalanine, glutamic acid, and histidine in all protein hydrolysates had faster absorption rates than free amino acid mixtures in diets without peptide hydrolysates. This evidence supports the concept that peptides not only have the ability to stimulate expression of peptide transporters and increase peptide absorption,

but can also stimulate amino acid transporter activity, resulting in the increase of free amino acid utilization.

Unfortunately, diarrhea caused by viruses (Zhang et al., 2019), and parasites (Sekikawa et al., 2003) or other pathogens affect Pept1 expression in the intestinal membrane, which may be the source of damage and inflammation to the mucosa layers.

The high N digestibility in PSB may be associated with the adequations that acidifiers make in strengthening the morphology and health status of the gut membrane as well as decreasing the pathogen effect on the intestinal mucosa. Improving health status of intestinal membrane cells will benefit the utilization of nutrients, including the bioactive peptide added to diets, and then these stimulate the PepT1 gene expression and amino acid transporter activity, resulting in greater protein utilization. Future research should be performed to clarify the mode of action by which acidifiers enhance the biological activity of peptide within cells of the intestinal membrane of nursery pigs.

Besides nutrient digestibility, BUN decreased in phase 2 and increased in phase 3 when peptide was excluded. Coma (1995) noted that a reduction of BUN means a higher N utilization and may be influenced by protein quality. A similar reduction pattern of BUN concentration was observed in all treatments and not just in those with peptide inclusion. Different feeding factors such as energy intake, lysine concentration, amino acid ratios, feeding management strategies, and blood collection time can highly impact BUN results (Cai, 1992). The reduction of BUN in all treatments during phase 2 may be associated with a compensation response of protein synthesis for tissue growth. Phase 1 is a challenging stage for nursery pigs where nutrient utilization is compromised, whereas in phase 2 pigs are more adapted and thus able to increase

amino acid utilization for muscle growth, and minimize amino acid deamination, which in turn decreases urea levels in the blood.

Neutrophils and lymphocytes have important roles in immune defense against pathogens. Neutrophils are closely linked to inflammatory responses (Malech et al., 2014), while lymphocytes act as mediators that help regulate the immune system (Cantor, 2014). Interestingly, the interaction of these 2 immune cells, defined as Neutrophil- Lymphocyte Ratio (NLR), has been described as an indicator that is closely related to systemic inflammation caused by a compromised health status (Imtiaz et al, 2012). Our results showed that NLR decreased as the level of peptide increased in diets where a high level of zinc was fed, whereas pigs fed only peptide (0.25% and 0.50%) had the highest NLR, and these correlated inversely to ADG. These results agreed with those observed by Wei et al. (2020), where peptide in combination with a high level of zinc reduced NLR, indicating that feeding peptide in high zinc-based diet may help to regulate the immune system and minimize stress. Furthermore, the red blood cell profile showed that pigs fed 0.50PZ and PSB had the highest level of hemoglobin among treatments. Interestingly, iron absorption in pigs fed 0.50PZ and PSB were higher than observed in pigs fed the PC diet by 10.29% and 10.75 % respectively. In contrast, pigs fed 0.50P had the lowest level of hemoglobin and also the lowest iron digestibility. Rincker et al. (2004) found that levels of hemoglobin can be raised by supplemental iron in nursery pigs. These results agreed with our hemoglobin response and iron digestibility. Peptide plus acidifiers or zinc oxide increased iron digestibility and this could possibly be used by the system to compensate for the lack of systemic iron, thus helping to stabilize healthy hemoglobin levels. In addition, Ishaya (2012) indicated that a hemoglobin level around 7 mg/dl is considered anemia, a condition that is closely related to decreased growth performance in pigs. Our results showed that, on average,

pigs fed PSB and 0.50PZ were the only ones that had levels of hemoglobin over 8 mg/dl. This suggest that the inclusion of just 0.25 % peptide plus acidifiers can alleviate the anemic status similar to that observed in pigs fed the 0.50 % peptide in diet containing high ZnO level. Similarly, pigs fed 0.50PZ and PSB had the highest hematocrit percentage. These results agreed with those by Knight (2006), where he found that hemoglobin levels were highly correlated with hematocrit concentrations in nursery pigs, indicating a close connection between level of hematocrit and hemoglobin concentration.

Volatile fatty acids (VFA) are categorized as essential metabolites produced by microbial fermentation of dietary fiber. They exert beneficial functions concerning health, nutrition, and immune status (Macfarlane and Macfarlene, 2012; Koh, 2016). Their concentrations are influenced by the microbiota community and diet. Our results found no difference in the levels of acetate, propionate, and butyrate production among pigs fed different dietary treatments. However, we observed differences in levels of iso-butyrate an iso-valerate, where pigs fed 0.50P had the highest level of these 2 VFA`s when compared to pigs fed PC, NC diets, and 0.25P. Acetate, propionate, butyrate account for 95% of total VFA production (den Besten et al., 2013), and are the VFA`s that have important roles serving as energy sources and gut health maintenance (Huang et al., 2017). Iso-butyrate and iso-valerate are mainly produced by bacterial fermentation of peptides and branched-chain amino acids that are not metabolized (Portune et al., 2016). These results may indicate that peptide alone or in combination with zinc or acidifiers may not influence important changes in general VFA production. In addition, the correlation inconsistency between iso-valerate and iso-butyrate production and nitrogen digestibility make it difficult to establish a possible explanation for the high level of these two VFA`s in pigs fed 0.50P.

Conclusion

A low dosage of peptide in combination with organic acids in nursery diets is as effective as peptide with high zinc diets on improving growth performance, nutrient digestibility, and nitrogen utilization in pigs. The mechanism of how peptide stimulates nitrogen utilization must be further studied.

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Appendix

Table 1. Nursery phase 1 diet composition.

Ingredients	PC	NC	0.25PZ	0.50PZ	0.25P	0.50P	PSB
Corn, Yellow Dent	44.0	46.38	46.13	45.88	46.38	46.13	45.78
Soybean meal, 48%, high prot	17.60	17.60	17.60	17.60	17.60	17.60	17.60
Poultry Fat	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Monocalcium P	0.65	1.04	1.04	1.04	1.04	1.04	1.04
Limestone	0.48	0.71	0.71	0.71	0.71	0.71	0.71
Salt	0.25	0.25	0.25	0.25	0.25	0.25	0.25
L-Lysine	0.36	0.36	0.36	0.36	0.36	0.36	0.36
DL-Methionine	0.21	0.21	0.21	0.21	0.21	0.21	0.21
L-Threonine	0.12	0.12	0.12	0.12	0.12	0.12	0.12
L-Tryptophan	0.04	0.04	0.04	0.04	0.04	0.04	0.04
L-Valine	0.09	0.09	0.09	0.09	0.09	0.09	0.09
L-Isoleucine	0.03	0.03	0.03	0.03	0.03	0.03	0.03
ZnO	0.26	0.26	0.26	0.26	0.00	0.00	0.00
T Mineral Premix (NB-8534)	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin Premix (NB-6508)	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Plasma (AP-920)	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Fish Meal, Menhaden	6.00	3.00	3.00	3.00	3.00	3.00	3.00
Whey Powder	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Peptide [1]	0.00	0.00	0.25	0.50	0.25	0.50	0.25
Ethoxiquin (Quinguard)	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Sodium Butyrate [2]	0.00	0.00	0.00	0.00	0.00	0.00	0.10
Benzoic Acid [3]	0.00	0.00	0.00	0.00	0.00	0.00	0.50
Lactose	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Ronozyme HiPhos 2700 (GT)	0.003	0.003	0.003	0.003	0.003	0.003	0.003

[1]Peptide = Peptiva ® (Vitech Bio-Chem Corp, Ca). [2]Sodium Butyrate = Villimax ® (DSM Nutritional Products, Parsippany, NJ). [3]Benzoic Acid = VevoVitall ® (DSM Nutritional Products, Parsippany, NJ).

Table 2. Nursery phase 1 diet calculated analysis.

Calculated Analysis	PC	NC	0.25PZ	0.50PZ	0.25P	0.50P	PSB
NSNG ME, Kcal/Kg	3487	3467	3466	3466	3475	3474	3455
CP (%)	21.155	19.482	19.583	19.685	19.604	19.706	19.555
Total P (%)	0.741	0.739	0.740	0.742	0.741	0.743	0.740
Available P (%)	0.541	0.539	0.539	0.539	0.539	0.539	0.539
Ca (%)	0.847	0.847	0.848	0.849	0.848	0.849	0.848
Na (%)	0.418	0.407	0.407	0.407	0.407	0.407	0.407
Zinc(ppm)	2026.568	2026.451	2026.409	2026.368	190.451	190.410	190.352
SID Lysine (%)	1.459	1.327	1.333	1.339	1.333	1.340	1.332
SID M + C (%)	0.846	0.789	0.789	0.788	0.789	0.789	0.787
SID Threonine (%)	0.875	0.811	0.814	0.818	0.815	0.818	0.814
SID Tryptophan (%)	0.278	0.261	0.263	0.264	0.263	0.264	0.262
SID Isoleucine (%)	0.803	0.736	0.739	0.742	0.740	0.742	0.738
SID Valine (%)	0.979	0.902	0.905	0.908	0.906	0.909	0.904
SID Leucine (%)	1.612	1.504	1.507	1.511	1.509	1.513	1.504
SID Histidine (%)	0.500	0.455	0.456	0.458	0.457	0.458	0.456
SID M+C:Lys	57.97	59.50	59.16	58.82	59.19	58.85	59.11
SID Thr:Lys	59.99	61.12	61.09	61.06	61.11	61.08	61.06
SID Trp:Lys	19.05	19.70	19.70	19.69	19.70	19.69	19.69
SID Ile:Lys	55.05	55.51	55.44	55.38	55.47	55.40	55.41
SID Val:Lys	67.07	67.96	67.87	67.79	67.91	67.82	67.82
SID Leu:Lys	110.46	113.35	113.07	112.79	113.19	112.91	112.91
SID His:Lys	34.24	34.28	34.22	34.17	34.25	34.19	34.19

Table 3. Nursery phase 2 diet composition.

Ingredients	PC	NC	0.25PZ	0.50PZ	0.25P	0.50P	PSB
Corn, Yellow Dent	54.48	56.86	56.61	56.36	56.81	56.56	56.21
Soybean meal, 48%, High Prot	24.25	24.25	24.25	24.25	24.25	24.25	24.25
Poultry Fat	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Monocalcium P	0.54	0.92	0.92	0.92	0.92	0.92	0.92
Limestone	0.79	1.03	1.03	1.03	1.03	1.03	1.03
Salt	0.50	0.50	0.50	0.50	0.50	0.50	0.50
L-Lysine	0.46	0.46	0.46	0.46	0.46	0.46	0.46
DL-Methionine	0.22	0.22	0.22	0.22	0.22	0.22	0.22
L-Threonine	0.16	0.16	0.16	0.16	0.16	0.16	0.16
L-Tryptophan	0.04	0.04	0.04	0.04	0.04	0.04	0.04
L-Valine	0.11	0.11	0.11	0.11	0.11	0.11	0.11
L-Isoleucine	0.02	0.02	0.02	0.02	0.02	0.02	0.02
ZnO	0.20	0.20	0.20	0.20	0.00	0.00	0.00
T Mineral Premix (NB-8534)	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin Premix (NB-6508)	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Plasma (AP-920)	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Whey Powder	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Fish Meal, Menhaden	3.00	0.00	0.00	0.00	0.00	0.00	0.00
Peptide[1]	0.00	0.00	0.25	0.50	0.25	0.50	0.25
Sodium Butyrate[2]	0.00	0.00	0.00	0.00	0.00	0.00	0.10
Benzoic Acid[3]	0.00	0.00	0.00	0.00	0.00	0.00	0.50
Ethoxiquin (Quinguard)	0.03	0.03	0.03	0.03	0.03	0.03	0.03
TiO2	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Ronozyme HiPhos 2700 (GT)	0.003	0.003	0.003	0.003	0.003	0.003	0.003

[1]Peptide = Peptiva ® (Vitech Bio-Chem Corp, Ca). [2]Sodium Butyrate = Villimax ® (DSM Nutritional Products, Parsippany, NJ). [3]Benzoic Acid = VevoVitall ® (DSM Nutritional Products, Parsippany, NJ).

Table 4. Nursery phase 2 diet calculated analysis.

Calculated Analysis	PC	NC	0.25PZ	0.50PZ	0.25P	0.50P	PSB
NSNG ME (kcal/kg)	3445	3424	3424	3423	3430	3430	3410
CP (%)	21.177	19.504	19.605	19.707	19.622	19.723	19.572
Total P (%)	0.609	0.605	0.607	0.609	0.608	0.610	0.606
Available P (%)	0.380	0.377	0.377	0.376	0.377	0.377	0.376
Ca (%)	0.751	0.750	0.750	0.751	0.751	0.751	0.750
Na (%)	0.377	0.366	0.366	0.366	0.366	0.366	0.366
Zinc(ppm)	1603.94	1600.81	1600.77	1600.73	190.0	190.0	190.0
SID Lysine (%)	1.419	1.287	1.293	1.299	1.293	1.300	1.292
SID M+C (%)	0.823	0.766	0.765	0.765	0.766	0.765	0.764
SID Threonine (%)	0.852	0.787	0.791	0.794	0.791	0.795	0.790
SID Tryptophan (%)	0.271	0.254	0.255	0.257	0.256	0.257	0.255
SID Isoleucine (%)	0.780	0.713	0.716	0.719	0.716	0.719	0.715
SID Valine (%)	0.951	0.873	0.877	0.880	0.877	0.880	0.875
SID Leucine (%)	1.569	1.461	1.465	1.468	1.466	1.470	1.461
SID Histidine (%)	0.493	0.448	0.449	0.450	0.449	0.451	0.448
SID M+C: Lys	59.97	59.54	59.19	58.84	59.22	58.87	59.13
SID Thr:Lys	60.02	61.20	61.16	61.13	61.18	61.15	61.13
SID Trp:Lys	19.09	19.76	19.75	19.75	19.76	19.75	19.75
SID Ile:Lys	54.98	55.44	55.37	55.31	55.39	55.32	55.33
SID Val:Lys	66.97	67.89	67.80	67.71	67.83	67.74	67.74
SID Leu:Lys	110.57	113.56	113.28	112.99	113.37	113.09	113.08
SID His:Lys	34.70	34.79	34.73	34.67	34.75	34.69	34.69

Table 5. Nursery phase 3 diet composition.

Ingredients	Common Diet
Corn, Yellow Dent	47.30
Soybean meal, 48%, high Protein, dehulled,	26.50
Corn DDGS, >6 and <9% Oil	20.00
Poultry Fat	3.00
Monocalcium P	0.35
Limestone	1.13
Salt	0.50
L-Lysine	0.50
DL-Methionine	0.14
L-Threonine	0.12
L-Tryptophan	0.04
Trace Mineral Premix (NB-8534)[1]	0.15
Vitamin Premix (NB-6508)[2]	0.25
Ethoxiquin (Quinguard)	0.03
Ronozyme HiPhos 2700 (GT)	0.003

[1]The vitamin premix provided per kg of complete diet: 397.5 mg of Ca as CaCO3, 11,022.9 IU of vitamin A, 1,77.9 IU of vitamin D_3, 44.09 IU of vitamin E, 0.0386 mg vitamin B_{12}, 4.41 mg of menadione, 8.27 mg of riboflavin, 27.56 mg of D-pantothenic acid, and 49.6 mg of niacin.

[2]The mineral premix provided per kg of complete diet: 84 mg of Ca as CaCO3, 165 mg of Fe as FeSO4, 165 mg of Zn as ZnSO4, 39.6 mg of Mn as MnSO4, 16.5 mg of Cu as CuSO4, 0.3 mg of I as CaI2, and 0.3 mg of Se as Na2SeO3.

Note: The vitamin premix and mineral premix used in phase 3 and shown in this table were also the same product and nutritional composition used in phase 1 and 2 diets (page 49 and 51, respectively).

Table 6. Nursery phase 3 diet calculated analysis.

Calculated Analysis	Common Diet
NSNG ME (kcal/kg)	3440
CP (%)	22.802
Total P (%)	0.505
Available P (%)	0.251
Ca (%)	0.648
Na (%)	0.290
Zinc(ppm)	197.041
Copper(ppm)	23.331
SID Lysine (%)	1.289
SID M + C (%)	0.748
SID Threonine (%)	0.775
SID Tryptophan (%)	0.246
SID Isoleucine (%)	0.774
SID Valine (%)	0.870
SID Leucine (%)	1.785
SID Histidine (%)	0.515
SID M+C:Lys	58.04
SID Thr:Lys	60.09
SID Trp:Lys	19.04
SID Ile:Lys	60.06
SID Val:Lys	67.47
SID Leu:Lys	138.45
SID His:Lys	39.94

Table 7. Effect of adding peptide alone, or in combination with high zinc oxide level or acidifiers on BW and ADG in nursery pigs (LS means).

									P-Value		
	PC	NC	0.25PZ	0.50PZ	0.25P	0.50P	PSB	SEM	Trt	Linear[1]	Quad[1]
BW, Kg											
d 0	5.84	5.88	5.88	5.85	5.84	5.83	5.82	0.350	0.080	0.298	0.377
Phase 1 (d 14)	6.89 [a]	7.05[a]	7.56[b]	7.72[b]	6.74[a]	6.77[a]	7.04[a]	0.397	0.0001	0.002	0.324
Phase 2 (d 27)	11.64[bc]	11.43[b]	12.27[c]	12.23[c]	11.37[ab]	10.68[a]	12.17[c]	0.598	0.001	0.037	0.184
Phase 3 (d 42)	18.65	18.35	19.6	19.15	18.2	17.69	19.08	0.920	0.109	0.236	0.151
ADG, kg											
Phase 1 (d 0-14)	0.075[a]	0.084[a]	0.120[b]	0.133[b]	0.064[a]	0.067[a]	0.087[a]	0.011	0.0001	0.001	0.384
Phase 2 (d 14-27)	0.36[bc]	0.337[b]	0.362[bc]	0.348[b]	0.356[b]	0.30[a]	0.394[c]	0.019	0.001	0.549	0.201
Phase 3 (d 27-42)	0.467	0.456	0.489	0.461	0.456	0.468	0.461	0.025	0.876	0.838	0.179
Phase 1&2	0.215[bc]	0.206[ab]	0.236[c]	0.236[c]	0.205[ab]	0.179[a]	0.235[c]	0.012	0.001	0.033	0.206
Overall	0.305	0.297	0.327	0.316	0.294	0.282	0.316	0.016	0.122	0.222	0.161

[a,b,c]Mean with different superscript differs significantly at $P \leq 0.05$

[1]Ortogonal contrast to determine linear and quadratic response effects of increased level of peptide in diets containing high level of zinc oxide.

Table 8. Effect of adding bioactive peptide alone, or in combination with high zinc oxide level or acidifiers on ADFI and FE in nursery pigs (LS means).

									P-Value		
	PC	NC	0.25PZ	0.50PZ	0.25P	0.50P	PSB	SEM	Trt	Linear[1]	Quad[1]
ADFI, Kg											
Phase 1 (d 0-14)	0.153[a]	0.182[ab]	0.202[bc]	0.230[c]	0.183[ab]	0.176[ab]	0.175[ab]	0.012	0.002	0.006	0.786
Phase 2 (14-27)	0.478[bc]	0.464[ab]	0.520[c]	0.502[bc]	0.477[b]	0.429[a]	0.491[bc]	0.024	0.009	0.097	0.062
Phase 3 (27-42)	0.761	0.727	0.769	0.735	0.718	0.714	0.732	0.036	0.582	0.808	0.194
Phase 1&2	0.310[a]	0.318[a]	0.355[bc]	0.361[c]	0.324[ab]	0.300[a]	0.327[ab]	0.016	0.005	0.014	0.282
Overall	0.471	0.464	0.503	0.495	0.465	0.446	0.472	0.022	0.141	0.147	0.193
G:F											
Phase 1 (d 0-14)	0.482[bc]	0.456[ab]	0.589[c]	0.580[c]	0.350[a]	0.354[a]	0.486[bc]	0.041	0.0004	0.039	0.158
Phase 2 (14-27)	0.748[bc]	0.711[abc]	0.695[ab]	0.694[a]	0.749[c]	0.700[abc]	0.807[d]	0.019	0.003	0.534	0.747
Phase 3 (27-42)	0.615	0.621	0.634	0.626	0.638	0.653	0.627	0.011	0.275	0.730	0.420
Phase 1&2	0.676[b]	0.636[ab]	0.663[b]	0.656[b]	0.633[ab]	0.596[a]	0.720[c]	0.016	0.0002	0.359	0.357
Overall	0.641	0.626	0.647	0.640	0.636	0.628	0.668	0.011	0.176	0.402	0.307

[a,b,c]Mean with different superscript differs significantly at $P \leq 0.05$

[1]Orthogonal contrast was used to determine linear and quadratic response in pigs fed NC, 0.25% peptide + high level ZnO, and 0.5% peptide + high level of ZnO.

Table 9. Effect of adding bioactive peptide alone, or in combination with high zinc oxide level or acidifiers on ATTD of nutrients in nursery pigs (LS means).

									P-value		
%	PC	NC	0.25PZ	0.50 PZ	0.25P	0.50P	PSB	SEM	Trt	Linear[1]	Quad[1]
DM	93.79^{bcd}	93.02^{d}	94.53^{abc}	94.41^{abc}	94.69^{ab}	93.67^{cd}	95.06^{a}	0.33	0.003	0.009	0.054
NDF	56.41^{a}	43.17^{b}	51.77^{ab}	52.35^{a}	53.11^{a}	53.11^{a}	58.71^{a}	3.18	0.056	0.046	0.305
Ash	43.83^{b}	33.55^{c}	47.55^{b}	47.83^{b}	48.21^{b}	28.78^{c}	58.27^{a}	2.35	<0.0001	0.0002	0.023
GE	78.16^{ab}	73.62^{c}	79.94^{a}	79.98^{a}	78.9^{ab}	75.78^{bc}	81.18^{a}	1.44	0.0097	0.003	0.080
N	58.47^{cd}	55.63^{d}	65.85^{b}	67.11^{ab}	62.87^{bc}	60.91^{bcd}	72.6^{a}	2.32	0.0003	0.001	0.119
P	65.82^{b}	58.61^{c}	68.3^{ab}	65.42^{b}	69.55^{a}	57.68^{c}	68.42^{ab}	1.33	<0.0001	0.001	0.0005
K	85.68^{ab}	82.55^{c}	86.36^{a}	85.65^{ab}	86.92^{a}	83.66^{bc}	86.87^{a}	0.84	0.0042	0.013	0.033
Ca	72.56^{bc}	69.36^{c}	80.16^{a}	76.09^{ab}	76.98^{ab}	72.31^{bc}	78.73^{a}	1.83	0.0018	0.014	0.002
Mg	38.57^{b}	29.32^{c}	43.20^{ab}	39.97^{ab}	46.16^{a}	28.54^{c}	43.24^{ab}	2.40	<0.0001	0.004	0.0063
S	76.56^{c}	71.84^{d}	78.24^{c}	77.66^{c}	83.26^{b}	78.79^{c}	86.52^{a}	1.02	<0.0001	0.0002	0.008
Na	87.04^{ab}	82.77^{b}	89.49^{a}	89.4^{a}	86.3^{ab}	85.84^{ab}	90.48^{a}	2.38	0.3026	0.054	0.244
Fe	33.3^{bc}	30.32^{c}	43.59^{ab}	43.78^{a}	44.25^{a}	24.78^{c}	44.05^{ab}	3.82	0.0013	0.015	0.160
B	85.82^{cd}	84.29^{d}	88.98^{a}	88.29^{ab}	88.24^{abc}	85.91^{bcd}	86.71^{abcd}	0.92	0.0059	0.003	0.016

^{a,b,c,d} Mean with different superscript differs significantly at $P \leq 0.05$

[1]Orthogonal contrast was used to determine linear and quadratic response in pigs fed NC, 0.25% peptide + high level ZnO, and 0.5% peptide + high level of ZnO.

Table 10. Effect of adding bioactive peptide alone, or in combination with high zinc oxide level or acidifiers on leukocyte count in nursery pigs (LS means).

									P- Value		
k/µl	PC	NC	0.25PZ	0.50PZ	0.25P	0.50P	PSB	SEM	Trt	Linear[1]	Quad[1]
WBC	13.48	14.69	13.63	13.16	15.06	13.61	13.96	0.956	0.784	0.254	0.804
Neutrophil,	4.75	5.90	5.34	5.08	6.35	6.05	5.73	0.564	0.399	0.298	0.831
Lymphocyte,	7.34	6.57	6.80	6.10	6.83	5.90	6.68	0.437	0.291	0.440	0.382
Monocyte,	0.37	0.34	0.34	0.46	0.38	0.33	0.33	0.053	0.567	0.112	0.289
Eosinophil, k	0.97	1.88	1.11	1.43	1.44	1.24	1.19	0.299	0.400	0.280	0.125
Basophil, k/µ	0.04	0.10	0.05	0.06	0.06	0.05	0.05	0.015	0.142	0.078	0.191
% Over WBC											
Neutrophil	36.48^{a}	39.96^{ab}	40.03^{ab}	39.48^{ab}	42.23^{bc}	45.47^{c}	42.00^{bc}	1.795	0.012	0.837	0.878
Lymphocyte	53.27^{c}	45.99^{ab}	49.28^{b}	46.40^{ab}	45.65^{ab}	43.94^{a}	47.03^{ab}	1.908	0.020	0.878	0.185
Monocyte	2.67^{ab}	2.41^{a}	2.42^{a}	3.19^{b}	2.50^{ab}	2.50^{ab}	2.37^{a}	0.268	0.288	0.033	0.227
Eosinophil	7.18	10.82	7.88	10.49	9.24	7.80	8.43	1.426	0.399	0.863	0.104
Basophil	0.28	0.53	0.38	0.44	0.38	0.30	0.32	0.077	0.187	0.345	0.251
NLR	73.31^{a}	106.35^{bc}	92.58^{abc}	89.59^{ab}	106.14^{bc}	113.8^{c}	100.99^{bc}	8.838	0.031	0.178	0.616

^{a,b,c}Mean with different superscript differs significantly at $P \leq 0.05$

[1]Orthogonal contrast was used to determine linear and quadratic response in pigs fed NC, 0.25% peptide + high level of ZnO, and 0.5% peptide + high level of ZnO.

Table 11. Effect of adding bioactive peptide alone, or in combination with high zinc oxide level or acidifiers on red blood cell characteristics in nursery pigs (LS means).

									P-Value		
	PC	NC	0.25PZ	0.50PZ	0.25P	0.50P	PSB	SEM	Trt	Linear[1]	Quad[1]
RBC, M/µl	6.74	6.68	6.69	6.95	6.49	6.42	6.96	0.215	0.425	0.347	0.620
Hemoglobin, g/dL	7.79[a]	7.96[abc]	7.82[ab]	8.38[c]	7.91[abc]	7.59[a]	8.32[bc]	0.203	0.047	0.115	0.136
Hematocrit, %	33.02[a]	34.05[abc]	33.10[a]	35.15[bc]	33.51[ab]	32.42[a]	35.57[c]	0.766	0.031	0.287	0.096
MCV	49.30	51.06	49.57	50.80	51.79	50.60	51.44	1.019	0.466	0.837	0.244
MCH, Pg	11.61	11.89	11.73	12.10	12.21	11.91	12.06	0.292	0.757	0.588	0.444
MCHC, g/dL	23.62	23.34	23.62	23.81	23.59	23.50	23.45	0.246	0.856	0.145	0.885
RDW, %	28.24	28.60	28.70	26.65	28.97	28.44	26.42	1.119	0.558	0.212	0.426
PLT, k/µl	545.71	515.2	478.36	488.48	471.68	543.45	477.28	39.81	0.586	0.598	0.596
MPV, fL	9.49	10.08	9.79	10.31	10.18	9.15	10.19	0.389	0.193	0.646	0.360

[a,b,c]Mean with different superscript differs significantly at $P \leq 0.05$

[1.] Orthogonal contrast was used to determine linear and quadratic response in pigs fed NC, 0.25% peptide + high level of ZnO, and 0.5% peptide + high level of ZnO.

Mean corpuscular volume (MCV): average of red cells.
Mean corpuscular hemoglobin (MCH): hemoglobin amount per red blood cell.
Mean corpuscular hemoglobin concentration (MCHC): hemoglobin amount relative to size of hemoglobin per red blood cell.
Red cell distribution width (RDW): calculation of variation in size of red blood cells.
Mean platelet volume (MPV): calculation average size of platelets.

Table 12. Effect of adding bioactive peptide alone, or in combination with high zinc oxide level or acidifiers on volatile fatty acids concentration (mM) in nursery pigs (LS means).

									P-Value		
	PC	NC	0.25PZ	0.50PZ	0.25P	0.50P	PSB	SEM	Trt	Linear	Quad
mM											
Acetate	68.75	73.94	66.93	61.85	69.75	69.30	67.80	3.529	0.184	0.005	0.793
Propionate	31.78	35.05	30.10	30.73	33.91	32.55	30.50	1.995	0.317	0.073	0.182
Butyrate	18.48	18.06	18.89	18.71	15.33	19.12	17.15	1.450	0.420	0.150	0.180
Iso-butyrate	2.19	2.49	2.86	2.53	2.30	3.43	2.70	0.309	0.129	0.941	0.352
Valerate	5.19	6.36	5.81	5.43	5.34	6.36	5.45	0.479	0.394	0.166	0.892
Iso-valerate	2.58	2.86	3.91	3.21	2.75	4.65	3.32	0.527	0.097	0.639	0.177
Total VFA	128.97	138.77	128.50	119.07	132.79	135.43	126.98	6.758	0.334	0.022	0.962
% Over VFA											
Acetate	53.11	53.82	51.52	51.61	51.99	50.83	53.05	1.040	0.501	0.355	0.537
Propionate	24.32	25.06	23.22	25.74	25.17	23.84	23.96	0.859	0.336	0.559	0.035
Butyrate	14.07	12.89	14.65	12.74	13.90	13.93	13.39	0.642	0.268	0.857	0.016
Iso-butyrate	1.68^{ab}	1.78^{ab}	2.17^{bc}	2.09^{bc}	1.73^{ab}	2.49^{c}	2.06^{bc}	0.185	0.039	0.251	0.291
Valerate	3.96	4.56	4.51	4.47	3.96	4.66	4.29	0.288	0.359	0.797	0.987
Iso-valerate	1.98^{a}	2.05^{ab}	2.99^{bc}	2.64^{abc}	2.03^{a}	3.39^{c}	2.52^{abc}	0.347	0.043	0.231	0.139

[a,b,c]Mean with different superscript differs significantly at $P \leq 0.05$

[1]Ortogonal contrast was used to determine linear and quadratic response in pigs fed NC, 0.25% peptide + high level of ZnO and 0.5% peptide + high level of ZnO.

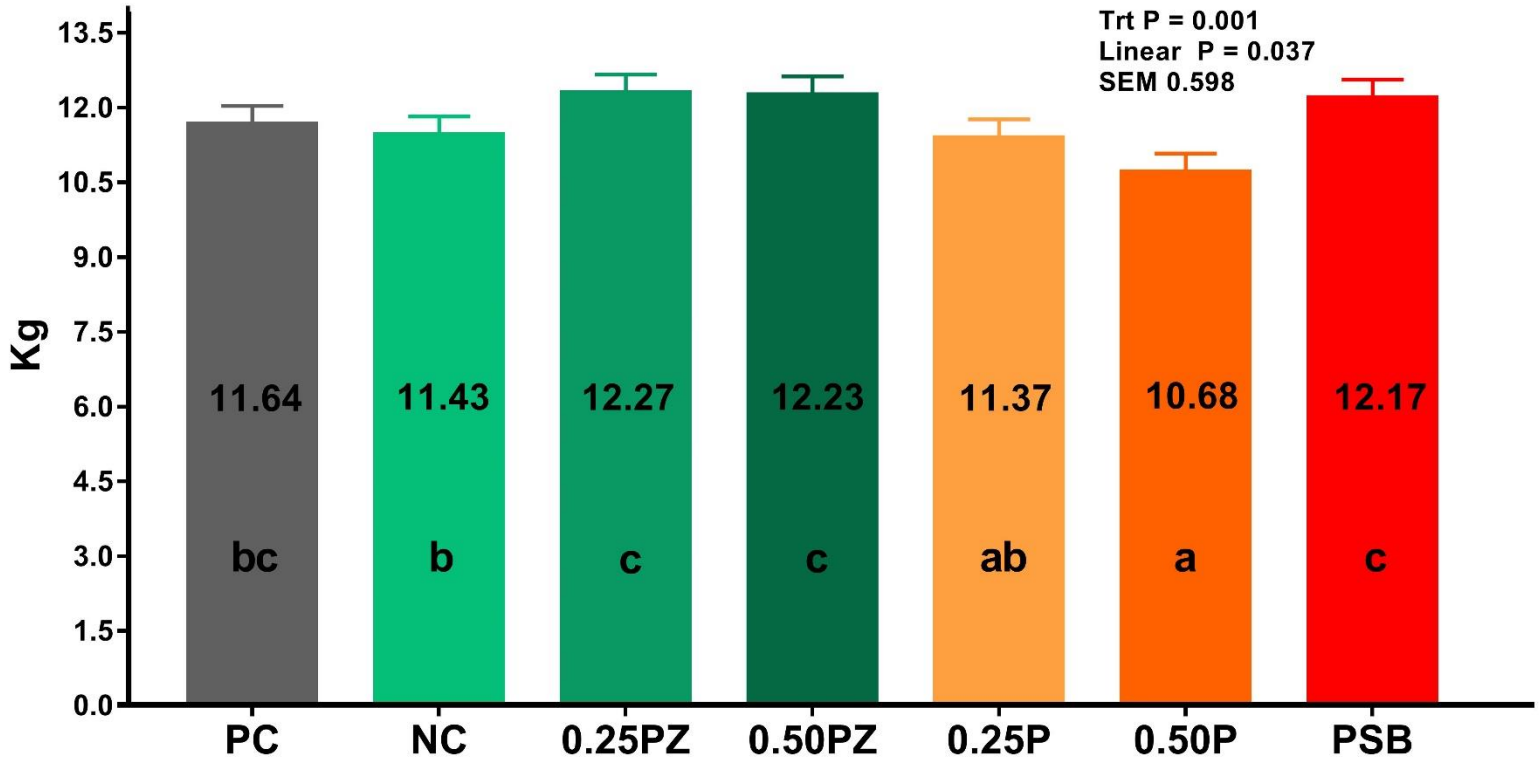

Figure 1. Effect of bioactive peptide alone, or in combination with zinc oxide or acidifiers on BW of nursery pigs in phase 2 (LS means).

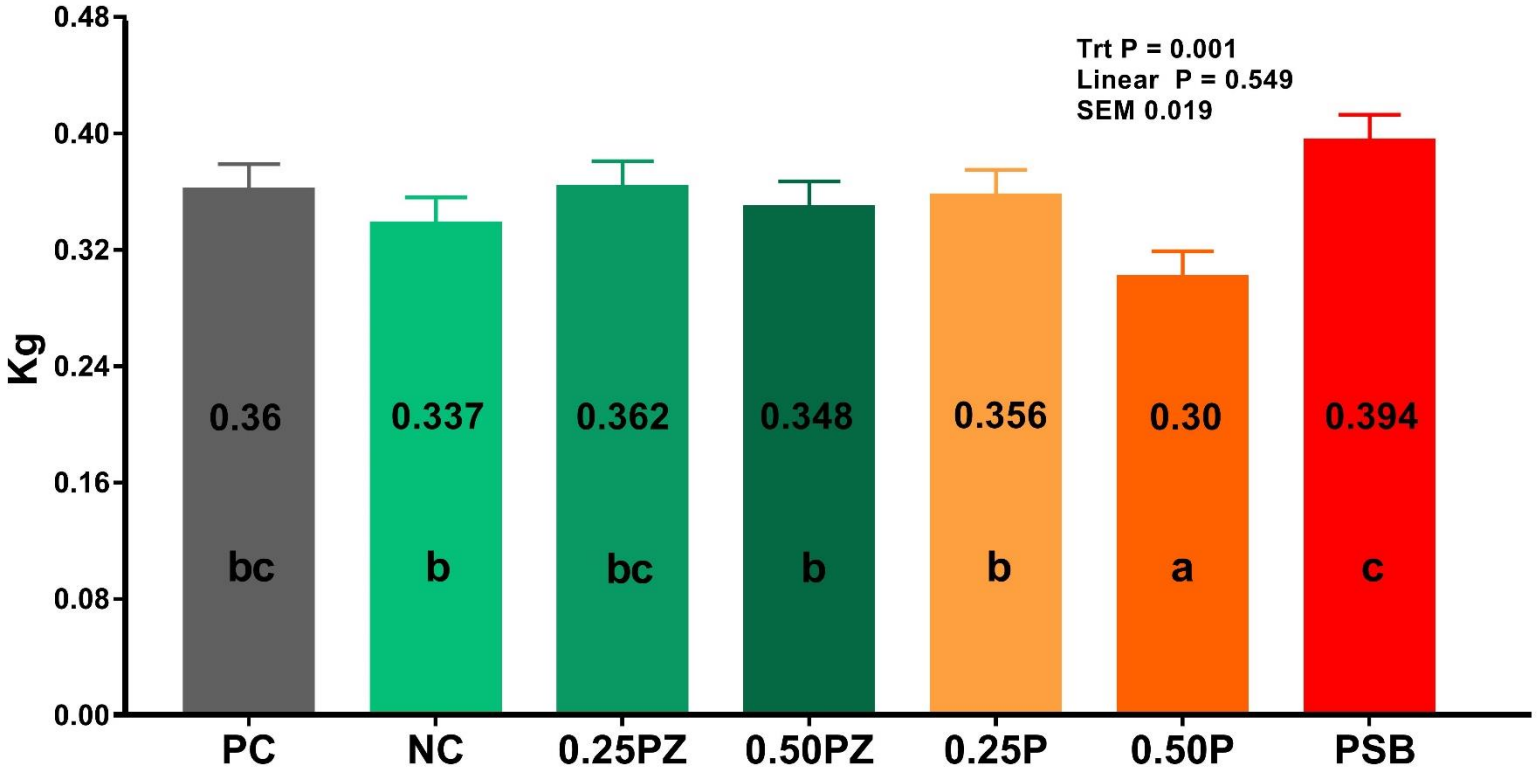

Figure 2. Effect of bioactive peptide alone, or in combination with zinc oxide or acidifiers on ADG of nursery pigs in phase 2 (LS means).

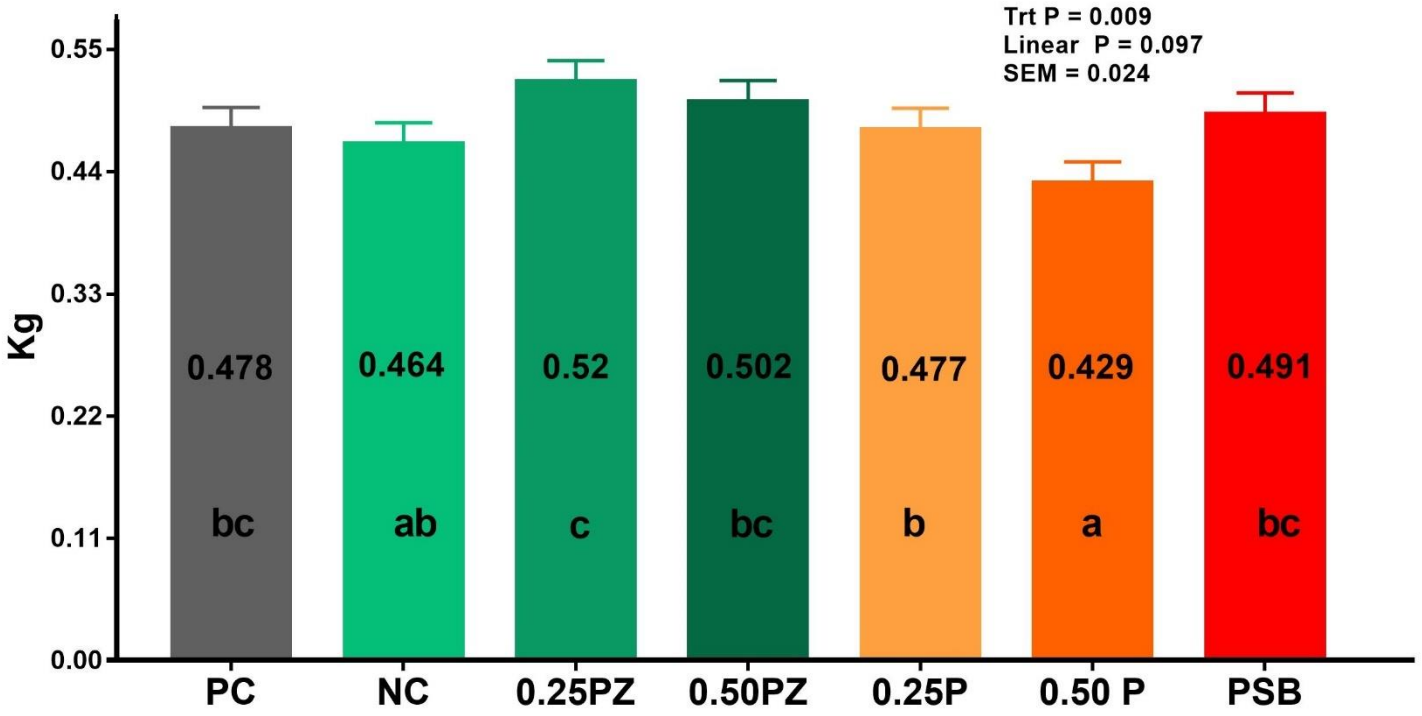

Figure 3. Effect of bioactive peptide alone, or in combination with zinc oxide or acidifiers on ADFI of nursery pigs in phase 2 (LS means).

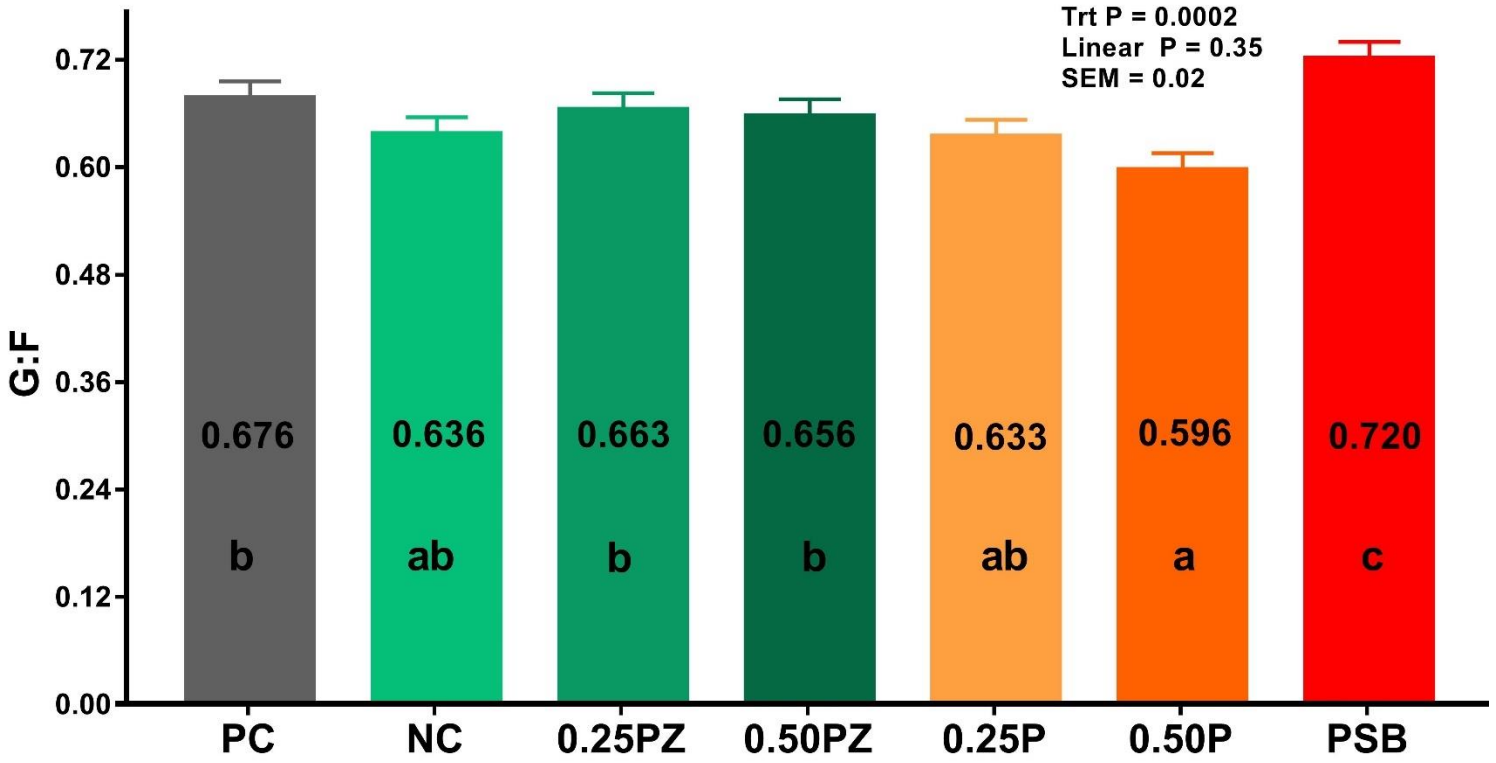

Figure 4. Effect of bioactive peptide alone, or in combination with zinc oxide or acidifiers on G:F ratio of nursery pigs in phase 2 (LS means).

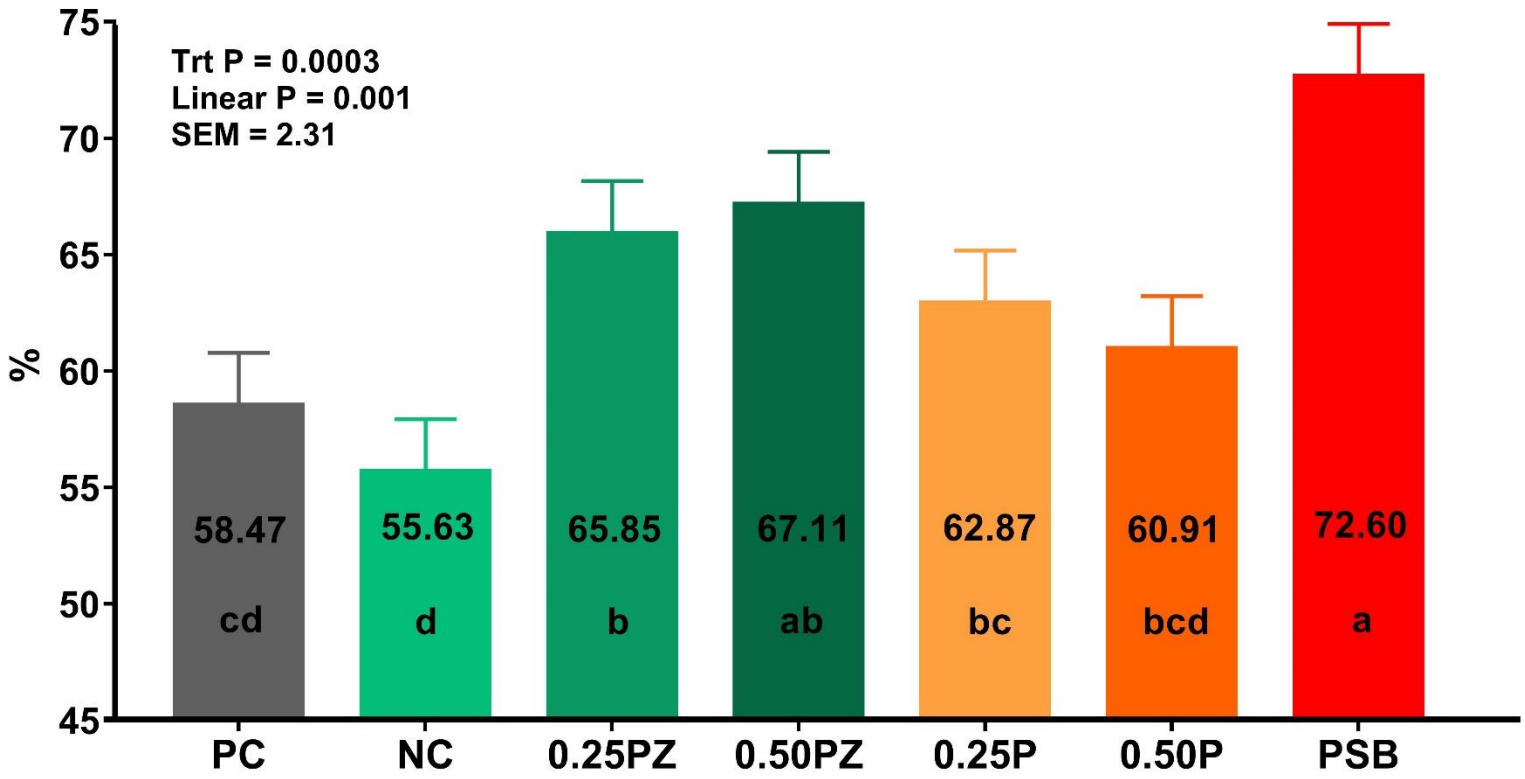

Figure 5. Effect of bioactive peptide alone, or in combination with zinc oxide or acidifiers on nitrogen digestibility in nursery pigs (LS means).

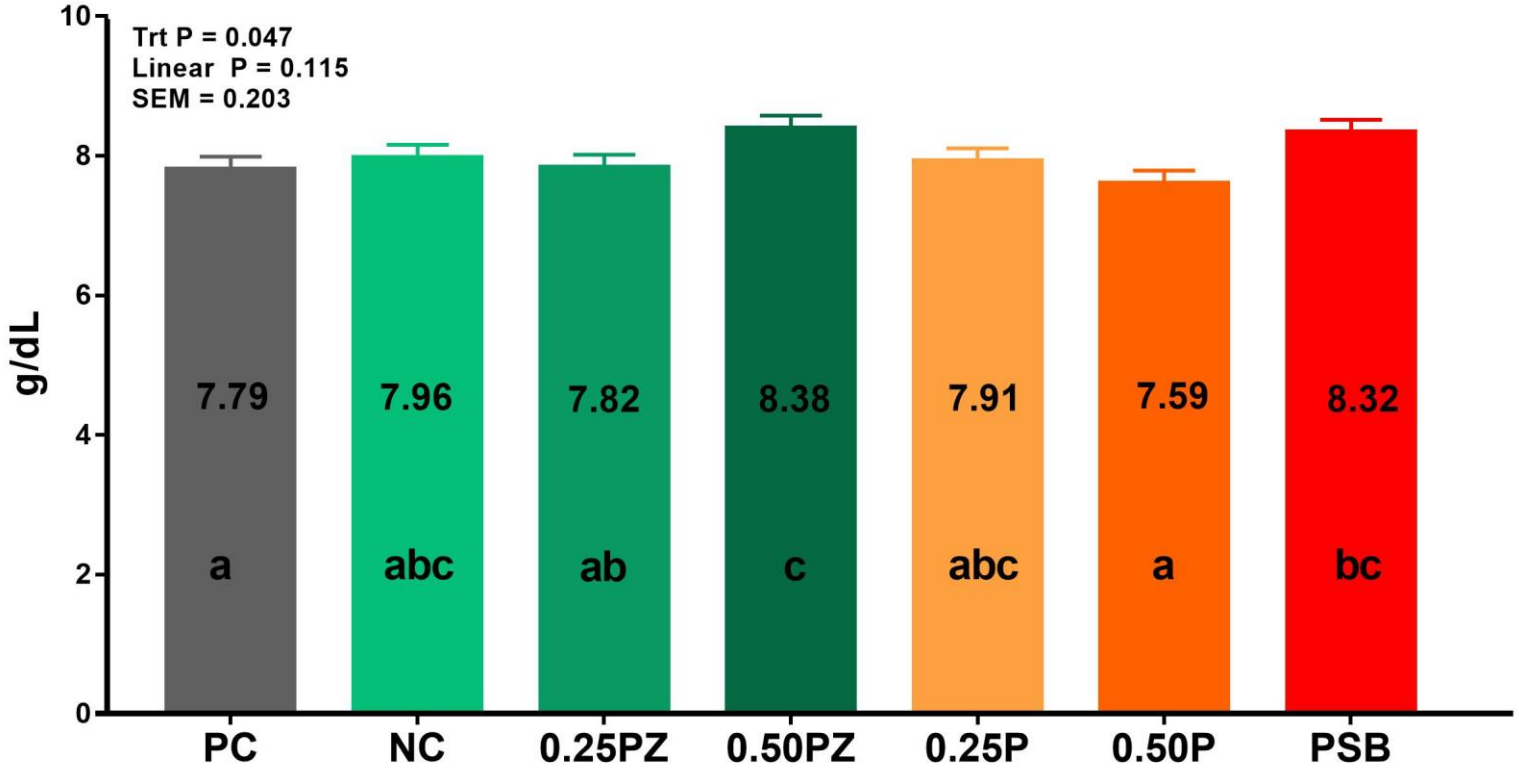

Figure 6. Effect of bioactive peptide alone, or in combination with zinc oxide or acidifiers on hemoglobin concentration in nursery pigs (LS means).

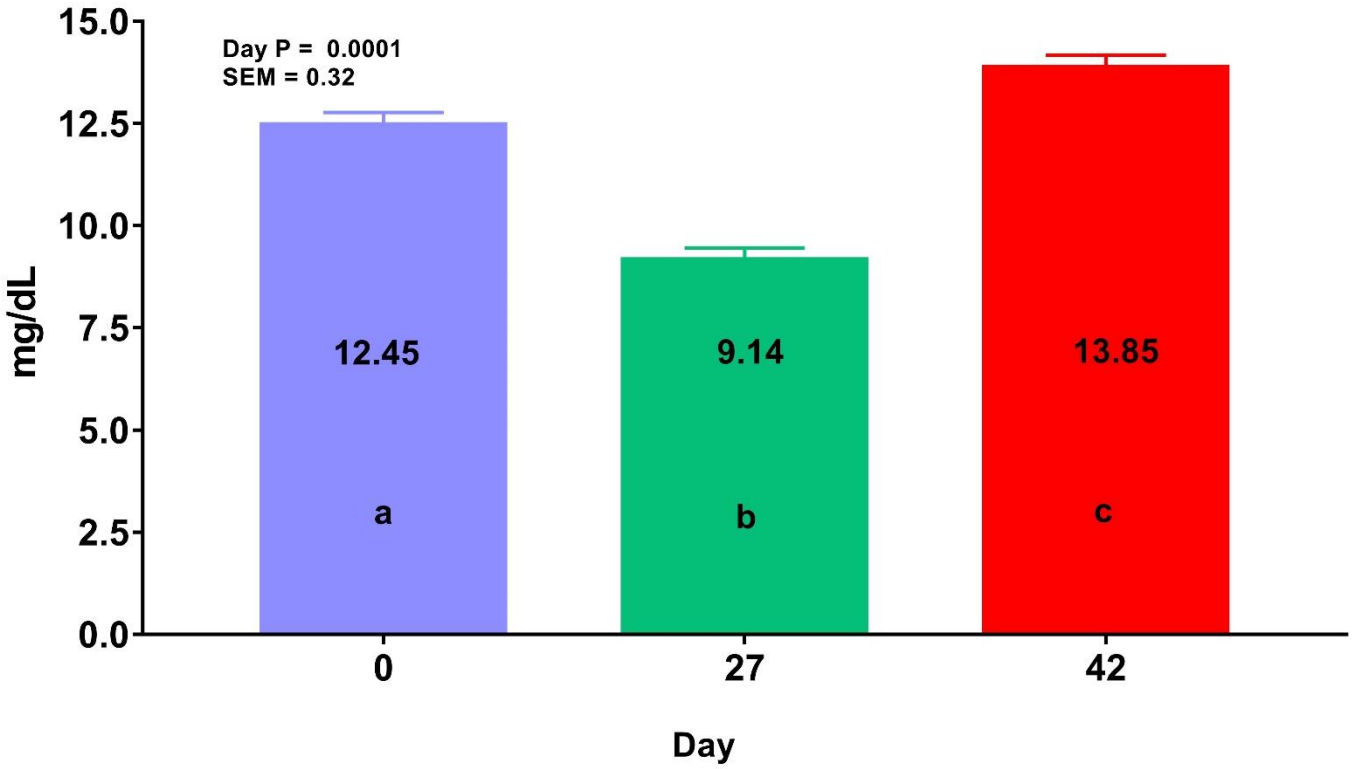

Figure 7. Effect of bioactive peptide alone, or in combination with zinc oxide or acidifiers on blood urea nitrogen (BUN) in nursery pigs.

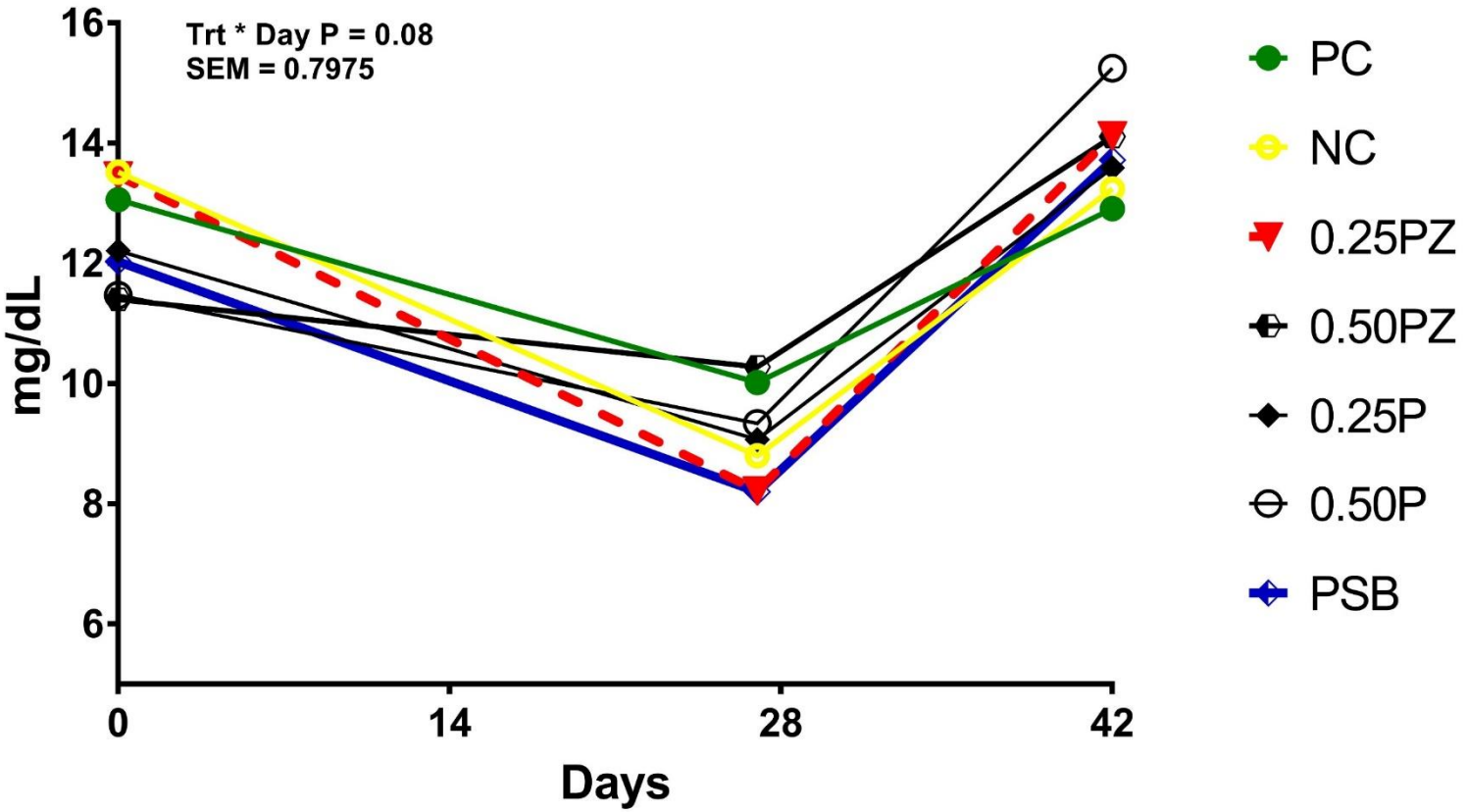

Figure 8. Treatment by day interaction effect of adding bioactive peptide alone or in combination with zinc oxide or acidifiers on blood urea nitrogen (BUN) in nursery pigs. (LS means).

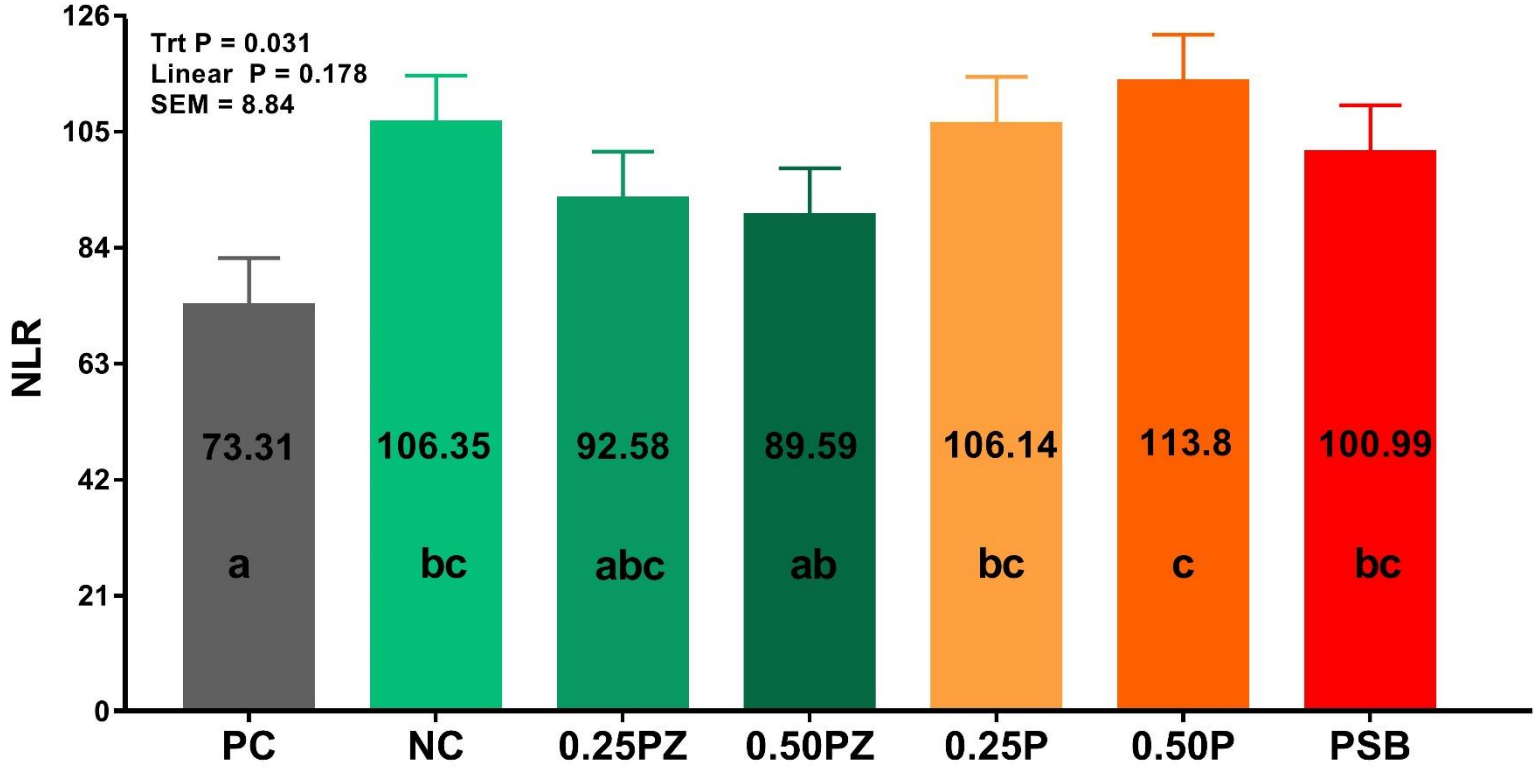

Figure 9. Effect of bioactive peptide alone, or in combination with zinc oxide or acidifiers on NRL in nursery pigs (LS means).

Chapter 3: Effect of Gossypol from Cottonseed Meal on Growth Performance, Complete Blood Cell Count, Plasma Gossypol and Reproductive Performance in Growing Gilts and Boars: Preliminary Study on Feral Hog Control

Effect of Gossypol from Cottonseed Meal on Growth Performance, Complete Blood Cell Count, Plasma Gossypol and Reproductive Performance in Growing Gilts and Boars: Preliminary Study on Feral Hog Control

Richard Mudarra, Tsungcheng Tsai, Christopher Hansen, Michael Dowd, Brittni Littlejohn, Charles Maxwell, Rick Rorie.

Abstract:

To evaluate the effect of cottonseed meal (CSM) on growth performance, plasma gossypol, hematology profile and semen quality in growing pigs, a total of 40 gilts (Exp 1) and 24 boars (Exp 2), 63 day of age (19.85±0.43 kg), were randomly allotted to 1 of 4 and 3 treatments with 2 replicates/treatments, respectively. Treatments for Exp 1 during phase 1 to 3 (14 d/phase) were a nutrient adequate control diet (NRC, 2012) without CSM (0% gossypol, G), and increasing levels of CSM was added to produce diets containing 0.01%, 0.02% and 0.04% G to form treatments 2 to 4, respectively. For Exp 2, treatments were the same as those in the gilt trail, except treatment 2 was removed (0.01% G). All pigs from both experiment were fed a common diet without CSM in phase 4. In both trials, individual body weight (BW), pen feed disappearance, and blood from 2 close-to-average pen-BW pigs were collected on d 0 and at the end of each phase to determine average daily gain, gain: feed ratio, plasma gossypol and complete blood cell count (CBC). Also, semen quality was evaluated in boars. Data were analyzed using the Mixed procedures of SAS (Cary, NC). ADG decreased linearly and quadratic ($P< 0.05$) with increasing level of CSM in gilts and boars, respectively in phase 3, while ADFI did not differ. Neutrophil concentration was higher while mean corpuscular volume (MCV) was lower in gilts fed CSM on d 42 than those fed control regardless level of inclusion

(Treatment*day, P <0.01). In boars, a Trt*Day interaction (P< 0.05) was observed for percentages of neutrophils, lymphocytes and basophils, RBC, hematocrit and MCV. Plasma gossypol increased with increasing level of CSM in both gilts and boars during phase 1-3 and was still higher than control after pigs were fed a common diet for 14 d (P< 0.05). There were not statistic differences in perm concentration/ml, motility and progressive sperm, and libido score in boars (P> 0.05). In conclusion, cottonseed meal derived gossypol impairs growth performance, stimulates CBC, and increase plasma gossypol in gilts and boars, but did not affect semen quality in boars.

Introduction

Cottonseed meal is the byproduct produced after the oil is extracted from the seed (Hall and Kononoff., 2011; Thirumalaisamy et al., 2016; Stein et al., 2016), and is considered a good nutritional source for livestock due to its high crude protein level (Stein et al., 2016; El-Sayed, 2020) energy and fiber content (Stewart, 2010). However, the main limitation of its uses is the presence of a phenolic compound, called gossypol. It is a toxic natural phenol that is produced by the pigment glands of the cottonseed plant (Gadelha et al., 2014). Monogastric animals are more susceptible to gossypol toxicity than ruminants (Jan, 2008).

Gossypol causes degeneration of hepatocyte cells (Gadelha et al., 2014), increased erythrocyte fragility and eryptosis, contributing to anemia in humans (Zbidah et al., 2012), ruminants and monogastric animals (Jan et al., 2008). Gossypol has been demonstrated to affect feed intake, body condition, and cardiac and respiratory functions in pigs (Haschek et al., 1989). Furthermore, gossypol has been reported to suppress spermatogenesis, degenerates seminiferous tubules (EL-Sharaky et al., 2010), and reduce sperm concentration and motility in various species (Dodou et al., 2005; Baker, 2019). Gossypol impairs Sertoli and Leydig cells functions, and reduces the synthesis of androgens, such as testosterone and 5α-dihydrotestosterone (Timurkanan, N. and Timurkaan, S. 2011; EL-Sharaky et al., 2010). The toxic effects of gossypol limit its utilization and inclusion in commercial pig diets (Rodriguez et al., 2013), but, because of its toxicity, cottonseed meal may be useful in controlling the feral hog population.

Feral hogs, one of the top 100 destructive invasive species in the world (Lowel et al., 2000), are widely distributed throughout several states of the US with an estimated population of over 6.9 million (Lewis et al., 2019). This species generates significant losses to the agricultural sector, with an estimated annual cost of over 1.5 billion in damages and control (USDA, 2020).

Besides the damages to agriculture, a main concern regarding feral hogs is the transmission of diseases to livestock animals and humans (Barrios-Garcia and Ballari, 2012; Brown et al., 2018). Several zoonotic diseases have been detected in wild hogs such as *Brucella suis* (Pedersen et al., 2012; Pedersen et al., 2014) *Leptospira spp* (Pedersen et al., 2017), *Salmonella spp* (Thakur et al., 2011), and hepatitis E virus (Ruiz-Fons, 2017).

Several strategies such as fencing, trapping and euthanasia, snares, ground and aerial shooting, injectable contraceptives and toxicants (sodium monofluoro acetate, warfarin) have been used to control the feral hog population. However, these efforts are not only expensive but also have been proven to be ineffective in bringing about total eradication. The use of several of these strategies are limited as they may not be legally approved in the US because of the possible effects on nontarget species (Massei, 2011). It appears that fertility control using GnRH vaccines have shown some progress. This fertility control strategy has high public acceptance because it does not impact wild hogs welfare and behavior (Killian et al., 2006,). However, it fails to be effective when use on a large scale.

The purpose of this project was to evaluate the effects of gossypol in cottonseed meal on growth performance and to determine if using it as a feed supplement, could adversely affects reproductive performance in boars. This could prove to be a promising fertility control measure against such an environmentally damaging species.

Materials and Methods

The protocols for this experiment were approved by the Institutional Animal Care and Use Committee at the University of Arkansas (Protocol number: 20001).

Animals and Experimental Design (Experiment 1)

A total of 40 growing gilts (PIC1050xDNA600), 63 days of age (20.22 ± 0.018 kg), were stratified by body weight and allotted to 1 of 4 treatments. Each treatment had 2 replicates with 5 pigs/replicate. Pigs were housed in 4.65 m^2 pens at the University of Arkansas conventional finisher facility. Pigs had access to feed and water during the 4 phases of the experiment, (14 days per phase).

Experimental Diets

Dietary treatments (Table 1) during phase 1 to 3 were: (1) a control diet (C), which was formulated to meet NRC, (2012) nutrient requirements, without cottonseed meal (CSM); (2) inclusion of 1.21% CSM (0.01% gossypol; G); (3) inclusion of 2.42 % CSM (0.02% G); and (4) diet formulated with 4.84 % CSM (0.04% G). During phase 4, gilts from 4 treatments were fed a common diet devoid of G. The 4 dietary treatments contained 177.0 ppm iron.

Sample Collection and Processing

Body weight (BW) was recorded on d 0. Feed intake (FI) and BW were measured at the end of each 4 phases to determine average daily feed intake, average daily gain, and gain:feed ratio by phase. Also, at the end of each phase, blood samples were collected from 2 pigs per pen. The same 2 pigs were used to collect blood samples during each of the 4 phases. Blood samples were collected in a 10 ml sodium heparin tube (BD Vacutainer, Becton, Dickinson and Company, Franklin Lakes, NJ). Whole blood was used to determine leukocyte differentiation and red blood cells characteristics, using a hematology analyzer (Hemavet 950 FS, Drew Scientific, Waterbury, CT). Samples were analyzed within 6 hr after collection.

Blood Gossypol Content.

Blood samples were centrifuged at 2500 x g for 15 min and 2 ml of plasma was collected

and stored at -80 ºC. Determination of gossypol content was quantified by high performance liquid chromatography by the Bullock et al, (2010) method.

Animals and Experimental Design (Experiment 2)

A total of 24 growing boars (PIC1050xDNA600), 63 days of age (19.36±0.13 kg), were stratified by BW and allotted to 1 of 3 treatments. Each treatment had 2 replicates with 4 pigs/replicate. Pigs were housed in 4.65 M^2 pens at the University of Arkansas conventional finisher facility. Pigs had access to feed and water during the 4 phases of the experiment, (14 days per phase).

Experimental Diets

Dietary treatments during phases 1 to 3 were: (1) control diet (C), which was formulated to meet NRC, (2012) nutrient requirements, without CSM; (2) diet with 2.42 % CSM (0.02% G); and (3) diet with 4.84 % CSM (0.04% G). Diets were the same as those used in the gilt study except the 0.01% G was removed (Table 13). During phase 4, boars from all treatments were fed a common diet devoid of gossypol. All diets contained 177.0 ppm iron.

Sample Collection and Processing

Feed intake was recorded during each phase of the experiment. Also, individual pigs were weighed on d 0 and BW and intake was measured at the end of each phase to determine average daily gain, average daily feed intake, and gain:feed ratio. Leukocyte differentiation, blood cell counts, and plasma gossypol were determined through the same methodology as described in Experiment 1.

Semen Collection and Analysis

Boars at 7 months of age were moved from group housing and then placed in individual 4.65 m^2 pens. Boars had free access to water but restricted feed intake at 2.5 kg per day of a diet

that met the NRC, (2012) nutrient requirements of their stage. A dummy was used for semen collection. After 2 successful semen collections, the boars were given 5 days of rest between each of the 3 additional collections. Semen collection was conducted achieved between 8:30 a.m to 11:30 a.m. Samples were diluted 1:1 ratio after collection using the extender BTS (Beltsville Thawing Solution) extender made according the methods described by Pursel and Johnson, (1975). Sperm concentration/ml, and percent motility and percent progressive motility were obtained using the computer-assisted sperm analysis system (IVOS 12.3C, Hamilton Thorne, Beverly, MA, USA).

Statistical Analysis

Data were analyzed using the PROC Mixed procedure of SAS (SAS Institute, Inc., Cary, NC) as a Randomized Complete Block Design with treatment as fixed effect, and BW block as random effect. Pen was the experimental unit for ANOVA. Also, orthogonal contrasts were used to determine linear, and quadratic effects of increased level of gossypol in diets. For semen analysis was used the same model, but treatment was the fixed effect. Also, Chi-Square analysis was used to evaluate boars that allowed semen collection or not.

Results

Experiment 1

Growth Performance

About experiment 1, the growth performance results are shown in table 15 and 16. The levels of gossypol used in the current study did not results in any death loss. A linear response in ADG ($P = 0.082$) and a quadratic response in G:F ratio ($P = 0.032$) were observed in phase 3 in gilts, resulting on a quadratic reduction in BW at the end of phase 3 ($P = 0.044$). Final BW

showed differences among treatment ($P < 0.05$), with gilts fed the 0.02% gossypol diet having the lowest BW. Average daily feed intake (ADFI) did not differ among treatments. Pigs fed the control diet in phase 3 had reduced G:F ratio when compared to those fed different levels of gossypol ($P < 0.05$), and G:F was linearly ($P < 0.05$) reduced with increasing dietary gossypol. In phase 4, when a diet devoid of gossypol was fed, pigs fed the control diet had a similar G:F ratio compared to pigs fed 0.02% G and 0.04 % G.

Complete Blood Cell count and Blood Cell Characteristics

Regarding the CBC results, there were not differences in leukocyte differentiation among treatments ($P > 0.05$), but a Trt*Day interaction was observed in WBC (Figure 13; $P = 0.018$), neutrophil, lymphocyte, monocyte, and eosinophil counts (Table 17, $P < 0.05$). Mean corpuscular volume (MCV) had a Trt*Day interaction ($P = 0.009$, Figure 15), whereas the percentage of hematocrit had a quadratic response ($P = 0.015$) with the lowest level observed in gilts fed 0.01% and 0.02% G (Figure 14).

Plasma Gossypol

Plasma gossypol corresponded to dietary gossypol content (Trt* Day $P < 0.0001$). Plasma gossypol increased after 14 days of feeding and peaked on d 28 in those fed 0.01 % and 0.04 % G, while gilts fed 0.02% G continued increasing until d 42. Although plasma gossypol decreased on d 56 from d 42 after the common diet was given, gilts fed 0.01 %, 0.02 %, and 0.04 % maintained the plasma gossypol at levels of 35 %, 30 %, and 29 %, respectively, compared to levels on d 42, (Figure 16).

Experiment 2

Growth Performance

Results of experiment 2 are shown in table 18 and 19. A quadratic response in ADG was observed in phase 2 and 3 (P = 0.028 and P = 0.0365, respectively), resulting on a quadratic response in final BW (P = 0.033). Boars fed 0.02% gossypol weighed less than boars fed the control diet and weight of boars fed 0.04 % G was intermediate (P < 0.05). There was no differences among treatment in ADFI in any phase (P > 0.05), whereas there was a quadratic reduction in G:F ratio for the overall study (d 0-42, P = 0.0489), with the lowest feed efficiency observed in boars fed the 0.02% G diets.

Complete Blood Cell count and Blood Cell Characteristics

White blood cell (WBC) and lymphocytes counts had quadratic responses (quadratic, P = 0.076; P = 0.028, respectively), with the lowest concentration observed in pigs fed 0.02% G. Monocyte percentage had a quadratic response (P = 0.044) with pigs fed 0.04% G having the lowest percentage. A Trt*Day interaction (P < 0.05) was observed RBC, hemoglobin, hematocrit, MCV and RDW, while PLT and MPV had statistic differences among treatments (P < 0.05) with pigs fed 0.02% G having the highest PLT, and the lowest MPV (Table 20).

Plasma Gossypol

Plasma gossypol increased with increasing dietary CSM content (Trt* Day P < 0.0001). Plasma gossypol in boars fed 0.04% G increased linearly until d 28, then decreased from d 28 to d 42, and kept decreased after d 42 when pigs where fed the common diet (Figure 21).

Semen Quality

There were no differences between sperm concentration/ml, percent motility, percent progressive motility and boars allowed collection or not (P > 0.05) among treatments, (Table 21, Figures 22, 23 and 24).

Discussion

Impaired growth performance was observed in both gilts and boars. Gilts fed increasing level of gossypol displayed a decrease in ADG and G:F ratio after phase 2 and consequently a reduced BW in phase 3 for gilts and BW in phase 4 for boars, in those fed 0.02% gossypol, while ADFI was not impacted in either gilts or boars. Several studies have revealed negative effects caused by gossypol on growth rate in the bovine (Pattanaik et al., 1992; Willard et al., 1995), birds (Elangovan et al., 2003; Zeng et al., 2014), and pigs (Haschek et al., 1989; Fombad and Bryant., 2004). However, the severity of the effects depends on the inclusion level of gossypol, and the duration of the feeding period (Haschek et al., 1989), and the CSM processing method (Jan, 2008).

Gilts and boars fed increasing levels of gossypol up to 0.02% G (200mg/kg) resulted in reductions to ADG and BW. Similar results were found by Fomband and Bryan (2004), who reported reduced ADG in 24 kg growing pigs fed cottonseed cake with 206 mg/kg gossypol. Also, Hermes et al. (1983) found that gossypol concentration over 200 mg/kg impaired growth performance of chickens. Interestingly, gilts and boars fed 0.04% G (400 mg/kg) were heavier by 2.5 kg and 7.2 Kg, respectively, than those fed 0.02% G. Hermes et al. (1983) and Nagalakshmi et al. (2007) concluded that the quality and quantity of dietary protein counteracts the negative effects of gossypol. Sterling et al. (2002) found that a slight increase in the level of dietary protein can help broilers maintain good growth performance by replacing soybean meal SBM with CSM. In our study, diet formulated with 4.21 % CSM (0.04% G) had a higher protein content than those formulated to achieve 0.02% G and control treatment. Increasing inclusion of CSM with (SBM) as the main protein source may have be helped to counteract the toxic effects

of gossypol. This may be the reason for the greater performance of pigs fed 0.04% G over those fed 0.02 % G.

The concentration of plasma gossypol in gilts and boars were well correlated to dietary gossypol inclusion. Concentrations of plasma gossypol in gilts and boars fed 0.04% G reached a plateau on d 28, decreasing slightly until d 42 and rapidly decreasing when given the common diet, while gossypol in pigs fed 0.02% G was still increasing until day 42 and only decrease when dietary treatments were switched to a common diet without gossypol. Boar plasma gossypol was lower than the gilts for the entire experiment. Plasma gossypol remained in blood 14 d after withdrawal of diet containing CSM in gilts and boars. Our results were similar to those reported by Mena et al. (2004), where plasma gossypol reached the highest level at d 28 in dairy cows fed CSM, and plasma gossypol was still detected 14 days after CSM withdrawal. Regardless of incremental increases in feed gossypol consumption over time, pigs fed the highest inclusion of CSM demonstrated a better capacity to reduce plasma gossypol than pigs fed diets with lower gossypol concentration. This may be due to the higher protein content in the diet formulated to achieve 0.04% G. Also, a small concentration of gossypol was detected in blood on d 28 for gilts fed the control diet. This small amount may be due to ingesting feces from adjacent pens. Small concentrations of boar plasma gossypol were detected on d 0 in pigs fed the control diet. These data were below the lowest point on the standard curve calibrated in the chromatograph. It may be possible that such samples were contaminated with small residues from a previous sample.

There were no statistical differences within the gilt and boar CSM treatments regarding leukocyte counts. Our results differed with those of Amao et al. (2012) who reported a reduction in neutrophils concentration as CSM was increased in the diet of male rabbits during a 22-week

period. Also, our results differed from those reported by Hu et al. (2015) who reported a reduction in the WBC count of juvenile black carp as they were fed increasing levels of CSM. NRC (1992) indicates that xenobiotics, such as toxic constituents in plants, can either stimulate or suppress the immune system. Stimulation is a result of toxic compounds acting as antigens that generate an immune response, while the immunosuppression is a response generated for high doses of toxin in the organism during a long period (NRC, 1992; Xu et al., 2009). Immune stimulation was observed in gilts where lymphocyte, neutrophil and WBCs reached their peak between d 28 and 42, and after replacing dietary CSM with the common diet, we observed a decrease in the presence of these cells on d 56. These results differed with those reported by Xu et al. (2009) where gossypol immunosuppressed lymphocyte proliferation by the inhibition of lymphoblastic transformation and stimulated cell apoptosis in mice. To Summarize, Dietary gossypol stimulated immune system in growing pigs, but the inclusion level used in these trials were not sufficiently high to cause a marked suppression of the immune system.

Berardi and Goldblatt (1980) indicated that anemia is one of the general symptoms of gossypol toxicity, and Makinde et al. (1997) reported that gossypol has cytocidal properties and the capacity to disrupt membrane enzyme activity. In our study, pigs did not show differences in hemoglobin concentration among treatments. Our results differed from those of Yildirim et al. (2003) who reported reductions in hemoglobin concentration and red blood cells (RBC) increased as level of gossypol in fish fed over 600 mg/kg. Interestingly, hematocrit percentage in gilts was reduced in those fed 0.01% G and 0.02% G, and MCV decreased in gilts over time regardless of dietary treatment until the withdrawal of dietary gossypol. These results agreed with those published by El-Mokadem et al. (2013) who found reductions in hematocrit level as well as MCV in rams fed 9 and 14 mg gossypol/Kg BW. The feeding period and gossypol

concentrations we subjected the gilts and boars to, appeared to have fallen short from that which would generate an acute RBC toxicity to the extent of causing anemia.

It is clear that gossypol toxicity depresses sperm motility via mitochondrial damages in sperm to the flagellum (Randel et al., 1989) decreasing ATP production (Druez, 1989). Sperm concentration in animals (Randel et al., 1998; Chenoweth et al., 2000) and humans (Hong et al., 1989) is reduced by blocking the gap junctional intercellular communication between Sertoli cells and germ cells (Zhou et al., 2008), affecting spermatogenesis. In our study, feeding boars different levels of CSM to achieve 0.01% 0.02% and 0.04% G for 6 weeks did not affect sperm concentration, percent motility, or percent progressive motility. Our results differed from those of (Baker, 2019) who reported significant reductions in sperm motility, sperm concentration and percentage of progressive sperm cells in domestic boars fed 0.74% gossypol for 10 weeks. These discrepancies between Baker and our results may be due to the different length of feeding period as well as inclusion level of gossypol. In addition, Arshami and Ruttle (1989) reported that gossypol toxicity in bull reproduction is time-dose dependent and the effects can be partially reversed 2 months after withdrawing from a gossypol diet. Besides the low dose of gossypol used in our study, another factor that may influenced the semen quality results in this study was that pigs were fed a common diet after CSM exposure for 4 months until semen was collected. Pigs may have had time to recover from gossypol toxicity after withdrawal of dietary gossypol.

Conclusion

Feeding growing pigs up to 0.02% gossypol for 42 days impaired their growth performance and stimulated complete blood cell count; however, there was no effect on semen quality later in life. In consideration of CSM as a method to control the feral hog population in

the US, future research should utilize increased concentration of gossypol and shorter period between dietary gossypol removal and semen collection.

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Appendix

Table 13. Growing pigs diet composition.

		% Gossypol		
Ingredients	PC	0.01	0.02	0.04
Corn, Aji Oct. 13	50.57	49.36	48.15	45.73
SBM, Aji, Oct. 13	24.00	24.00	24.00	24.00
CDDGS, Aji Oct 13	20.00	20.00	20.00	20.00
Fat (Darling, Yellow Grease)	2.750	2.75	2.75	2.75
Calcium phosphate (monocalcium)	0.160	0.16	0.16	0.16
Limestone, 2012 NRC	1.250	1.25	1.25	1.25
Sodium chloride	0.50	0.50	0.50	0.50
L-Lysine	0.333	0.333	0.333	0.333
DL-Methionine	0.081	0.081	0.081	0.081
L-Threonine	0.075	0.075	0.075	0.075
L-Tryptophan	0.025	0.025	0.025	0.025
[1]T Mineral Premix (NB-8534)	0.10	0.10	0.10	0.10
[2]Vitamin Premix (NB-6508)	0.125	0.125	0.125	0.125
Ronozyme 10000	0.003	0.003	0.003	0.003
Ethoxiquin (Quinguard)	0.030	0.03	0.03	0.03
Cottonseed, Fullfat	0.000	1.209	2.419	4.839

Dietary treatments for Experiment 1 and 2 were the same, except the 0.01% gossypol was not included in Experiment 2.

[1]The mineral premix provided per kg of complete diet: 84 mg of Ca as CaCO3, 165 mg of Fe as FeSO4, 165 mg of Zn as ZnSO4, 39.6 mg of Mn as MnSO4, 16.5 mg of Cu as CuSO4, 0.3 mg of I as CaI2, and 0.3 mg of Se as Na2SeO3.

[2]The vitamin premix provided per kg of complete diet: 397.5 mg of Ca as CaCO3, 11,022.9 IU of vitamin A, 1,77.9 IU of vitamin D_3, 44.09 IU of vitamin E, 0.0386 mg vitamin B_{12}, 4.41 mg of menadione, 8.27 mg of riboflavin, 27.56 mg of D-pantothenic acid, and 49.6 mg of niacin.

Table 14. Growing pigs diet calculated analysis.

		% Gossypol		
Calculated Analysis	C	0.01	0.02	0.04
ME (Kcal/Kg)	3436	3432	3424	3419
CP (%)	21.30	21.50	21.70	22.09
Total P (%)	0.456	0.461	0.466	0.475
Available P (%)	0.201	0.203	0.204	0.208
Ca (%)	0.601	0.609	0.610	0.614
Na (%)	0.288	0.288	0.287	0.287
Copper (ppm)	17.56	17.52	17.48	17.40
Iron (ppm)	177.89	177.66	177.44	177.00
Gossypol	0.000	0.010	0.020	0.040
Gossypol:Iron	0.00	1.777	0.887	0.442
Total Lysine (%)	1.261	1.269	1.277	1.292
SID Lysine (%)	1.085	1.090	1.094	1.103
SID Methionine (%)	0.377	0.378	0.380	0.382
SID Threonine (%)	0.708	0.711	0.714	0.720
SID Tryptophan (%)	0.222	0.223	0.225	0.228
SID Isoleucine (%)	0.739	0.742	0.746	0.753
SID Valine (%)	0.806	0.812	0.817	0.828
SID Leucine (%)	1.659	1.661	1.662	1.665
SID Histidine (%)	0.465	0.469	0.472	0.479
SID M+C:Lys	60.1	60.1	60.1	60.2
SID Thr:Lys	65.2	65.3	65.3	65.3
SID Trp:Lys	20.4	20.5	20.5	20.7
SID Ile:Lys	68.1	68.1	68.1	68.2
SID Val:Lys	74.3	74.5	74.7	75.0
SID Leu:Lys	152.8	152.4	151.9	150.9
SID His:Lys	42.9	43.0	43.1	43.4

Table 15. Effect of increasing levels of dietary gossypol on body weight (BW) and average daily gain (ADG) of growing gilts (LS means).

	% Gossypol					P-Value		
	Control	0.01	0.02	0.04	SEM	Trt	Linear	Quad
BW, Kg								
d 0	20.25	20.20	20.24	20.23	0.016	0.354	0.856	0.458
Phase 1 (d 14)	28.64	27.60	26.49	26.42	1.27	0.615	0.293	0.559
Phase 2 (d 28)	39.69	38.44	33.84	35.97	1.164	0.109	0.082	0.105
Phase 3 (d 42)	50.46^{c}	47.97^{bc}	41.66^{a}	44.09^{ab}	1.283	0.049	0.031	0.056
Phase 4 (d 56)	64.5^{c}	60.51^{bc}	53.25^{a}	55.75^{ab}	1.498	0.040	0.022	0.044
ADG, Kg								
Phase 1(d 0-14)	0.600	0.528	0.447	0.442	0.090	0.615	0.293	0.564
Phase 2 (d 4-28)	0.789	0.774	0.529	0.682	0.080	0.257	0.301	0.199
Phase 3 (d 28-42)	0.770	0.680	0.559	0.579	0.038	0.079	0.035	0.104
Phase 4 (d 42-56)	1.003	0.896	0.828	0.83	0.088	0.552	0.277	0.439
Overall (d 0-42)	0.719^{c}	0.661^{bc}	0.510^{a}	0.568^{ab}	0.031	0.051	0.031	0.057
Overall (d 0-56)	0.790^{c}	0.719^{bc}	0.590^{a}	0.634^{ab}	0.027	0.039	0.022	0.045

Table 16. Effect of increasing level of dietary gossypol on average daily feed intake (ADFI) and gain: feed ratio (G:F) of growing gilts (LS means).

	% Gossypol					P-Value		
	Control	0.01	0.02	0.04	SEM	Trt	Linear	Quad
ADFI, Kg								
Phase 1(d 0-14)	1.030	1.071	0.993	0.955	0.086	0.732	0.390	0.767
Phase 2 (d 4-28)	1.563	1.618	1.300	1.273	0.085	0.138	0.061	0.714
Phase 3 (d 28-42)	1.662	1.697	1.401	1.494	0.099	0.290	0.206	0.433
Phase 4 (d 42-56)	2.255	2.434	1.954	2.117	0.191	0.538	0.424	0.698
Overall (d 0-42)	1.418	1.46	1.231	1.234	0.065	0.168	0.083	0.654
Overall (d 0-56)	1.628	1.676	1.412	1.455	0.053	0.150	0.079	0.391
G:F								
Phase 1(d 0-14)	0.579	0.493	0.443	0.474	0.058	0.492	0.313	0.294
Phase 2 (d 4-28)	0.505	0.483	0.429	0.542	0.071	0.733	0.721	0.387
Phase 3 (d 28-42)	0.462^{b}	0.397^{a}	0.396^{a}	0.390^{a}	0.009	0.028	0.017	0.032
Phase 4 (d 42-56)	0.447^{b}	0.302^{a}	0.424^{b}	0.395^{b}	0.012	0.052	0.770	0.080
Overall (d 0-42)	0.507^{c}	0.452^{ab}	0.416^{a}	0.464^{bc}	0.010	0.035	0.088	0.012
Overall (d 0-56)	0.485	0.406	0.419	0.436	0.013	0.123	0.200	0.060

Table 17. Effect of increasing levels of dietary gossypol on complete blood cell (CBC) count in growing gilts (LS means).

		% Gossypol							
	Control	0.01	0.02	0.04	SEM	Trt	Trt*Day	Linear	Quad
WBC	12.469	13.494	12.81	13.319	1.423	0.928	0.018	0.732	0.864
Neutrophil	4.622	5.092	5.055	4.318	0.643	0.790	0.001	0.618	0.380
Lymphocyte	6.305	6.616	5.625	6.683	0.6	0.589	<0.001	0.802	0.418
Monocyte	0.729	0.703	0.656	0.736	0.082	0.899	0.017	0.945	0.480
Eosinophil	0.758	1.007	0.814	1.332	0.234	0.207	0.002	0.073	0.580
Basophil	0.054	0.0768	0.0723	0.0578	0.011	0.409	0.706	0.926	0.123
NLR	37.056	37.707	38.53	32.685	2.32	0.300	0.252	0.153	0.214
% over WBC									
Neutrophil	50.389	48.853	49.131	51.314	1.65	0.700	0.652	0.553	0.315
Lymphocyte	5.836	5.262	5.466	5.724	0.577	0.894	0.306	0.960	0.515
Monocyte	6.282^{a}	7.648^{ab}	6.327^{a}	9.829^{b}	1.138	0.107	0.335	0.043	0.403
Eosinophil	0.434	0.535	0.503	0.418	0.072	0.627	0.417	0.662	0.257
Basophil	0.754	0.863	0.858	0.653	0.093	0.343	0.407	0.308	0.131
RBC, M/µl	7.545	7.396	7.545	7.792	0.287	0.517	0.643	0.235	0.431
Hemoglobin, g/dL	10.69	9.965	10.363	10.847	0.458	0.403	0.758	0.486	0.201
Hematocrit, %	37.535^{b}	35.14^{a}	35.816^{ab}	37.54^{b}	1.4	0.055	0.150	0.536	0.015
MCV	49.765	47.51	47.785	48.092	0.73	0.135	0.009	0.240	0.074
MCH, Pg	14.19	13.478	13.751	13.92	0.295	0.385	0.070	0.836	0.176
MCHC, g/dL	28.675	28.565	28.97	29.142	1.285	0.880	0.078	0.078	0.047
RDW, %	21.735^{b}	22.358^{b}	21.706^{b}	20.639^{a}	0.389	0.025	0.001	0.013	0.115
PLT, k/µl	275.43	278.73	310.46	314.12	30.433	0.719	0.001	0.305	0.792
MPV, fL	9.423	9.018	9.227	8.95	0.212	0.368	0.249	0.194	0.752

WBC: White Blood Cells; NLR: Neutrophil to Lymphocyte Ratio; RBC: Red Blood Cells; MCV: Mean Corpuscular Volume. MCH: Mean Corpuscular Hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red cell distribution width. MPV: Mean Platelet Volume.

Table 18. Effect of increasing levels of dietary gossypol on body weight (BW) and average daily gain (ADG) in growing boars (LS means).

		% Gossypol			P-Value		
Variable	Control	0.02	0.04	SEM	Trt	Linear	Quad
BW, Kg							
d 0	19.42[a]	19.45[a]	19.22[b]	0.0283	0.050	0.0385	0.0667
Phase 1 (d 14)	27.5	25.51	27.5	0.6448	0.2405	1	0.1285
Phase 2 (d 28)	37.85	33.54	37.08	1.4851	0.2944	0.7505	0.1635
Phase 3 (d 42)	50.43	42.58	48.73	1.4991	0.1164	0.5066	0.0624
Phase 4 (d 56)	63.87[a]	54.26[b]	61.46[a]	1.2862	0.0621	0.3163	0.0334
ADG, Kg							
Phase 1(d 0-14)	0.5771	0.4333	0.5913	0.0475	0.2281	0.8524	0.1221
Phase 2 (d 4-28)	0.7391	0.573	0.6844	0.0607	0.3402	0.5897	0.2033
Phase 3 (d 28-42)	0.8991[a]	0.646[b]	0.8323[a]	0.0306	0.0517	0.2630	0.0280
Phase 4 (d 42-56)	0.9598[a]	0.8342[b]	0.9092[ab]	0.0161	0.0608	0.1558	0.0365
Overall (d 0-42)	0.7384	0.5507	0.7026	0.0362	0.1168	0.5574	0.0621
Overall (d 0-56)	0.7938[a]	0.6217[b]	0.7543[a]	0.0234	0.0629	0.3545	0.0335

Table 19. Effect of increasing level of dietary gossypol on average daily feed intake (ADFI) and gain:feed ratio (G:F) in growing boars (LS means).

		% Gossypol			P-Value		
Variable	Control	0.02	0.04	SEM	Trt	Linear	Quad
ADFI, kg							
Phase 1(d 0-14)	1.1725	0.9922	1.1728	0.0336	0.094	0.9966	0.0482
Phase 2 (d 4-28)	1.4295	1.258	1.3762	0.0707	0.3934	0.6471	0.2363
Phase 3 (d 28-42)	1.7896	1.5495	1.6322	0.0913	0.3589	0.3471	0.2855
Overall (d 0-42)	1.4639	1.2666	1.3937	0.0562	0.2404	0.4709	0.1428
G:F							
Phase 1(d 0-14)	0.4965	0.4328	0.5042	0.0511	0.6297	0.9247	0.3933
Phase 2 (d 4-28)	0.518	0.4524	0.4968	0.0279	0.4092	0.6449	0.2483
Phase 3 (d 28-42)	0.5011	0.4209	0.5109	0.0224	0.1703	0.7867	0.0899
Overall (d 0-42)	0.5053	0.4331	0.5042	0.0134	0.0954	0.9586	0.0489

Table 20. Effect of increasing levels of dietary gossypol on complete blood cell (CBC) count in growing boars (LS means).

		% Gossypol			P-Value			
	Control	0.02	0.04	SEM	Trt	Trt*Day	Linear	Quad
Concentration, k/µl								
WBC	12.77	11.25	13.49	0.84	0.171	0.3768	0.550	0.076
Neutrophil	4.00	3.42	4.55	0.45	0.229	0.0696	0.394	0.136
Lymphocyte	6.80	6.03	7.12	0.33	0.070	0.3328	0.489	0.028
Monocyte	0.85	0.87	0.80	0.10	0.89	0.9077	0.733	0.734
Eosinophil	1.05	0.86	0.92	0.20	0.791	0.1259	0.660	0.604
Basophil	0.09	0.08	0.09	0.01	0.482	0.206	0.692	0.256
NLR	61.26	59.85	66.16	8.06	0.810	0.0201	0.633	0.664
% over WBC								
Neutrophil	30.74	30.66	33.53	2.42	0.616	0.0419	0.403	0.609
Lymphocyte	54.29	53.67	53.27	3.25	0.961	0.027	0.782	0.972
Monocyte	6.66	7.51	5.83	0.49	0.068	0.6102	0.240	0.044
Eosinophil	7.66	7.48	6.67	1.63	0.863	0.1037	0.614	0.851
Basophil	0.64	0.68	0.70	0.11	0.930	0.006	0.705	0.983
RBC, M/µl	7.19	7.10	7.39	0.26	0.718	<0.0001	0.582	0.553
Hemoglobin, g/dL	10.28	9.58	10.06	0.49	0.585	0.0553	0.747	0.328
Hematocrit, %	34.68	31.96	34.11	1.48	0.401	<0.0001	0.788	0.188
MCV	48.43	45.15	46.119	2.06	0.52	0.0098	0.434	0.406
MCH, Pg	14.38	13.55	13.59	0.65	0.594	0.5779	0.39	0.590
MCHC, g/dL	29.63	29.98	29.50	0.37	0.522	0.2007	0.772	0.273
RDW, %	21.74	21.98	23.18	0.91	0.352	0.0188	0.179	0.604
PLT, k/µl	264.18[a]	336[b]	313[ab]	20.5	0.049	0.2415	0.097	0.064
MPV, fL	9.81[b]	8.63[a]	9.25[ab]	0.27	0.013	0.0885	0.143	0.009

Table 21. Effect of increasing level of dietary gossypol on sperm characteristics (LS means).

	Control	% Gossypol		SEM	P-Value				
		0.02	0.04		Trt	Day	Trt*Day	Linear	Quad
[1]Conc/ml	281.68	261.57	218.15	28.66	0.353	0.579	0.442	0.169	0.730
[2]Motility %	79.88	82.75	74.25	2.93	0.154	0.018	0.611	0.230	0.120
[3]Prog %	56.25	60.67	58.25	6.61	0.887	0.018	0.371	0.84	0.661

[1]Concentration of sperm/ml.
[2]Percent motility.
[3]Percent progressive motility.

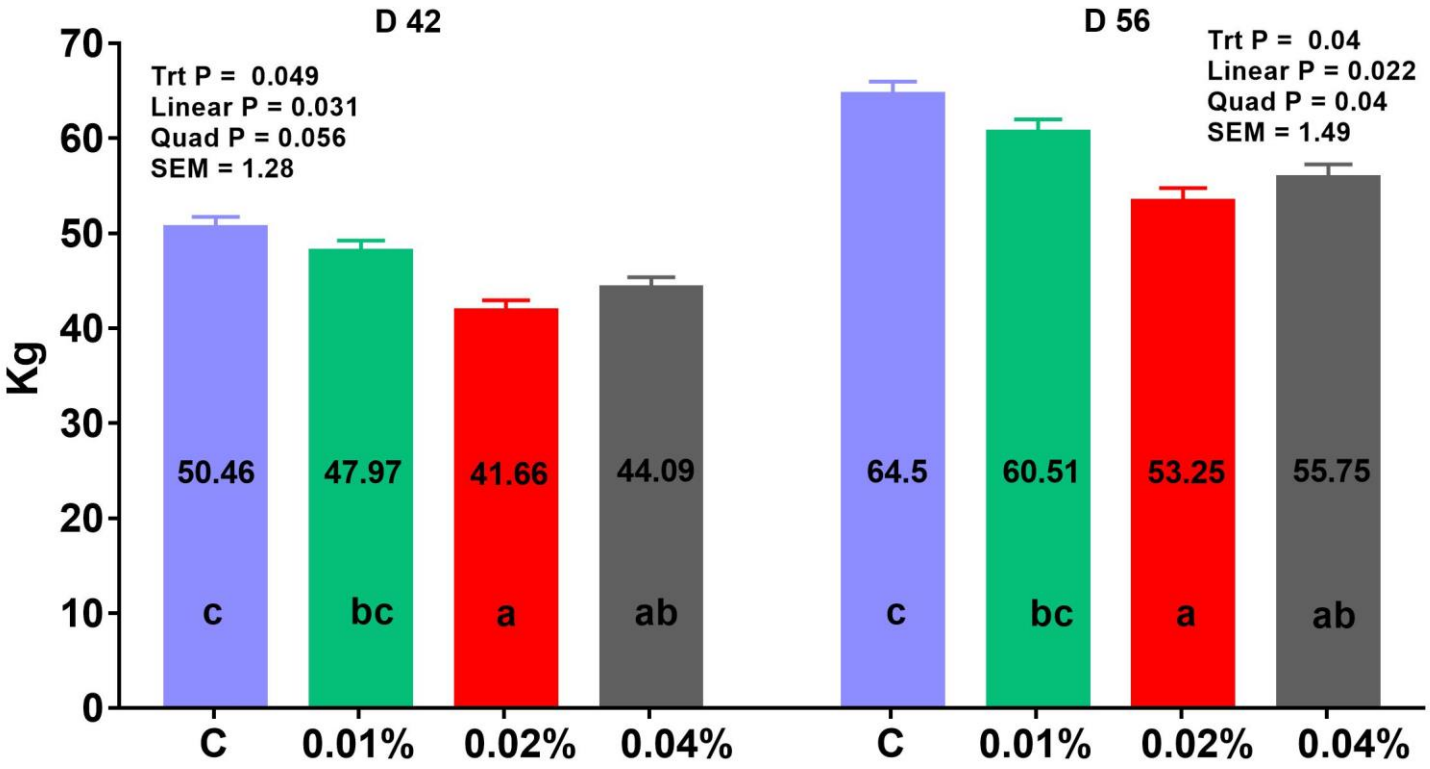

Figure 10. Effect of increasing level of dietary gossypol on BW of growing gilts in phase 3 (d42) and 4 (d 56), (LS means).

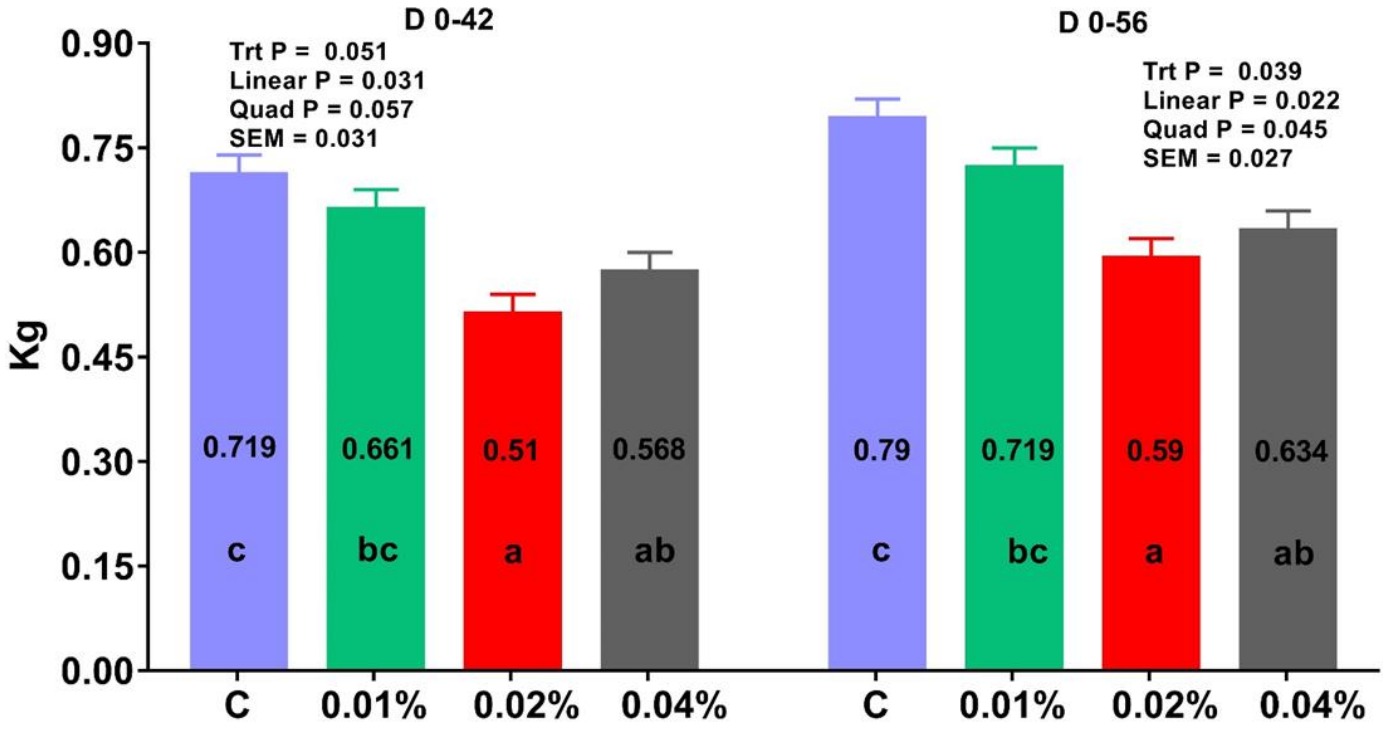

Figure 11. Effect of increasing level of dietary gossypol on ADG of growing gilts in overall (d 0-42) and (d 0-56) phases, (LS means).

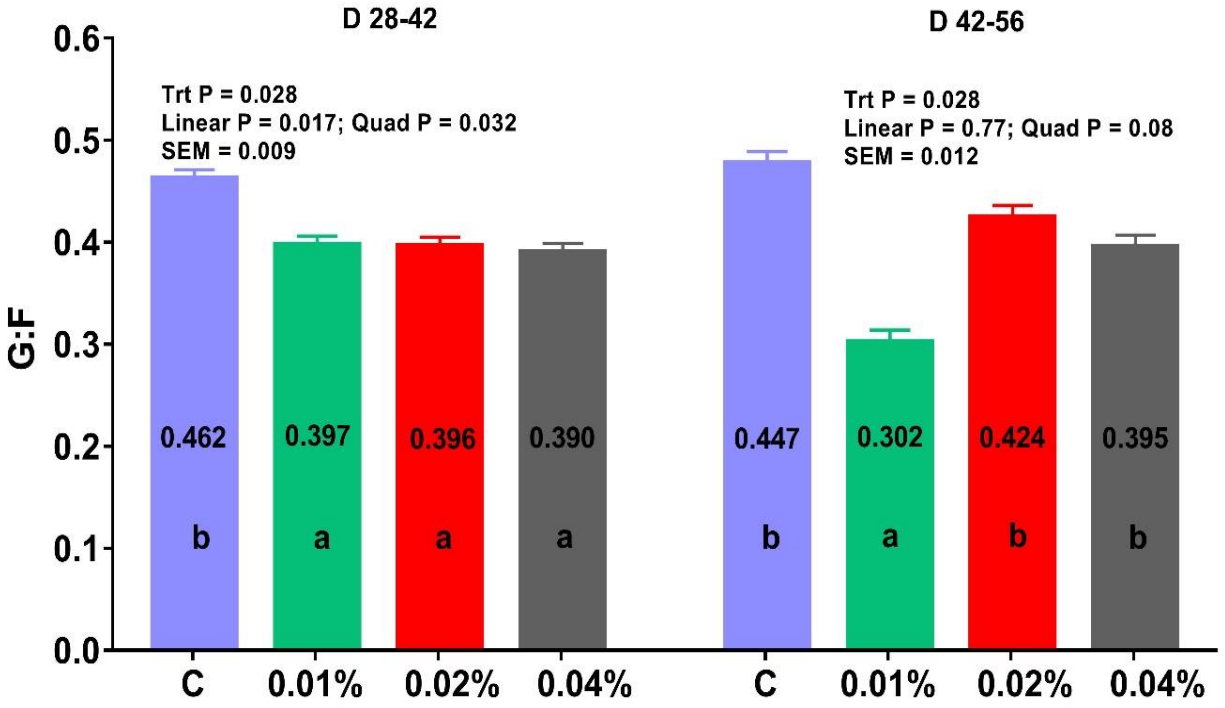

Figure 12. Effect of increasing level of dietary gossypol on G:F ratio of growing gilts in phase 3 (d 28-42) and 4 (d 42-56), (LS means).

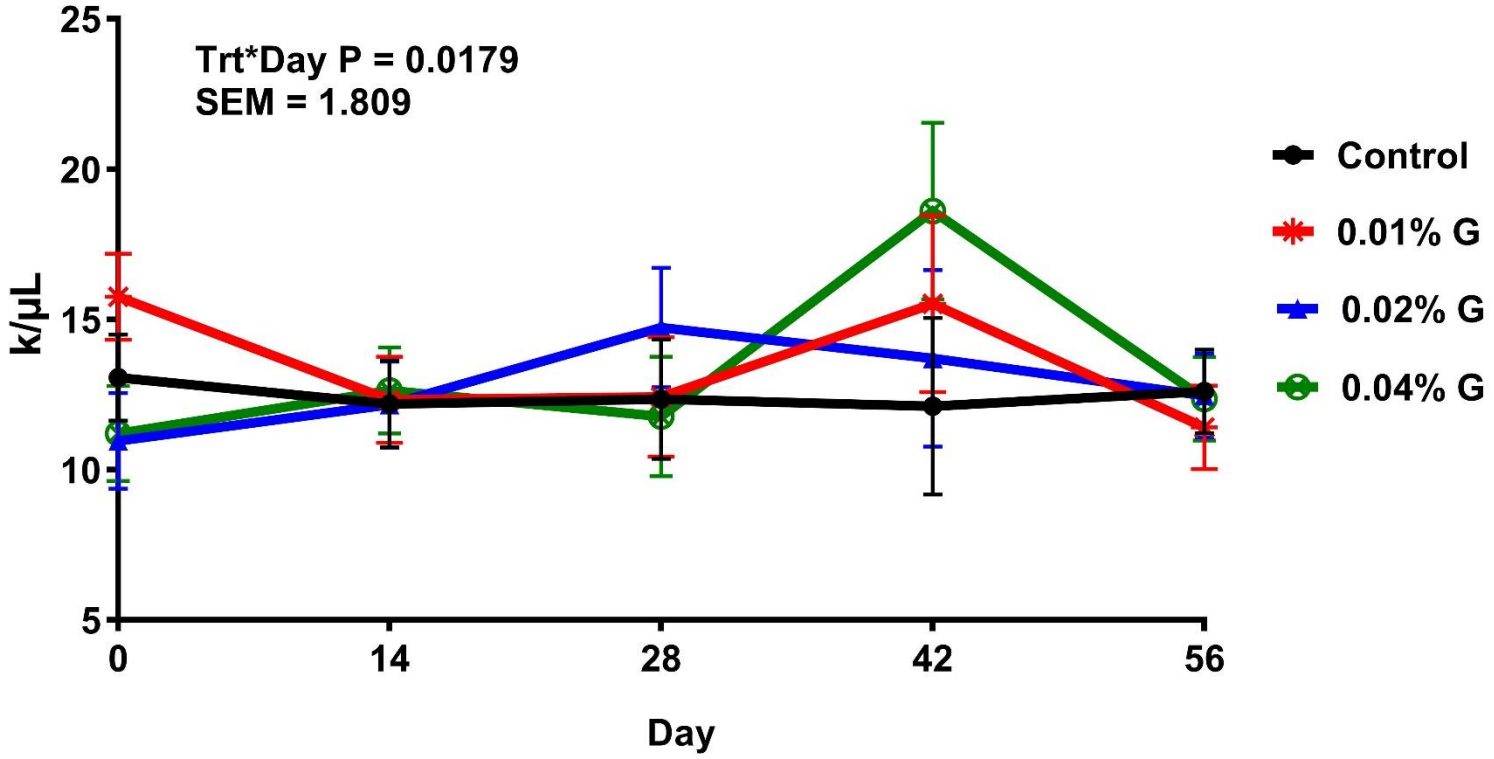

Figure 13. Effect of increasing level of dietary gossypol on white blood cell concentration of growing gilts (LS means).

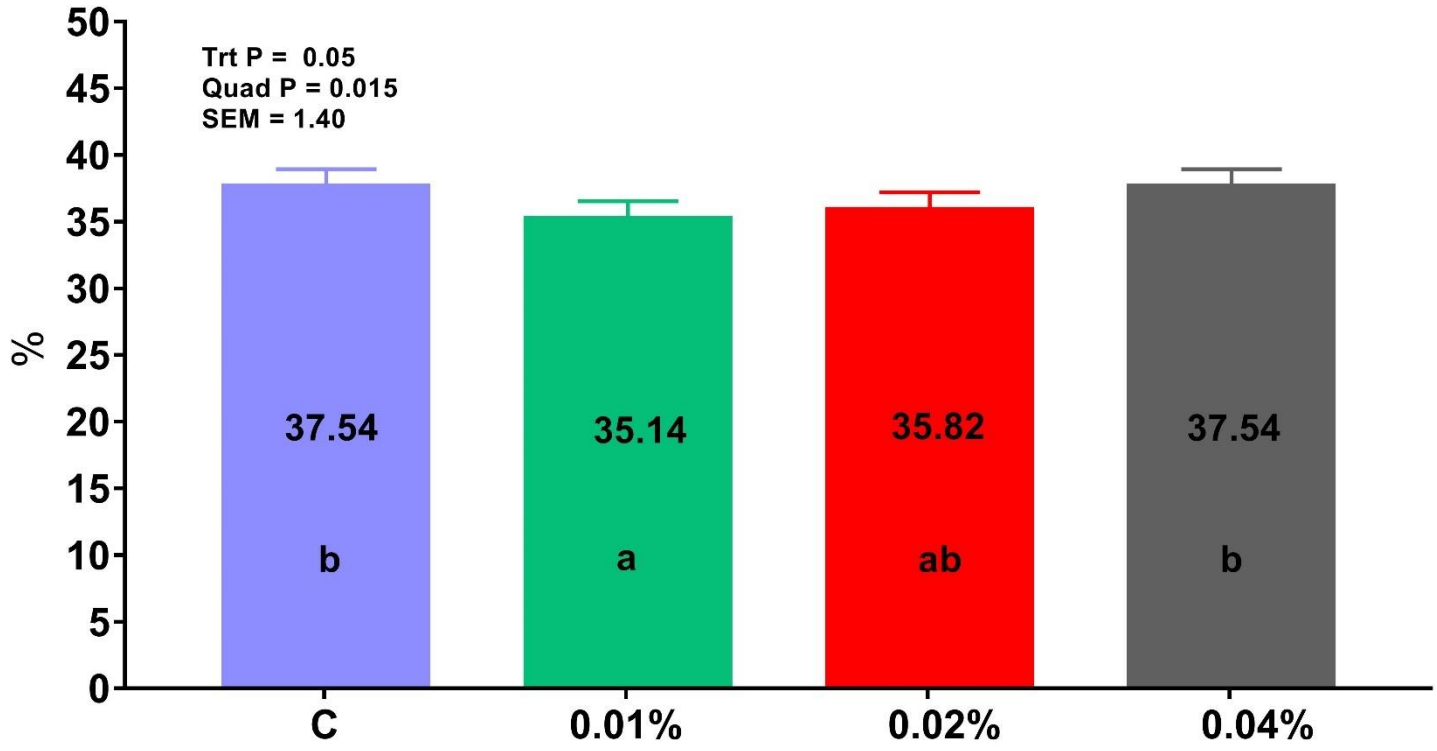

Figure 14. Effect of increasing level of dietary gossypol on hematocrit percentage of growing gilts (LS means).

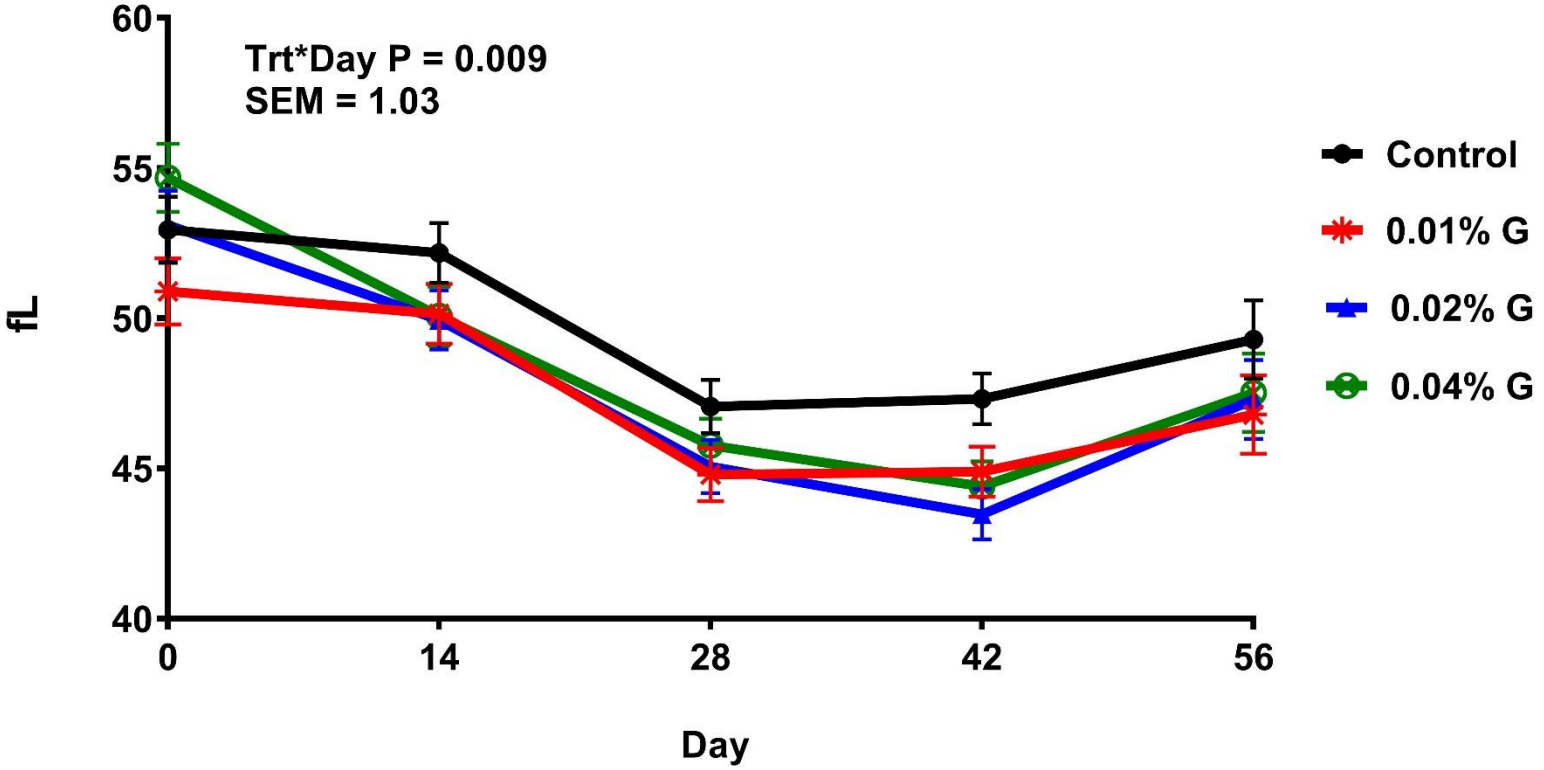

Figure 15. Effect of increasing level of dietary gossypol on MCV of growing gilts (LS means).

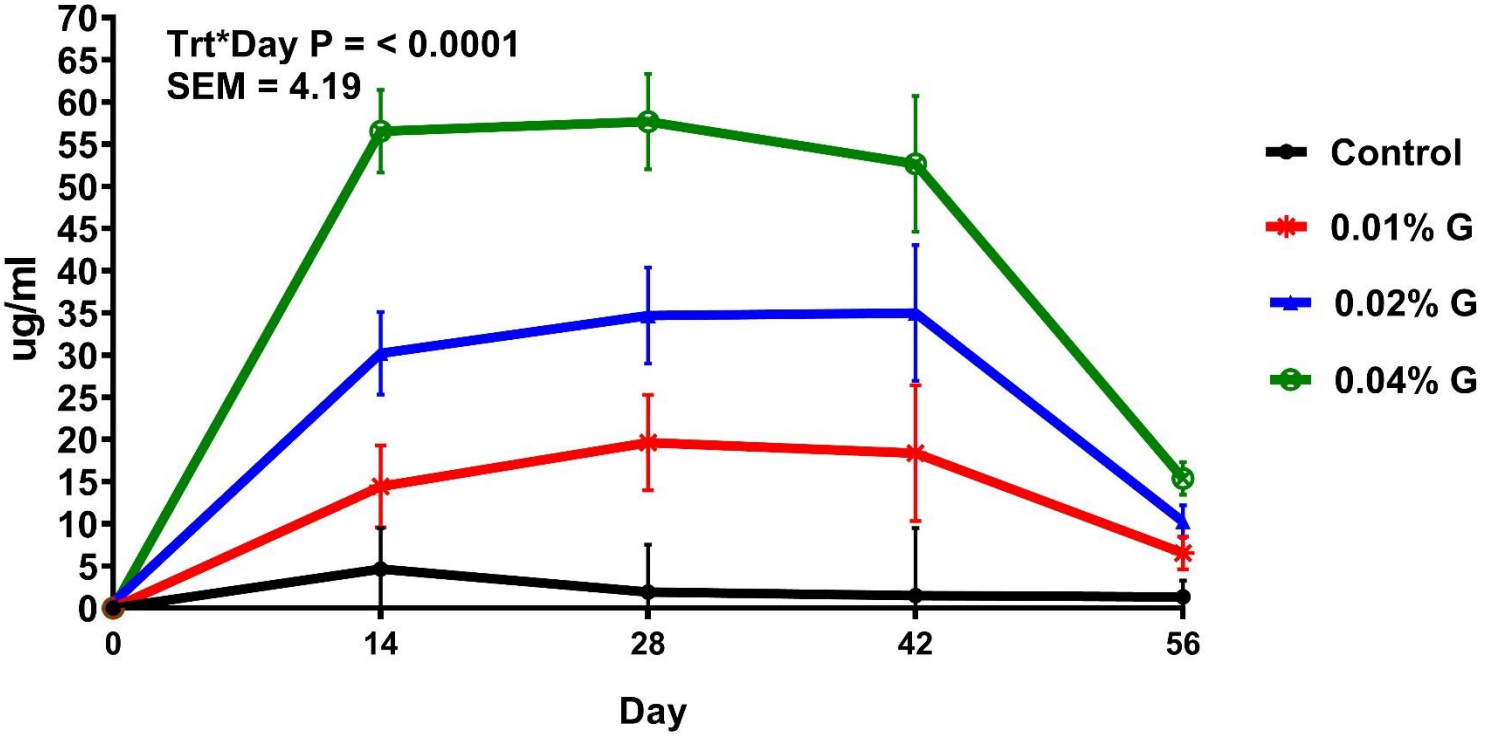

Figure 16. Effect of increasing level of dietary gossypol on plasma gossypol of growing gilts (LS means).

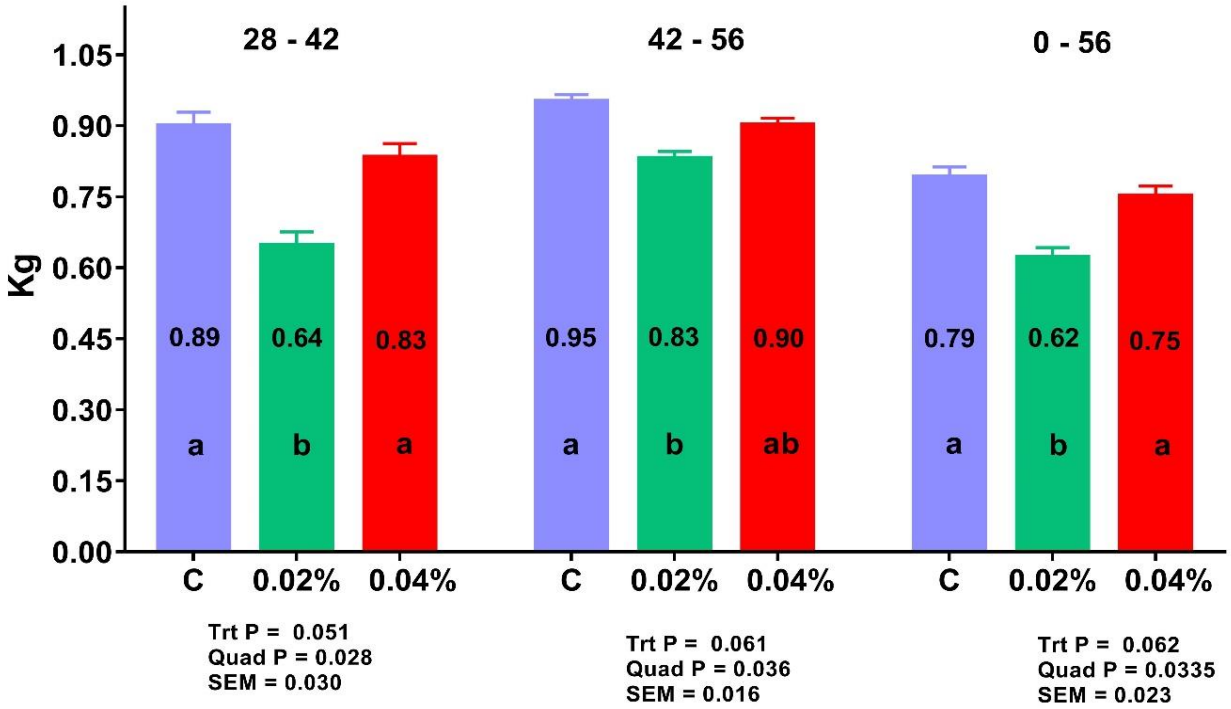

Figure 17. Effect of increasing level of dietary gossypol on ADG of growing boars in phase 3 (d 28-42), 4 (d 42-56) and overall (d 0-56), (LS means).

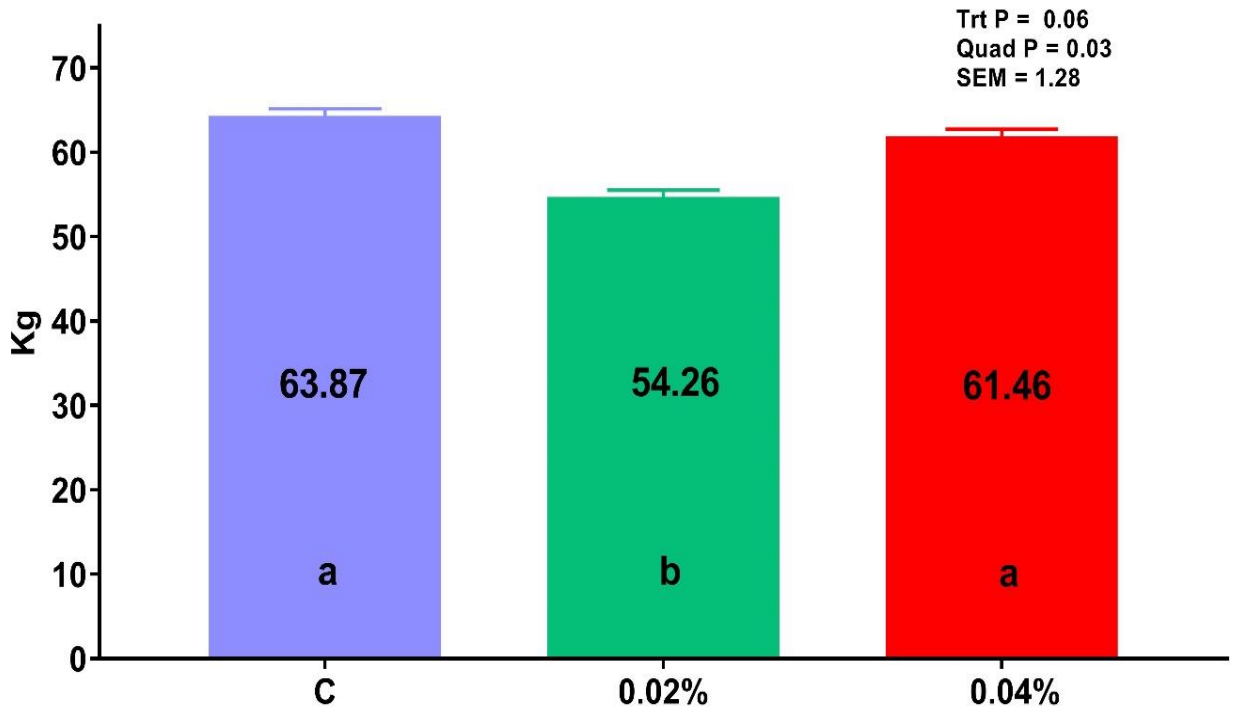

Figure 18. Effect of increasing level of dietary gossypol on BW of growing boars in overall (d 0-56) phase, (LS means).

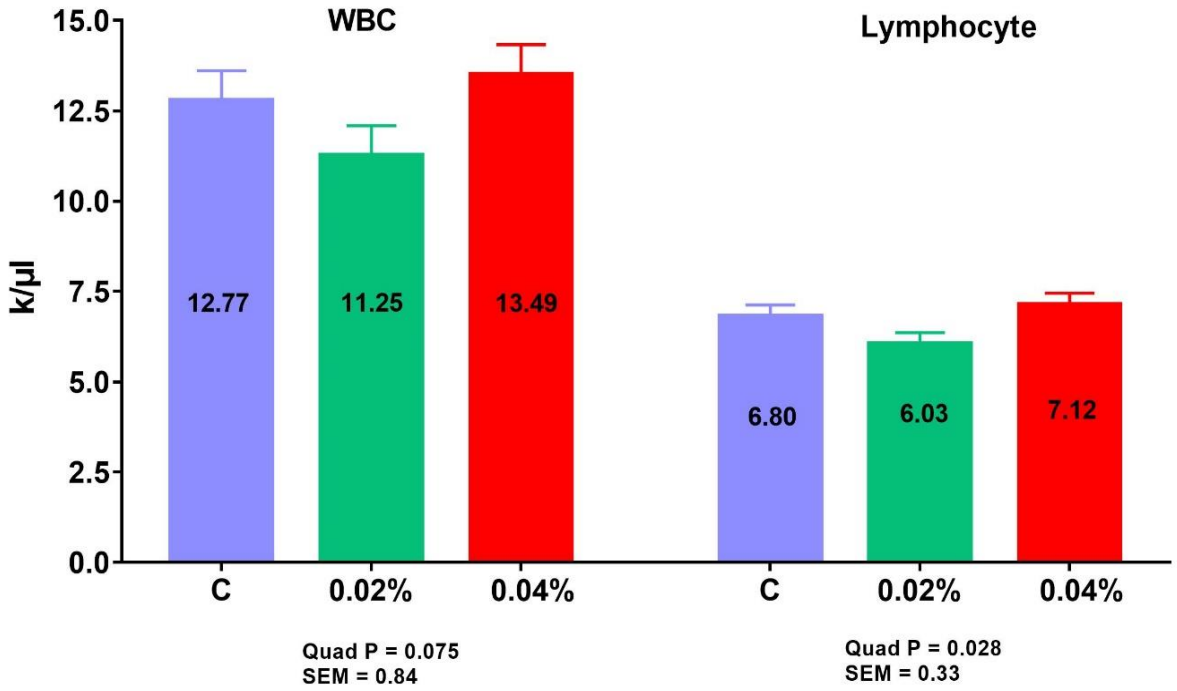

Figure 19. Effect of increasing level of dietary gossypol on WBC and lymphocyte concentration of growing boars (LS means).

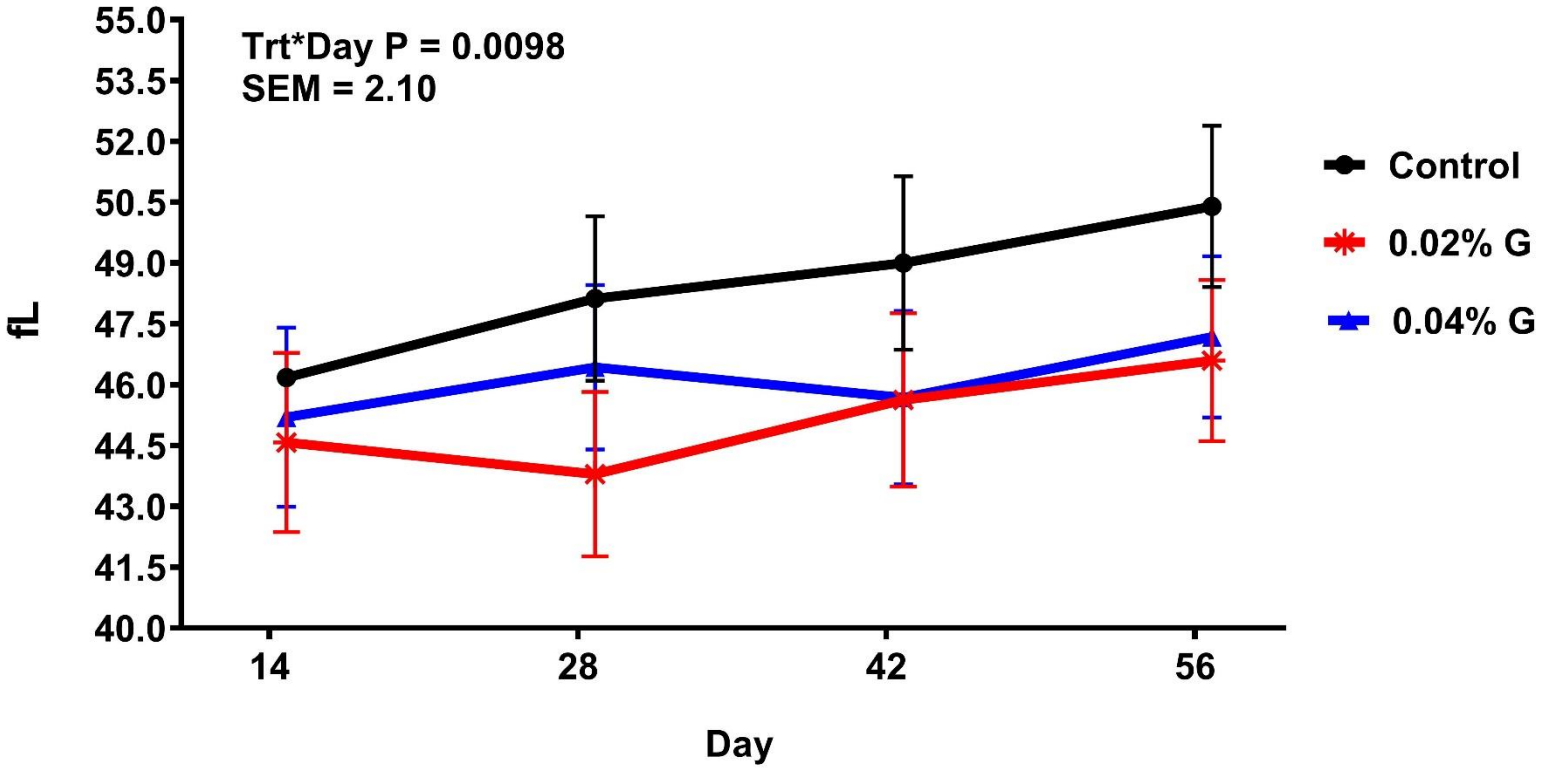

Figure 20. Effect of increasing level of dietary gossypol on MCV of growing boars (LS means).

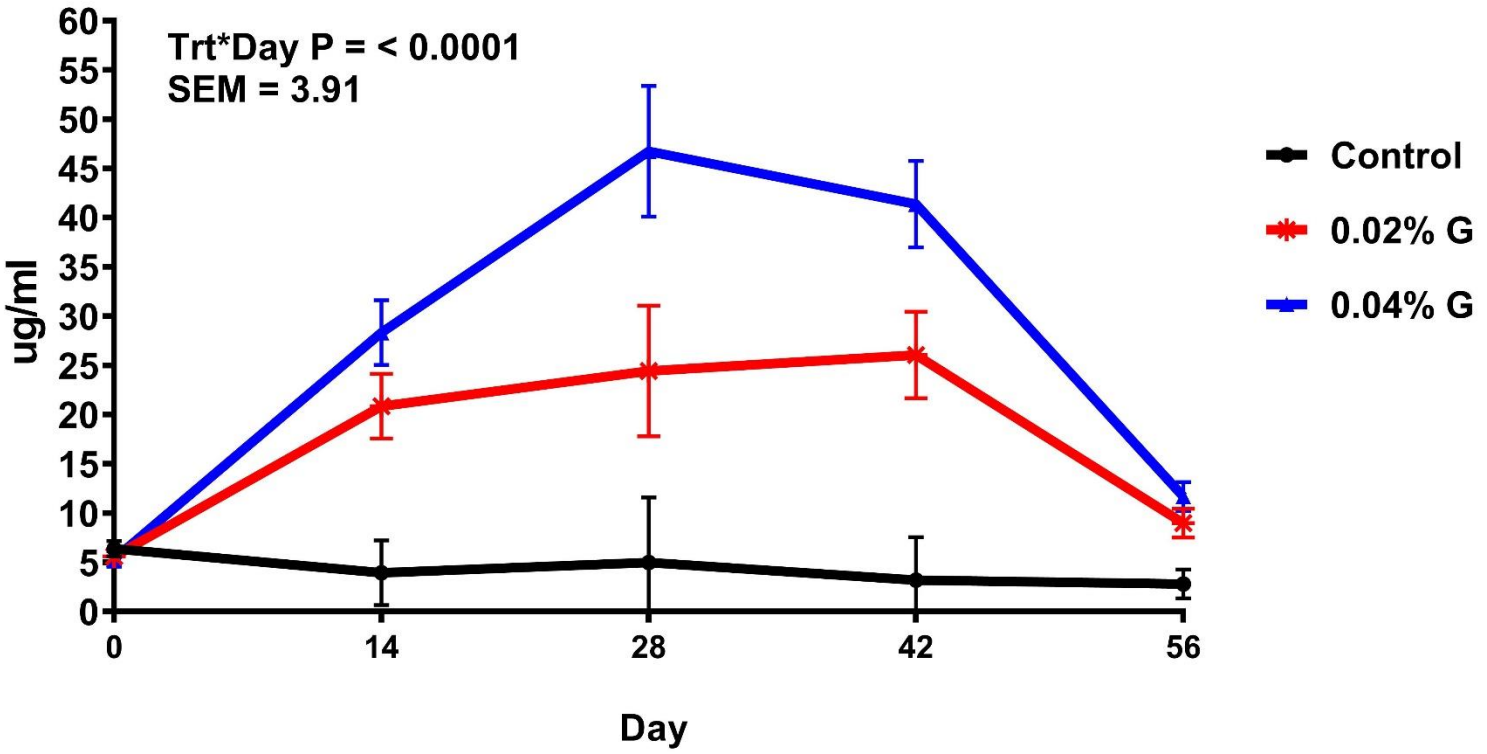

Figure 21. Effect of increasing level of dietary gossypol on plasma gossypol of growing boars (LS means).

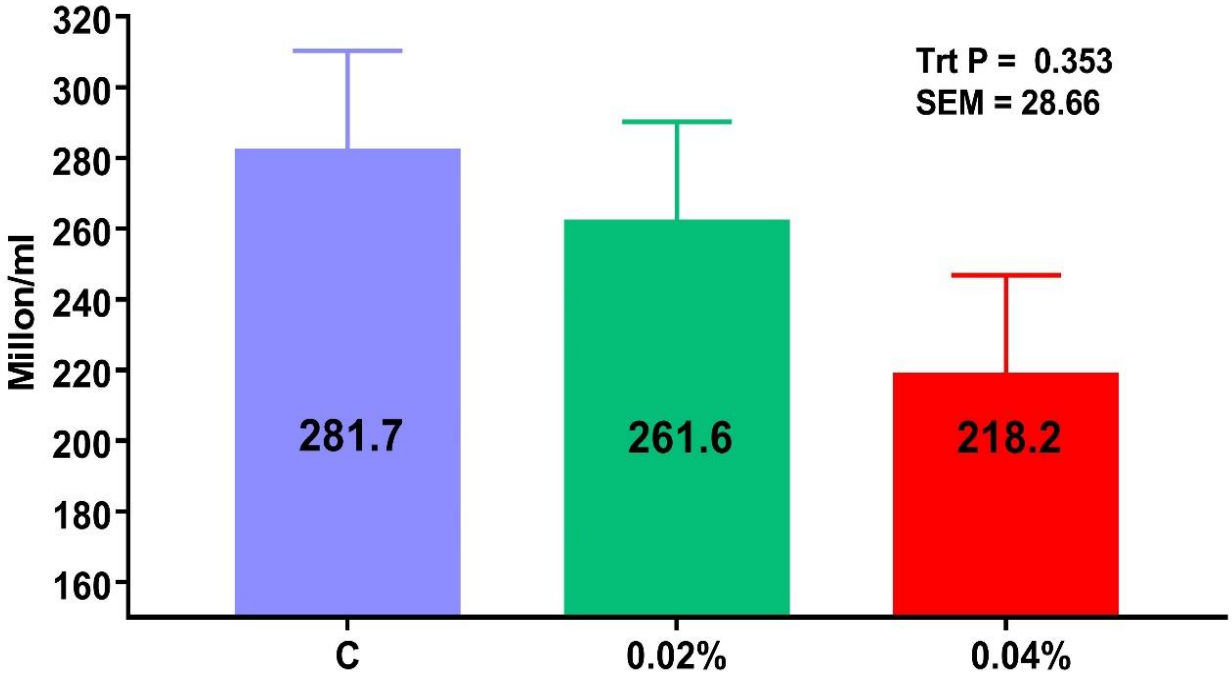

Figure 22. Effect of increasing level of dietary gossypol on sperm concentration/ml of boars (LS means).

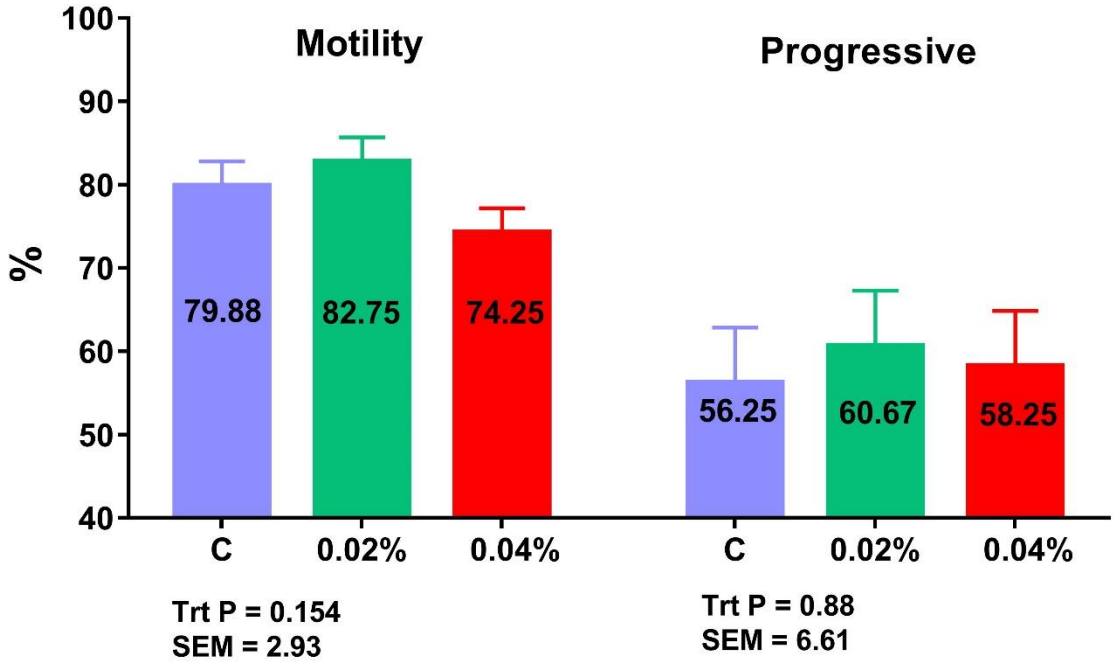

Figure 23. Effect of increasing level of dietary gossypol on percentage of motility and progressive sperms in boars (LS means).

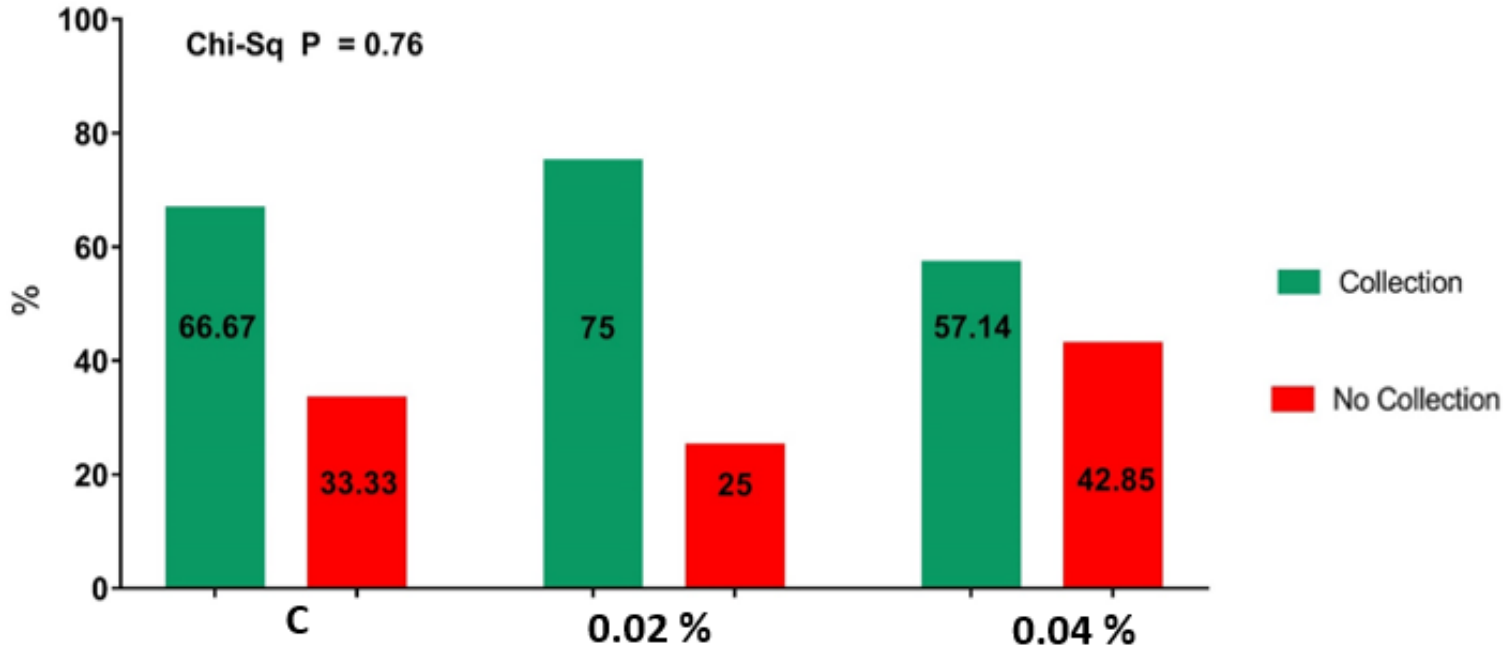

Figure 24. Effect of increasing level of dietary gossypol on pigs allowed collection or not (LS means).

Chapter 4: Conclusion

Feed additives such as bioactives peptides demonstrated in this study that it may be an alternative to replace high inclusion of zinc oxide in reduced protein level diets in nursery pigs without affecting their growth performance. Gossypol from cottonseed meal at inclusion levels up to 0.02% impaired growth performance and stimulated complete blood cell count in gilts and boars, but it was not enough to affect semen quality in boars later in life.

To: Rick Rorie
Fr: Billy Hargis - Ag-IACUC Chair
Date: January 2nd, 2020
Subject: IACUC Approval
Expiration Date: July 19th, 2021

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your personnel addition(s) of Richard Mudurra Hernandez to protocol # **20001:** *Evaluation of Feral Swine Control Measures Using Domestic Swine as a Model.*

In granting its approval, the Ag-IACUC has approved only the addition of the personnel listed. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the change.By policy the Ag-IACUC cannot approve a study for more than 3 years at a time.

The Ag-IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

BMH/tmp

Figure 25. IACUC Approval (Feral hog Control).

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Charles Maxwell
Fr:	Craig Coon
Date:	December 10th, 2019
Subject:	IACUC Approval
Expiration Date:	October 24th, 2022

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **20034:** *Effect on growth performance of adding Peptiva as a replacement for fish meal in nursery pigs - 2.*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond October 24th, 2022 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Charles Maxwell, Tsung-Cheng Tsai, Joshua Knapp, Chris Hart, Lensey Watson, Xiaofan Wang, Hayden King, Hannah Crabb, Brianna Freeze, Owen Hossack, Anita Maya, Elizabeth Bredlow, and David Price. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

Figure 26. IACUC Approval (Bioactive peptide).

To: Charles Maxwell
Fr: Billy Hargis - Ag-IACUC Chair
Date: March 4th, 2020
Subject: IACUC Approval
Expiration Date: October 24th, 2022

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your personnel addition(s) of Richard Mudurra to protocol # **20034:** *Effect on growth performance of adding Peptiva as a replacement for fish meal in nursery pigs - 2.*

In granting its approval, the Ag-IACUC has approved only the addition of the personnel listed. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the change.By policy the Ag-IACUC cannot approve a study for more than 3 years at a time.

The Ag-IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

BMH/tmp

Figure 27. IACUC Approval (Bioactive peptide).