ABSTRACT

ZHENG, LAN. Use of Functional Feed Additives to Enhance Intestinal Health and Growth of Nursery Pigs. (Under the direction of Dr. Sung Woo Kim).

Weaning causes morphological and functional changes of the small intestine of pigs where most of the nutrients are being digested and absorbed. Functional feed additives may promote growth of nursery pigs by enhancing nutrient digestion, intestinal morphology, immune status, and by restoring intestinal balance. The functional feed additives, such as fermented or enzyme-treated soy oligopeptides and soybean meal, lysophospholipids, and fermented rice bran extracts, were evaluated for their effects focused on intestinal health of pigs by conducting a total of 7 animal experiments.

Two experiments (Chapter 2) were conducted to evaluate effects of fermented soy oligopeptides (FSO) on growth performance, intestinal morphology, and crude protein digestibility in nursery pigs. In Exp.1, 120 pigs were allotted to 3 treatments (0.0, 1.0 or 2.0% of FSO). In Exp. 2, 40 pigs were allotted to 4 treatments (0.0, 0.5, 1.0, or 1.5% FSO). Results showed that FSO supplementation at 2.0% showed positive effect on reducing diarrhea incidence, however, negative effect was found on BW, whereas, FSO supplementation at 1.0% increased feed efficiency without affecting feed intake. Supplementation of FSO up to 1.5% may promote growth performance by enhancing intestinal morphology, and improving crude protein digestibility in a dose-dependent manner.

Two experiments (Chapter 3) were conducted to evaluate effects of enzyme-treated soy oligopeptides (ESO) on feed preference, growth performance, diarrhea incidence, intestinal morphology, and immune response of nursery pigs. In a 27-d feed preference study (Exp. 1), 24 pigs were allotted to 6 pens. Pigs were allowed to choose between two different diets (basal vs. 2.0% ESO diet). In Exp. 2, 128 pigs were allotted to 4 treatments (0.0, 1.0, 2.0, or 3.0% of ESO).

Results showed pigs preferred basal diet (0.0% ESO) instead of 2.0% ESO as the feeding day increased. Supplementation of ESO showed a potential to improve feed efficiency during d 11 to 32, however, ADG and ADFI were decreased with increasing levels of ESO during 32-d experimental period. ESO supplementation at the level of 0.74% may be most effective in improving the growth performance. Supplementation of ESO reduced diarrhea incidence without altering immune status, and may potentially enhance intestinal morphology.

Experiment 5 (Chapter 4) was conducted to evaluate the effects of enzyme-treated soybean meal (ESBM), fermented soybean meal (FSBM), and fermented soybean meal containing probiotic microorganisms (PFSBM) when replaced a conventional soybean meal (SBM) on diarrhea incidence, intestinal morphology, intestinal microbiota, and immune response in nursery pigs. Forty-eight pigs were allotted to 4 treatments. Results showed G:F of pigs fed ESBM diet was higher than that of pigs fed SBM diet during d 5 to 15, whereas, no differences in growth performance were found among the treatments during 27-d experimental period. The PFSBM inclusion increased mucosa-associated *Lactobacillus*, and it may can be considered as an effective carrier of probiotics.

Experiment 6 (Chapter 5) was conducted to evaluate the supplemental effect of lysophospholipid complex (LPL) on growth performance, fat utilization, and intestinal health in nursery pigs. Twenty-four pigs were allotted to 2 treatments (0.0 or 0.1 % LPL). Results showed LPL supplementation positively affected growth of nursery pigs and showed a potential to enhance lipid digestibility, and improved intestinal morphology and barrier function of nursery pigs.

Experiment 7 (Chapter 6) investigated effects of fermented rice bran extracts (FRBE) on growth performance, diarrhea incidence, intestinal morphology, mucosa-associated microbiota,

and immune response in nursery pigs. Thirty pigs were allotted to 3 treatments (0.0, 0.5, or 1.0% of FRBE). Results showed supplementation of FRBE may have beneficial effects on growth performance by enhancing morphology in a dose-dependent manner. Pigs fed 0.5% FRBE had distinctly different jejunal mucosa-associated microbiota from fed 0.0% FRBE diet.

Overall, functional feed additives tested in this study showed a potential to promote intestinal health and growth performance of nursery pigs.

Use of Functional Feed Additives to Enhance Intestinal Health and Growth of Nursery Pigs

by
Lan Zheng

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APPROVED BY:

_____	_____
Dr. Sung Woo Kim Committee Chair	Dr. Peter Ferket
_____	_____
Dr. Jeffrey Hansen	Dr. Jack Odle

BIOGRAPHY

Lan Zheng is a native of Liaoning, China. She attended Shandong Agricultural University, where she received a Bachelor of Science in Animal Science and a Bachelor of Arts in English Literature. She went to Konkuk University in South Korea to pursue a Master of Science in Poultry Nutrition. After her graduation, she worked as a technical marketing specialist at CJ CheilJedang Co., Seoul, South Korea. A part of her work was to design feeding trials and make marketing materials to support international amino acid sales. Through the industry experience, she was able to see that there is a strong need for a technical support in order to achieve better production of animal products. She decided to pursue a Ph.D. in Animal Nutrition at North Carolina State University. This has allowed her to learn and apply animal nutrition knowledge to solving animal production challenges. Her work was focused on the use of various functional feed additives on intestinal health and growth in pigs. She intends to continue her support of animal production in her professional career.

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CHAPTER 1. LITERATURE REVIEW

INTRODUCTION

Weaning is considered as one of the most critical periods in pig management. It is associated with environmental, social, and diet stress (Lallès et al., 2004), and those various stressors result in low feed intake, body weight loss, and a high incidence of diarrhea, which consequently, can lead to mortality (Hampson, 1986; Heo et al., 2013). Nowadays, weaning age on commercial farms are in the range of 3 to 4 weeks old, whereas pigs are naturally weaned at an age of 14 to 17 weeks (Jensen, 1986). Weaning under the commercial conditions, pigs are confronted by multiple changes such as separation from the sow to a new environment, relocation with new littermates, and most notably, pigs encounter the abrupt change of diet from liquid sow milk to solid feed. Post-weaning anorexia or inadequate feed intake after weaning result in insufficient dietary nutrients utilization and local inflammation (Pluske et al., 1997; McCracken et al., 1999). As a consequence, weaning causes profound changes in the gastrointestinal tract (GIT) of pigs. Intestine, an important part of GIT, is a major site of nutrient digestion and absorption. Intestinal disorders after weaning is caused by alterations in intestinal architecture and function and with mostly evident in villus atrophy and crypt hyperplasia and increased in intestinal permeability (Spreeuwenberg et al., 2001). Last but not least, intestinal microbiota disruption is possibly linked to diarrhea and pathogenic infections after weaning (Lallès et al., 2007; Gresse et al., 2017).

WEANING ASSOCIATED INTESTINAL FUNCTIONAL CHANGE

Morphological change

Enterocytes are composed of villi projecting into the lumen, and a folded cell monolayer structured into crypts. Villi are mainly lined by absorptive, goblet and enteroendocrine cells, and

the crypts are the main site of cell production containing stem cells, proliferative and undifferentiated cells, and a subset of differentiated secretory cells (Paneth, goblet and enteroendocrine cells). When stem cells divide, they are believed to go through a cell division into a new stem cell and a committed daughter cell (Potten, 1998). The differentiation and maturation of each cell type happens as the cells move either migrate up the crypt–villus axis (enterocytes, mucous, and enteroendocrine cells) or downwards to the bottom of the crypt (Paneth cells) (Umar, 2011). In the mammalian small intestine, active enterocyte proliferation is restricted to the crypts at the base of the villi (Cheng and Leblond, 1974). Stem cells in the crypts undergo cell division and differentiation to form mature absorptive enterocytes, mucus-producing goblet cells, and enteroendocrine cells, and those cells migrate along crypt-villus axis toward the villus tip, where they are exfoliated into the intestinal lumen (Wong and Wright, 1999).

After weaning, a consistent series of intestinal alterations occurs. Architectural alterations associated with weaning reported in previous studies are presented in Table 1. Villus height was shown to reduce to around 75% of pre-weaning values within 24 hours of weaning (Hampson 1986). The reduction in the villus height is a result of an increase in cell loss and/or reduction in crypt cell production (Hampson 1986). The villus atrophy and the reduction in crypt cell production during the post-weaning period result in loss of mature enterocytes, which causes decrease in nutrient absorption (Hedemann et al., 2003; Moeser et al., 2007). Reduced activity of brush-border enzymes, such as lactase and peptidases and nutrient transporters, have been observed to be correlated with shortened villus height (Hampson and Kidder, 1986; Tsukahara et al., 2013).

Barrier function

Tight junction proteins between epithelial cells form the barriers, which seals the paracellular space between epithelial cells regulating the paracellular permeability (Ulluwishewa et al., 2011). These proteins consist of transmembrane proteins occludin and claudins, as well as cytoplasmic proteins such as the zonula occludens (ZO) (Hartsock and Nelson, 2008). As a barrier between the luminal and basolateral compartments, tight junction proteins control the passive diffusion of ions and other small solutes, through the paracellular pathway (McKay and Baird, 1999). These tight junction proteins serving as a filter in order to allow important dietary nutrients, electrolytes, and water to translocate from the lumen of the intestine into circulation (Kunzelmann and Mall, 2002; Blikslager et al., 2007; Broer, 2008). Increases in intestinal permeability can result in inflammatory response by allowing the entry of toxins, allergenic compounds or bacteria (Asmar et al., 2002; Arrieta et al., 2006). Intestinal barrier function can be compromised by various factors, such as age, diet, pathogens, and diseases (Mullin et al., 2002; Sander et al., 2005).

Weaning induced impaired barrier function of epithelial cells promotes the entering of pathogenic bacteria and allergenic compounds from lumen into the body (Spreeuwenberg et al., 2001; Pié et al., 2004). Weaning causes compromised paracellular barrier function (Wijtten et al., 2011). Active absorption decreases when pigs are weaned at 3 weeks of age or earlier as a process of natural intestinal maturation stimulated by weaning; however, if pigs are weaned after 3 weeks of age, the active absorption is no more affected by weaning indicating weaning at an early age can disrupt barrier function (Wijtten et al., 2011).

Mucosal immunity at weaning

Up to 70% of the immune cells are localized in the mucosa and submucosa of the intestine (Castro and Arntzen, 1993; Furness et al., 1999). The gut-associated lymphoid tissue (GALT) consists of both isolated and aggregated lymphoid follicles forming Peyer's patches (PP) and mesenteric lymph nodes (Neutra et al., 2001). The induction of intestinal immune reactions starts with antigen presentation by microfold cells (M cells) (Mabbott et al., 2013). Lamina propria serves as a mucosal compartment for regulation of immune responses (predominantly IgA), with few T-cells or dendritic cells, but with myeloid cells with the characteristics of macrophages and granulocytes (Artis, 2008). The production of secretory antibodies, mostly secretory IgA and IgM, is the major defending characteristics of the mucosal immune system. These antibodies are actively transported by immature epithelial cells in the crypts and immune exclusion is carried out by the generated in cooperation with innate non-specific defense mechanisms (Brandtzaeg et al., 1999). Two important periods of maximum exposure to antigens occur, immediately after birth and at weaning. At weaning, the abrupt changes in the diet and environment induce alterations in the mucosal immune response (Gresse et al., 2017). Immune system in the intestine of pigs reaches adult-like structure at 7-wk-old age (Stokes et al., 2004). Conventionally, weaning of pigs is done in the range of 3 to 4 weeks old, when cytotoxic (CD8+) T cells are largely absent (Stokes et al., 2004). Weaning also affects the systemic development of innate and adaptive immunity largely as a consequence of the withdrawal of milk (Gallois et al., 2009). Up-regulated expression of pro-inflammatory cytokines are observed in pigs at weaning (Pié et al., 2004). Recent studies have shown that pro-inflammatory cytokines including tumor necrosis factor-α, interferon-γ, interleukin-1β induce disturbance in intestinal barrier and increase intestinal epithelial permeability (Al-Sadi et al., 2009; Capaldo and Nusrat, 2009). The

appropriate development of intestinal immune system is essential for optimum growth and performance of the pigs. Controlling the intestinal inflammation by the over expression of intestinal pro-inflammatory cytokines may alleviate subsequent intestinal disorders induced by the weaning stress.

Intestinal microbiota and intestinal health

In pigs, the hindgut is the major site of microbial fermentation, and the microbial population in the small intestine is less diverse than the hindgut (Kelly et al., 2017). The small intestine is a major place for nutrient absorption, and microbiota present in the outer mucosal layer of the small intestine are more susceptible to dietary influence (Levesque et al., 2012; Levesque et al., 2014). The small intestinal mucosa is frequently exposed to various exogenous antigens and microbial components from feed ingredients. Changes in mucosa-associated microbiota may have enormous effect on host growth and development. Most of the past studies are focused on the dietary intervention on luminal and fecal microbiota, few studies evaluated on mucosa-associated microbiota. Post-weaning dietary intervention showed a long lasting effect on ileal mucosa-associated microbiome, but not on that of the ileal digesta (Levesque et al., 2012). Microbial community within the outer layer of the mucosa is closely connected with host tissues, mucosa-associated bacteria are in direct competition with substrates with the host (Zoetendal et al., 2012). Distinct microbial populations present throughout the gastrointestinal tract due to the different physicochemical conditions and substrate availability (Zhao et al., 2015). Fecal microbiome is distinctly different from that of the luminal of the small intestine. The similarity index of fecal microbiome and luminal microbiome of the large intestine was 0.75, whereas it was only 0.38 when comparing fecal and luminal microbiome of the small intestine (Zhao et al., 2015). Mucosa-associated microbiota of cecum was distinctively different from that of the

digesta in the cecum (Kelly et al., 2017). From the outer mucosal layer into the lumen, a rapid declining oxygen gradient exists which generating distinct microenvironment between mucosal tissue and lumen (Albenberg et al., 2014). Mucosa-associated microbiota provides a line of defense against pathogens and modulates host's immune status (Brandtzaeg, 2007; Macfarlane et al., 2011; Mayer et al., 2015). The microbiota induces production of IgA by mucosal immune system which is secreted into the lumen to limit bacterial colonization and prevent penetration of bacteria through the epithelial layer (Benveniste et al., 1971; Macpherson et al., 2005; Brandtzaeg, 2007). Several studies have shown the effects of dietary intervention on microbiome composition in pigs. One of the most frequently employed product is prebiotics (Berding et al., 2016). Many studies focusing on prebiotic oligosaccharides such as fructooligosaccharides, galactonoligosaccharides, and mannanoligosaccharides, proved the link between prebiotics consumption and restoring intestinal balance (Castillo et al., 2008). Studies with fructooligosaccharides showed that supplementing with fructooligosaccharides caused a shift in intestinal microbial composition via modulating short chain fatty acids production, which provide substrates and promote normal proliferation and differentiation of intestinal cells (Rossi et al., 2005; Scott et al., 2014). At weaning, the abrupt changes in the diet and environment induce alterations in the intestinal microbiome (Konstantinov et al., 2006; Gresse et al., 2017). Reductions in *Lactobacilli* is one of the most evident change after weaning (Konstantinov et al., 2006). It was postulated the alterations in the composition and activity of the GIT microbial community is correlated with pathogenic infections after weaning (Hopwood and Hampson, 2003). A lower stability of microbial community structure was observed in ileal digesta of weaned pigs than that of unweaned pigs (Konstantinov et al., 2006). The intestinal bacterial community composition was shown to become stable at 6 month of age (Zhao et al., 2015).

FUNCTIONAL FEED ADDITIVES

To assist in overcoming the weaning-associated intestinal dysfunction and depressed growth, effective dietary strategies need to be explored. Feed additives including protein hydrolysates, emulsifiers, prebiotics, probiotics, feed enzymes, organic acids, and/or trace minerals are commonly used in the nursery pig diets to promote growth and intestinal health.

Protein hydrolysates

Protein hydrolysates are produced from a variety of protein sources by chemical, microbial or enzymatic hydrolysis to eliminate or reduce anti-nutritional factors (Pasupuleki and Braun, 2010). Typical protein hydrolysates used in animal diets are animal protein hydrolysates (such as salmon viscera and porcine intestines) and plant protein hydrolysates (such as soybean protein hydrolysates) (Hou et al., 2017). Through the production of protein hydrolysates, anti-nutritional factors are totally or partially hydrolyzed which make those hydrolysates a high quality protein source for nursery pigs (Zhu et al., 1998; Hong et al., 2004). Digestion of protein is mainly completed in the small intestine (Daniel, 2004). After weaning, decreased enzymatic activity of peptidases (aminopeptidase N and dipeptidylpeptidase IV) were detected (Hedemann et al., 2003). Improvements in crude protein digestibility by soy protein hydrolysates supplementation have been reported in nursery pigs (Kim et al., 2007; Zhou et al., 2011). Spray-dried plasma is a commonly used animal protein hydrolysate in nursery pig diets. It has been shown to increase growth performance (Pierce et al., 2005), enhance intestinal barrier function (Peace et al., 2011), and modify intestinal immune function (Nofrarías et al., 2007) when fed to newly weaned pigs. Additionally, some peptides derived from protein hydrolysis especially milk and soy protein possess various biological functions including antimicrobial, antihypertensive, and immunomodulatory activities (Nongonierma and FitzGerald, 2016).

Emulsifiers

Animal fats and vegetable oils are commonly added to meet energy concentration in the diet. In order to be absorbed in the gastrointestinal tract, dietary fat has to be emulsified by detergent action of the endogenous emulsifiers (such as bile salts) and hydrolyzed by lipase into fatty acids and mono- and diglycerides. Sow's milk contains approximately 40% fat on a dry matter basis (DeMan and Bowland, 1963; Hurley 2015); whereas, typical nursery diets include fat from 3 to 6% as a maximum level (Maxwell and Carter 2001). Digestibility of fat from sow's milk in suckling pigs is over 90%, however, digestibility of fat from solid feed in newly weaned pigs is as low as 73% (Frobish et al., 1967, 1969) and increases gradually return to the preweaning level ranging from 4 to 6 weeks postweaning (Wiseman, 1984; Cera et al., 1988). The form of the milk fat presents as micelles and consequently aid digestion (Salentinig et al., 2013) by pancreatic lipase, whereas, fat in solid diets is not in an easily accessible form. The synthesis of hepatic bile acid is low at weaning in pigs (Lewis et al., 2000), therefore, the emulsification process is a rate-limiting step in the digestion of dietary fat during this period.

Prebiotics

Prebiotics has been widely used for improving beneficial microbial populations in the intestines. The definition of prebiotics was first introduced by Gibson and Roberfroid (1995) as "Nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health." This concept has been refined during the past 20 years, and the recent definition to date was defined by Bindels et al. (2015) as "a prebiotic is a nondigestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host." Bindels et al.

(2015) indicated the metabolic benefits attributed to prebiotics do not require a selective fermentation, which was mentioned in the earlier concept. The revised definition instead focused on the concept of ecological and functional characteristics of the microbiota to be relevant for host physiology, such as ecosystem diversity, and the support of broad microbial consortia. Additionally, regardless of bacterial fermentation, prebiotic oligosaccharides (such as fructooligosaccharides and galactooligosaccharides) were shown to exert an anti-inflammatory effect or have anti-adhesive activity to inhibit binding pathogens (Quintero et al., 2011; Zenhom et al., 2011).

Probiotics

Probiotics is defined as "living microorganisms that, on ingestion in sufficient numbers, exert health benefits beyond basic nutrition". Prebiotics and probiotics exert their beneficial effects in a similar manner, through the modulations in the intestinal microbiota. Probiotics affect the microbiota via beneficial microorganisms, whereas prebiotics alter the microbiota by the supply of a substrate. Cultures commonly used in feed are lactic acid bacteria, *Bacillus* and yeasts (Simon, 2005). The beneficial microbes play an important role in maintaining the host health. They reduce the colonization and invasion of pathogens, maintain epithelial integrity, and enhance immune function (Ouwehand et al., 1999; Herich and Levkut, 2002). Probiotics used in pig diets showed beneficial effects including reduced diarrhea incidence and improved in growth performance (Simon, 2005; Lallès et al., 2007). The combination use of prebiotics and probiotics as synbiotics beneficially affects microenvironment of the intestines to improve the survival and colonization of live beneficial microorganisms in the GIT (Pandey et al., 2015).

Feed enzymes

The major goal of the use of feed enzymes is to eliminate anti-nutritional factors to better utilize nutrients in the feed (Simon, 1998). Carbohydrase has been widely used for their roles in breaking down non-starch polysaccharides (NSP) present in most of the vegetable ingredient (Kim et al., 2003; Ao et al., 2011). The use of NSP enzymes showed to improve feed efficiency, and energy and amino acid digestibility in nursery pigs (Kim et al., 2003). Protease breaks down peptide bonds in protein and polypeptides were shown to increase protein digestibility and feed efficiency in nursery pigs (Tactacan et al., 2016). Another commonly used feed enzyme is phytase. Phytase catalyzes the phytate hydrolysis and releases phosphorous and phytate-bound nutrients (Yin et al., 2007). The use of phytase increased phosphorus digestibility, bone characteristics, and growth performance (Kies et al., 2006; Yáñez et al., 2013).

Organic acids

Organic acids have been used in the pig diets to decrease gastric pH (Thompson and Lawrence, 1981), prevent pathogenic bacterial growth (Risley et al., 1992), improve nutrient digestion (Blank et al., 1999), and improve growth performance (Partanen and Morz, 1999). Gastric pH in weaned pigs are in the range between 2.6 and 5.0, whereas, the optimum gastric pH for vegetable protein digestion is in the range of 2.0 to 3.5. Inclusion of organic acids such as fumaric and citric acids are shown to have beneficial effects when fed to newly weaned pigs (Falkowski and Aherne, 1984; Henry et al., 1985).

Trace minerals

Zinc, copper, iron, manganese, selenium, and iodine are commonly included in trace mineral premixes for pig diets. In general, zinc oxide is added to the nursery pig diets to prevent diarrhea and to promote growth performance (Poulsen, 1995; Case and Carlson, 2002; Shelton et

al., 2011). According to NRC 1998 and 2012, weaned pigs require 100 mg Zn/kg of diet, however, a high dosage of Zn (up to 3000 mg/kg) have been added to prevent diarrhea after weaning (Katouli et al., 1999; Højberg et al., 2005). Zinc oxide supplemented at 2500 mg/kg showed a lower bacterial activity in digesta of weaned pigs when compared to those fed 100 mg/kg (Højberg et al., 2005). Although it can reduce diarrhea and improve growth performance, the usage of high dosage of zinc oxide is restricted in some areas because of the environmental concerns (Jondreville et al., 2003).

SELECTED FUNCTIONAL FEED ADDITIVES

Considering the scope of current research, only soy protein hydrolysates produced from fermented or enzymatic hydrolysis (protein hydrolysate), lysophospholipids (emulsifier), and fermented rice bran extracts (prebiotics) will be further explored.

Soy protein hydrolysates

Soybean meal is one of the most commonly used ingredients in animal feed, however, digestive disturbances are often observed when it is fed to young animals especially newly weaned pigs (Li et al., 1990; Baker, 2000). Soybean meal contains various anti-nutritional factors including trypsin inhibitors, lectins, indigestible carbohydrate complexes, and soybean globulins (Li et al., 1990; Hong et al., 2004; Singh et al., 2015). Trypsin inhibitors and lectins can be inactivated by a proper heat treatment and fat extraction (Baker, 2000; Lallès, 2000). However, the presence of indigestible carbohydrate complexes, antigenic soybean globulins, and residual trypsin inhibitor limits its use in young pig diets (Li et al., 1990; Li et al., 1991; Lallès, 2000). Glycinin and β-conglycinin, antigenic proteins, are the major anti-nutritional factors cause allergic responses in young animals (Miller et al., 1984; Li et al., 1990). These proteins can cause

hypersensitivity that induce abnormal intestinal morphological change and diarrhea when fed to young pigs (Li et al., 1990; Dréau et al., 1994; Sun et al., 2008). Fermented soybean meal using microorganisms such as *Aspergillus oryzae*, *Bacillus subtillis*, and *Lactobacillus casei* and enzyme-treated soybean meal are showed to have reduced anti-nutritional factors and increased concentrations of CP and AA than conventional soybean meal (Cervantes-Pahm and Stein, 2010; Kim et al., 2010). Through the microbial fermentation or enzymatic treatment of soybean meal, the antigenic proteins are hydrolyzed into small size peptides and the glycosidic bonds in the carbohydrate fraction in soybean meal are broken down by enzymes produced by fungus and bacteria, or by a mixture of enzymes (Zhu et al., 1998; Middelbos and Fahey, 2008). Fermented and enzyme-treated soybean meal have been shown to improve growth performance and feed efficiency of nursery pigs when partially replaced conventional soybean meal in the diets (Kim et al., 2010; Yuan et al., 2017). Soy oligopeptides, a soy protein hydrolysate, was shown to improve amino acid absorption compared to an intact soy protein or corresponding amino acid mixtures in a human study (Maebuchi et al., 2007). Amino acid absorption in the portal blood from a soy protein hydrolysate was more efficient than the constituent amino acids from an amino acid mixture and those from an intact soy protein in rats (Kodera et al., 2006). In addition, enhanced intestinal morphology was observed when fed soy protein hydrolysates to nursery pigs (Yun et al., 2005; Zhu et al., 2017).

Lysophospholipids

Phospholipids, nature's principal surface-active agents, performs as an excellent emulsifying agent. Main constituents of the phospholipid mixture are phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, and phosphatidic acid (Li et al., 2015). The majority of the phospholipid in the small intestine is derived from bile with a smaller component

coming from the diet. Phospholipase A_2, a pancreatic enzyme secreted in bile, hydrolyzes the ester bond at the sn-2 position of the phospholipid, yielding a free fatty acid and lysophospholipids with different head group, which are then incorporated into micelles for subsequent absorption (Wendel, 2000; Joshi et al., 2006; Van Nieuwenhuyzen and Tomas, 2008). On a commercial scale, lysophospholipids are often produced by the modification of soybean phospholipids (chemical or enzymatic methods) using phospholipase A_2 which yields a mixture of lysophospholipids with different head groups depending on the source of the phospholipids (e.g. lysophosphatidylcholine, lysophosphatidylinositol, lysophosphatidylethanolamine, and lysophosphatidic acid) (Wendel, 2000; Cabezas et al., 2015). Hydrophilic-lipophilic balance (HLB) values are assigned to emulsifiers from 0 to 20, higher values are assigned to those are more hydrophilic. Soybean lysophospholipids have HLB value of 19 (Pokorný, 2006), whereas the native soybean phospholipids have values of 5 (Estiasih et al., 2013). In addition, lysophospholipids have been reported to involve in various biological processes such as cell growth, proliferation and differentiation mediated by specific G-protein coupled receptors (Moolenaar 1999; Gardell et al., 2006; Choi and Chun, 2013). Lysophospholipids supplemented in the diet showed to increase crypt cell mitosis and enhance villus morphology in broiler chickens (Boontiam et al., 2017).

Fermented rice bran extracts

Rice bran, a co-product obtained during rice milling process, is rich in cell wall materials such as hemicellulose and cellulose containing neutral detergent fiber in the range of 19 to 34% (Shi et al., 2015). The high fiber content is a major limitation of its use in young animal diets especially in newly weaned pigs. Fermentation and enzymatic treatment (Tangendjaja, 1993; Kompiang et al., 1995; Wizna et al., 2012) have been applied to improve rice bran nutritional

value. Prebiotic properties of rice bran were reported in the studies with mice (Henderson et al., 2012) and pigs (Herfel et al., 2013). Glucooligosaccharides, one of the emerging prebiotics was shown to be assimilated by *Bifidobacterium* species, but not by pathogenic species including *Clostridium* and *Salmonella* (Monsan and Paul, 1995). Rice bran oligosaccharides, mainly composed of glucooligosaccharides, was reported to possess prebiotic potential (Kurdi and Hansawasdi, 2015). The rice bran glucooligosaccharides was shown to be able to promote the growth of *Lactobacillus* species, which was not hydrolyzed by human intestinal conditions.

SCOPE OF CURRENT RESEARCH

At weaning, pigs are confronted by multiple stressors such as separation from the sow, a new environment, separation from littermates and cohabitation with new pigs, and the abrupt change of diet from liquid sow milk to solid feed. Weaning causes morphological and functional changes of the small intestine of pigs where most of the nutrients are being digested and absorbed. These changes can result in severe diarrhea and even cause mortality.

In order to minimize weaning-associated depressed growth, the need for developing effective dietary strategies is critical. Functional feed additives which have positive influence on enhancing intestinal health will aid in amelioration of the depressed growth and intestinal dysfunction associated with weaning. The functional feed additives such as fermented soy oligopeptides, enzyme-treated soy oligopeptides, fermented soybean meal, enzyme-treated soybean meal, lysophospholipids, and fermented rice bran extracts were evaluated their roles in promoting intestinal health and growth of nursery pigs to allow a better nutritional management during the crucial postweaning period. The evaluations on how these feed additives affect intestinal architectural structure, intestinal barrier function, mucosal immunity, and intestinal microbial community can provide valuable information to formulate optimized nursery diets.

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Table 1. Intestinal morphological changes of pigs after weaning

Weaning age (days)	Intestinal section	Results	References
21	Small intestine	Decreased villus height and increased crypt depth during d 11 postweaning	Hampson (1986)
21 or 35	Jejunum	Decreased villus height during d 3 postweaning when weaned at 21 or 35 days	Cera et al. (1988)
14	Small intestine	Decreased villus height to crypt depth ratio at d 7 postweaning	Kelly et al. (1991)
28	75% of small intestine	Increased crypt depth at d 5 postweaning	Pluske et al. (1996)
26	Small intestine	Decreased villus height at d 2 and 4 and decreased villus height to crypt depth ratio at d 2 and 4 postweaning	Spreeuwenberg et al. (2001)
29	Jejunum	Decreased villus height from d 2 postweaning with minimal length was observed at d 3 postweaning and increased crypt depth at d 5 postweaning	Hedemann et al. (2003)
21	Jejunum and ileum	Decreased villus height at d 4 postweaning in both the jejunum and ileum, decreased villus to crypt depth ratio in the ileum and increased crypt depth in the ileum; Increased crypt depth at d 11 postweaning in the jejunum and in the ileum and decreased villus height to crypt depth ratio in the ileum	Moeser et al. (2012)

CHAPTER 2. EFFECTS OF FERMENTED SOY OLIGOPEPTIDES ON GROWTH PERFORMANCE, DIARRHEA INCIDENCE, INTESTINAL MORPHOLOGY, AND APPARENT ILEAL DIGESTIBILITY OF CRUDE PROTEIN IN NURSERY PIGS

ABSTRACT: Two experiments were conducted to evaluate the effects of fermented soy oligopeptides (FSO, soy protein hydrolysates) on growth performance, diarrhea incidence, intestinal morphology, and apparent ileal digestibility of crude protein in nursery pigs. In Exp.1, 120 pigs (60 barrows and 60 gilts at 5.62 ± 0.10 kg BW) were randomly allotted to 3 treatments in a randomized complete block design (RCBD) with sex and initial BW as blocks. Pigs were fed a basal diet supplemented with FSO at the level of 0.0, 1.0, or 2.0% based on 2 phases (11 and 21 d, respectively). BW and feed disappearance were recorded at the end of each feeding phase. Fecal scores were recorded for the first 14 d to measure diarrhea incidence. On d 32, blood samples were collected for immune response analysis. In Exp. 2, 40 pigs (20 barrows and 20 gilts at 5.33 ± 0.10 kg BW) were randomly allotted to 4 treatments in a RCBD with sex and initial BW were used as blocks. Pigs were fed a basal diet supplemented with FSO at the level of 0.0, 0.5, 1.0, or 1.5% based on 3 phases (7, 10, and 10 d, respectively). BW and feed consumption were recorded on d 7, 17, and 27. In Exp. 1, pigs fed 1% FSO tended to have a higher ($P = 0.054$) ADG than those fed the 2.0% FSO diet, and had a higher ($P < 0.05$) G:F than those fed the basal diet and 2.0% FSO diet during the entire 32 d. Pigs fed 2.0% FSO tended to have a lower ($P = 0.058$) ADFI than those fed the basal diet, and had a lower ($P < 0.05$) BW than those fed the basal diet and 2.0% FSO diet at d 32 postweaning. Supplementation of FSO up to 2.0% reduced ($P < 0.05$) diarrhea incidence. In Exp. 2, increasing supplementation of FSO changed (quadratic, $P < 0.05$) G:F during phase 1 and tended to change ADG (quadratic, $P = 0.056$) and ADFI (quadratic, $P = 0.084$) during phase 2. Increasing levels of FSO tended to change ($P = 0.083$) villus height in the duodenum and changed (quadratic, $P < 0.05$) that in the jejunum. Increasing levels of FSO changed (quadratic, $P < 0.05$) Ki-67 positive cells in the duodenal crypts. Increasing levels of FSO decreased (linear, $P < 0.05$) tumor necrosis factor-α in

serum and tended to reduce (linear, $P = 0.098$) that in the jejunal mucosa. Increasing levels of FSO tended to change (quadratic, $P = 0.074$) interleukin-10 in the duodenal mucosa. In conclusion, the FSO supplementation up to 2.0% showed positive effect on reducing diarrhea incidence, however, negative effect was found on BW. The FSO supplementation at 1.0% increased feed efficiency without affecting feed intake. Supplementation of FSO up to 1.5% showed a potential to promote growth performance, enhanced intestinal morphology, and improved crude protein digestibility in a dose-dependent manner.

Key words: fermented soy oligopeptides, growth performance, intestinal health, nursery pigs

INTRODUCTION

Soybean meal is the most commonly used protein source in the animal feed industry (Baker, 2000). However, it contains a variety of anti-nutritional factors, such as trypsin inhibitor, lectins, indigestible carbohydrate complexes, and soybean globulins (glycinin and β-conglycinin), which have limited the application of its use in the diets for young animals, especially newly weaned pigs (Singh et al., 2015). Heating process and fat extraction can remove most of the trypsin inhibitor and lectins (Baker, 2000; Lallès, 2000), however, indigestible carbohydrate complexes, antigenic soybean globulins, and residual trypsin inhibitor can cause digestive disturbances and inhibit growth when fed to young pigs (Li et al., 1990; Li et al., 1991; Lallès, 2000). Soy protein hydrolysates are produced by enzymatic, or microbial hydrolysis to eliminate or reduce anti-nutritional factors in soybean meal (Pasupuleki et al., 2010; Hou et al., 2017). Fermented soybean meal has been used as a feed source for its high profile of amino acid and reduced anti-nutrient factors (Hong et al., 2004; Cervantes-Pahm and Stein, 2010; Kim, 2010). Kim et al. (2007) showed pigs fed fermented soybean meal can improve villus height and crypt depth after weaning compared with pigs fed conventional soybean meal. In addition, Kodera et al. (2006) showed amino acids absorption in the portal blood from soy protein hydrolysates was more efficient than the constituent amino acids from an amino acid mixture and those from an intact soy protein in rats. The potential reason would be explained by the advantage in absorption in the intestines than the supplementation of intact protein and free amino acids (Adibi, 1971; Steinhardt and Adibi, 1986). Thus, small size peptides supplementation into a diet would enhance animal growth and development. Furthermore, in previous studies, soy peptides supplementation enhanced immune status such as the modulation

of immune systems by immune-modulatory peptides in mice (Tsuruki et al., 2003) and in pigs (Young et al., 2012).

Weaning of pigs causes profound changes in intestinal morphology and function. Villus atrophy and crypt hyperplasia, and increases in intestinal permeability are mostly evident at weaning (Spreeuwenberg et al., 2001). In addition, immune function can be altered at weaning (Gallois et al., 2009). Up-regulated expression of pro-inflammatory cytokines are observed in pigs after weaning (Pié et al., 2004).

The fermented soy oligopeptides used in the present study was produced by fermentation treatment of soybean meal using *Bacillus subtilis*. Previous studies showed the fermentation process enabled soy proteins degraded into smaller peptides and reduced or eliminated antigenic soybean globulins (Hong et al., 2004; Kwon et al., 2011). It was hypothesized that fermented soy oligopeptide (FSO) supplementation would be useful as a supplement to nursery diets to promote protein digestion, intestinal morphology, and improve growth performance. Therefore, the objective of this study was to evaluate the effects of FSO on growth performance, intestinal morphology, crude protein digestibility, and immune response of nursery pigs.

METERIALS AND METHODS

The experimental protocol was approved by the Institutional Animal Care and Use Committee at North Carolina State University (Raleigh, NC).

Fermented Soy Oligopeptides (FSO)

The FSO, a soy protein hydrolysates, produced by fermentation of soybean meal was obtained from Yunong, Co. Ltd, China. Briefly, soybean meal was steamed at 120°C for 30 min, cooled to 36°C before inoculation with *Bacillus subtilis* and incubated at 36°C for 24 h. After the

enzymatic reaction, the peptide solution was filtrated and the supernatant was sterilized and spray dried. The molecular weight of FSO was analyzed using an analytical ultracentrifuge (ProteomeLab XL-A, Beckman Coulter, Fullerton, CA) according to the method described by Schuck et al. (2002). Briefly, all interference data were collected at a speed of 40,000 rpm in an An-60 Tirotor at 4°C. Homogenized samples were centrifuged at 10,000 rpm for 15 minutes at 4°C (Eppendorf MiniSpin, Eppendorf AG, Hamburg, Germany). A set of 200 scans was collected at 6-min intervals. The proteins were prepared with 2 mL of 20 m*M* Tris-HCl buffer including 0.1% sodium dodecyl sulfate, and 5 m*M* dithiothreitol at pH 7.4. Aliquots (110 uL) of sample solution were loaded into 6 sector sample cells. Absorbance was monitored at 280 nm for the loaded samples. Sedimentation velocity data were analyzed using the software program SEDFIT (version 11.8) (National Institutes of Health, Bethesda, MD) to generate the sedimentation coefficient distribution of protein samples.

Experiment 1

Experimental Diets and Pigs

A total of 120 pigs (PIC 337 x Camborough 22) (60 barrows and 60 gilts at 5.62 ± 1.0 kg BW) were used. Pigs were grouped by BW and sex into five blocks and randomly assigned to three treatments with four pigs per pen and ten replicate pens per treatment in a randomized complete block design. Pigs were housed in pens (4.0 × 1.4 m) equipped with a feeder and a nipple drinker. Pigs had free access to feed and water. FSO was added at the level of 0.0, 1.0, or 2.0% into a basal diet (Table 2) to replace the equal amounts of soybean meal in the diets. All diets were made to meet or exceed requirement estimates for nursery pigs (NRC, 2012). The experimental period was divided into 2 phases (11 and 21 d, respectively). Individual BW and feed disappearance of each pen were recorded at the end of each feeding phase.

Diarrhea Incidence

The incidence of diarrhea of piglets were recorded every day for the first 14 d of the experimental period. Pig feces were scored according to the modified method of Liu et al. (2010). Fecal scores were 1, normal, firm feces; 1.5, possible slight diarrhea; 2, moderate liquid consistency; 2.5, definitely unformed and fluid feces; 3, very watery and frothy feces. The occurrence of diarrhea was defined as maintenance of feces at Level 2.5 or Level 3 for two continuous days. Diarrhea incidence was calculated according to the formula reported by Sun et al. (2008): diarrhea incidence (%) = number of pigs with diarrhea/(number of pigs × total experimental days) × 100, where "number of pigs with diarrhea" was the total number of pigs with diarrhea observed each day, and "total experimental days" was 14 d.

Immune Response Parameters

At d 32, blood (7 mL) was taken from the jugular vein with BD sterile vacutainers (BD, Franklin Lakes, NJ) for obtaining serum. Blood samples were centrifuged at 3,000 × g for 15 min at 4°C to obtain the supernatant. The serum samples were stored at -80°C until analyzed for immune responses. Tumor necrosis factor-α (TNF-α) was measured in serum according to the method described by the manufacturer using a Porcine TNF-α Colorimetric ELISA kit (Pierce Biotechnology, Rock- ford, IL). Briefly, 50 μL of standard plus dilute or 100 mL of sample was added to microplate wells which were already coated with capture antibody in conjunction with biotinylated antibody reagent. Detection occurred by the use of a color reagent substrate and a stop solution of diluted hydrochloric acid, and absorbance was read at 450 nm and 540 nm. The measured protein concentration was used to determine the amounts of immunological subset per gram or milligram of protein of each tissue type. The immunoglobulin subset immunoglobulin G

(IgG) was measured according to the method described by the manufacturer using ELISA kit (Bethyl, Montgomery, TX).

Experiment 2

Experimental Diets and Pigs

A total of forty pigs (PIC 337 x Camborough 22; 20 barrows and 20 gilts at 5.33 ± 0.10 kg BW) were randomly allotted to 4 treatments in a randomized complete block design. Sex and initial BW were used as blocks. Pigs were housed individually in pens (0.7 × 1.5 m) equipped with a feeder and a nipple drinker. Pigs had free access to feed and water. A corn-soybean meal-based diet was formulated to meet or exceed requirement estimates for nursery pigs (NRC, 2012). Experimental diets were made by adding FSO at the level of 0.0, 0.5, 1.0, or 1.5%, respectively, to the basal diet (Table 5). The experimental period was divided into 3 phases (7, 10, and 10 d, respectively). Individual BW and feed disappearance of each pen were recorded at the end of each feeding phase.

Intestinal Morphology and Proliferation of Enterocytes

Duodenum and jejunum samples were rinsed with a 0.9% saline solution, fixed with a 10% buffered formalin solution immediately after slaughter. Tissue samples then embedded in paraffin (Paraplast-Sigma) and paraffin sections of 5 µm thick tissue were taken on rotary microtome. The sections were stained with haematoxylin and eosin. Villus height, villus width, and crypt depth of duodenum and jejunum were measured at 40 × magnification under an Infinity 2-2 digital CCD camera attached to an Olympus CX31 microscope (Lumenera Corporation, Ottawa, Canada) as previously described (Shen et al., 2009). Lengths of 10 well-oriented intact villi and their associated crypt were measured in each slide. To count the percentage of Ki-67 antigen forming cells present in the duodenum, immunohistochemistry

staining on histological sections was performed using the modified method of Rekiel et al. (2010). The intact crypt was cropped and Image JS software was used for calculating the ratio of Ki-67 positive cells to total cells in duodenal crypt (Almeida et al., 2012). Crypt cell proliferation, % = Ki-67 positive cells/ Total cells × 100%.

Digestibility Assay

Diets and freeze dried digesta were analyzed for DM (Method 930.15; AOAC International, 2007) and titanium (Myers et al., 2004). Nitrogen levels in the feed and digesta were measured using TruSpec N Nitrogen Determinator (LECO Corp., St. Joseph, MI) to calculate crude protein (6.25 × N). Apparent ileal digestibility (AID, %) of DM and CP were calculated using the titanium concentration in the diets and digesta by using AID = 100 – [(ND/NF) × (TiF/ TiD) × 100], where ND is the DM or CP concentration present in the ileal digesta, NF is the concentration of DM or CP present in the feed, TiF is the titanium concentration in the feed, and TiD is the titanium concentration in the ileal digesta.

Immune Response Parameters

Tumor necrosis factor (TNF)-α concentration in serum was measured in serum according to the method described by the manufacturer using a Porcine TNF-α Colorimetric ELISA Kit (Pierce Biotechnology, Rock- ford, IL). Briefly, 50 μL of standard plus dilute or 100 mL of sample was added to microplate wells which were already coated with capture antibody in conjunction with biotinylated antibody reagent. Detection occurred by the use of a color reagent substrate and a stop solution of diluted hydrochloric acid, and absorbance was read at 450 nm and 540 nm. Concentrations of TNF-α in the duodenum and jejunum were also analyzed using the ELISA kit. Upon analysis, protein concentrations in tissues were measured using a commercial kit (Thermo Fisher Scientific) according to Smith et al. (1985). The measured

protein concentration was used to determine the amounts of immunological subset per gram or milligram of protein of each tissue type. The immunoglobulin subsets immunoglobulin A (IgA) and immunoglobulin G (IgG) were measured according to the method described by the manufacturer using ELISA kits (Bethyl, Montgomery, TX, USA). Interleukin-10 concentrations in serum and mucosa were measured according to the method described by the manufacturer using ELISA kit (R&D System, MN).

Statistical Analysis

Data analysis was performed using SAS version 9.3 (SAS Inc, Cary, NC, USA). Data for diarrhea incidence were analyzed for the effect of supplemental FSO using FREQ procedure with Pearson's chi-square test. All other data were analyzed as a randomized complete block design using the Mixed procedure with pen as the experimental unit. Fixed effect was the treatment, and random effects were initial BW and sex blocks. In Exp. 1, the LSMEANS procedure was used to calculate mean values for all treatments. When treatment effect was significant or tended to be significant, least squares means among treatments were compared in a pairwise manner using the probability of differences (PDIFF) option with a Tukey adjustment of SAS. In Exp. 2, orthogonal polynomials were used to determine linear and quadratic effects of treatments. The alpha level used for determination of statistical significance was 0.05 and levels between 0.05 and 0.10 were considered as tendency.

RESULTS

Molecular Weight (MW) Distribution of Fermented Soy Oligopeptides (FSO)

The 93.3% of average MW of soluble protein in FSO was 859 Da and the rest 6.7% was 27,700 Da (Table 1).

Experiment 1

Growth Performance

No differences were found in BW among the treatments at d 11 postweaning. Pigs fed 2.0% FSO diet had a lower (P <0.05) BW than those fed the basal diet and 1.0% FSO diet at d 32 postweaning (Table 3). Pigs fed 1.0% FSO diet tended to have a higher ADG than those fed 2.0% FSO during d 11 to 32 postweaning (P = 0.077) and during the entire 32 d (P = 0.054), respectively, whereas no differences were observed when compared to those of the basal diet. Pigs fed 2.0% FSO diet tended to have a lower ADFI than those fed the basal diet during d 11 to 32 postweaning (P = 0.069) and during the entire 32 d (P = 0.058), whereas ADFI of pigs fed 1% FSO did not differ with those fed the basal diet. Pigs fed 1.0% FSO diet had a higher G:F than those fed the basal diet and 2.0% FSO diet during d 11 to 32 postweaning (P < 0.05) and during the entire 32 d (P < 0.05), respectively.

Diarrhea Incidence and Immune Response Parameters

During 14 d observation period after weaning, diarrhea incidence of pigs fed FSO was lower (P < 0.05) than those fed the basal diet (Table 4). At 32 d postweaning, supplementation of FSO at 1.0% or 2.0% did not affect IgG and TNF-α levels in serum.

Experiment 2

Growth Performance

Increasing levels of FSO did not affect BW (Table 6). From d 0 to 7, increasing levels of FSO changed (quadratic, P < 0.05) G:F. No differences were observed in other phases, except a tendency for a quadratic response in ADG (P = 0.056) and ADFI (P = 0.084) during d 17 to 27 postweaning, respectively. During the entire 27 d, increasing levels of FSO did not affect ADG, ADFI, and G:F.

Intestinal Morphology and Proliferation of Enterocytes

Increasing levels of FSO tended to change (quadratic, $P = 0.083$) villus height in the duodenum (Table 7). Villus width, crypt depth, and villus height to crypt depth ratio of duodenum were not affected by the dietary treatment. In the jejunum, increasing levels of FSO changed (quadratic, $P < 0.05$) villus height and tended to change (quadratic, $P = 0.098$) villus width of the jejunum. Crypt depth and villus height to crypt depth ratio of jejunum were not affected by the dietary treatment. Increasing levels of FSO changed (quadratic, $P < 0.05$) percentage of positive reactions measured by Ki-67 staining in the duodenal crypts.

Apparent Ileal Digestibility of DM and CP

Increasing levels of FSO tended to increase (linear, $P = 0.092$) AID of DM, and increased (linear, $P < 0.05$) AID of CP (Table 8).

Immune Response Parameters

At d 27 postweaning, increasing levels of FSO did not affect IgA levels in the duodenal and jejunal mucosa (Table 9). IgG in serum, duodenum and jejunum were not affected by the dietary FSO. Increasing levels of FSO decreased (linear, $P < 0.05$) TNF-α in serum and tended to reduce (linear, $P < 0.05$) that in the jejunal mucosa. Increasing levels of FSO tended to change (quadratic, $P = 0.074$) IL-10 in the duodenal mucosa.

DISCUSSION

Growth Performance

The main objective of Exp. 1 was to evaluate the effects of dietary FSO on growth performance of nursery pigs when fed at 1.0% and 2.0%. Based on the depressed BW observed at 2.0% supplemental level in Exp. 1, as a follow up study, FSO supplemental level was adjusted

to 1.5% as a highest level in Exp. 2. In addition, the effects of FSO supplementation on intestinal health and crude protein digestibility were evaluated.

In Exp. 1, decreased BW showed at 2.0% FSO treatment may largely due to depressed feed intake. Supplementation of FSO at 1.0% did not alter ADG or ADFI, whereas G:F was increased when compared to that of the basal diet. In Exp. 2, when the supplemental level was adjusted to 1.5% as a highest level, a quadratic change in G:F was found with greatest value at 0.5% treatment during d 0 to 7 postweaning period (phase 1). No differences were shown in ADG and ADFI by the dietary treatments, except tendencies for quadratic responses in ADG and ADFI during d 7 to 17 postweaning (phase 2). The beneficial effect showed on G:F in Exp. 1 and partially (during phase 1) in Exp. 2 is in agreement with data from Jiang et al. (2009) who reported that broiler chickens fed soy peptides (average MW was 300 to 700 Da) at the level of 120 and 200 mg/kg of diets promoted growth with improved feed conversion ratio. Similarly, in a study with yellow catfish, soy peptides (MW was less than 10 kDa) supplementation up to 16% increased growth performance with improved weight gain and feed efficiency (Zhao et al., 2016). In the present study, FSO (93% average MW was 859 Da and 7% was 27.7 kDa) supplementation at the level of 2.0% in the diet tended to decrease feed intake during 32 d after weaning. When FSO was fed up to 1.5%, no differences among the treatments in feed intake were observed, except the tendency for a quadratic effect of FSO levels, in which 1.5% treatment showed the lowest value during d 7 to 17 postweaning. Kim et al. (2010) reported that fermented soybean meal (86.6% MW was less than 20 kDa) could be supplemented up to 10% with no adverse effect on feed intake for 14 d after weaning. The bitterness of protein hydrolysates has been proposed to be linked with their MW as well as the release of peptides containing hydrophobic amino acid residues. It is believed that the lower MW has a higher bitterness and

hydrophobic amino acids at the C- and N-terminal position of peptides correlates with bitterness (Ney, 1971; Matoba and Hata, 1972; Guigoz and Solms, 1976). Pigs have larger olfactory epithelium surfaces and higher number of olfactory sensory neurons which involve in the smelling sensitivity than humans and chickens (Roura et al., 2008). Pigs also have 3 to 4 folds more taste buds than humans (Chamorro et al., 1993). These may partially explain the potential negative effect on feed intake showed in the present study. The supplementation level should be taken into account in feed formulations. The nutritional values of small size peptides would be possibly related to the molecular weight (degree of hydrolysis) and primary sequence. Future research investigating MW range of peptides that may negatively affect feed intake and elucidate which fraction of the peptides are needed to enhance its nutritional value.

Intestinal Morphology and Proliferation of Enterocytes

The intestinal morphology and proliferation of enterocytes were measured to establish a possible mechanism of promoting growth performance (Exp. 2). In previous studies, antigenic proteins present in the soy beans stimulated a transient hypersensitivity associated with abnormal morphology of the small intestine in weaned pigs (Li et al., 1990). In the present study, beneficial effects of FSO on intestinal morphology was observed. Tendencies for quadratic changes were found on villus height of duodenum and villus width of jejunum, and the villus height of jejunum changed with increasing levels of FSO. The present results are in agreement with a previous study conducted by Jiang et al. (2009) who reported that soy peptides supplementation improved duodenal villus height to crypt depth ratio of broilers. In a murine model of dextran sodium sulphate-induced colitis, peptides derived from β-conglycinin (β-conglycinin hydrolysates) showed the abilities for the prevention and repair of intestinal mucosa injury in the colon. Reductions in villus height and increases in crypt depth are the most evident

changes in intestinal morphology after weaning (Pluske et al., 1997; Fan et al., 2004). Stem cells in the crypts undergo cell division and differentiation to form mature absorptive enterocytes, and those enterocytes migrate along crypt-villus axis toward the villus tip, where they are eventually exfoliated 2 to 5 d after emerging from the crypt (Wong and Wright, 1999). In the present study, increasing levels of FSO positively affected the percentage of Ki-67 positive cells in the duodenal crypts. A quadratic response of FSO levels on Ki-67 positive cells, in which 0.5% treatment showed the highest value was observed. No differences were found in crypt depth of duodenum indicating that the effect showed in duodenal enterocyte proliferation might not be related to crypt elongation. Results from the present study suggest that supplementation of FSO up to 1.5% can positively affect villus architectural structure and epithelial cell proliferation.

Apparent Ileal Digestibility of Crude Protein

Protein digestion is largely completed in the small intestine (Daniel, 2004). Villus atrophy with the loss of mature enterocytes after weaning are shown to be correlated with declined peptidases activity (Hedemann et al., 2003). Dietary protein undergoes a series of degradative steps carried out by the digestive enzymes located in the stomach, pancreas, and small intestine, and absorbed by the enterocytes in the small intestine (Erickson et al., 1995). Three major peptidases such as aminopeptidase A, aminopeptidase N, and dipeptidyl peptidase IV play important roles in the final digestion into di-, and tripeptides, and free amino acids (Erickson et al., 1995). After weaning, decreased enzymatic activities of aminopeptidase N and dipeptidylpeptidase IV were detected (Hedemann et al., 2003). This has been ascribed to the loss of mature enterocytes as well as immature enterocytes lack of brush-border enzyme activity (Hampson and Kidder, 1986). In the present study, crude protein digestibility was changed with increasing levels of FSO. This is in agreement with previous study reported by (Feng et al.,

2007) in which fermented soybean meal fed pigs had a higher crude protein digestibility compared to those fed a conventional soybean meal. It was ascribed to the increased activities of total protease and trypsin in the duodenum and jejunum of pigs when fed fermented soybean meal than those fed the conventional soybean meal. Small peptides produced from fermentation process would require less digestion and may improve protein utilization.

Diarrhea Incidence and Immune Response

In Exp. 1, pigs fed FSO had lower diarrhea incidence, whereas in Exp. 2, none of the piglets showed diarrhea (data not shown). The diet fed in Exp. 2 was formulated with both antibiotics and zinc oxide which would contribute in preventing diarrhea. It was expected to find changes in intestinal immunity and humoral immune status which may provide an explanation for the potential mechanism leading to decreased diarrhea in Exp.1. Decreases in the allergenic proteins after soybean meal fermentation may have helped reduce diarrhea in pigs (Song et al., 2010). No differences were found on the pro-inflammatory cytokine, serum TNF-α, among the dietary treatments at d 32 postweaning when provided FSO at 1.0 or 2.0% in the diet (Exp. 1). On the other hand, when fed up to 1.5%, a linear reduction was observed in serum TNF-α, and there was a tendency for a linear decrease in the jejunal mucosal TNF-α at d 27 postweaning in Exp. 2. Additionally, IL-10, anti-inflammatory cytokine, was tended to change in the duodenal mucosa by increasing FSO supplementation. In previous studies, soy peptides supplementation have been shown to have immunomodulatory effects. It was thought that a tetra-peptide, Met-Ile-Thr-Leu, being released in the intestine after soybean consumption showed immunostimulatory effect in rats (Tsuruki et al., 2003). Similarly, it was reported that di- and tripeptides derived from soybean exerted anti-inflammatory activity in pigs in those had dextran sulfate sodium-induced colitis (Young et al., 2012). Jiang et al. (2009) also reported immunomodulatory effect

of soy peptides, it was reported that supplementation of soy peptides increased numbers of IgA-forming cells in the duodenal mucosa in broiler chickens. Results from the present study suggest FSO supplementation levels up to 2.0% are beneficial in reducing diarrhea after weaning without altering immune status, and may have beneficial effect on immune function when it was fed up to 1.5%.

CONCLUSION

Increasing supplemental level of FSO from 0 to 1.5% after weaning showed a potential to promote growth performance, enhanced intestinal morphology, and improved crude protein digestibility. The FSO supplementation up to 2.0% showed positive effect on reducing diarrhea incidence, however, negative effect was found on BW, may largely due to depressed feed intake.

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Table 1. Molecular weight distribution of fermented soy oligopeptides (FSO) measured by analytical ultracentrifuge

	Molecular weight, Da	Percentage, %
FSO	27,700	6.7
	859	93.3

Table 2. Composition of experimental diets (Exp. 1)

Item	Phase 1 (d 0 to 11 postweaning) FSO[1] (%)			Phase 2 (d 11 to 32 postweaning) FSO (%)		
	0.0	1.0	2.0	0.0	1.0	2.0
Ingredient, %						
Corn, yellow dent	43.13	43.16	43.23	54.45	54.49	54.53
Soybean meal, dehulled	20.00	19.00	18.00	23.50	22.50	21.46
Whey permeate	20.00	20.00	20.00	10.00	10.00	10.00
Poultry meal	5.00	5.00	5.00	5.00	5.00	5.00
Blood plasma	4.00	4.00	4.00	2.30	2.30	2.30
Fish meal	4.50	4.50	4.50	0.00	0.00	0.00
FSO	0.00	1.00	2.00	0.00	1.00	2.00
Poultry fat	1.30	1.30	1.25	1.80	1.80	1.80
L-Lys HCl	0.47	0.47	0.46	0.49	0.49	0.49
DL-Met	0.21	0.21	0.22	0.19	0.20	0.21
L-Thr	0.15	0.15	0.14	0.15	0.14	0.14
L-Trp	0.01	0.01	0.01	0.00	0.00	0.00
L-Val	0.03	0.02	0.01	0.04	0.02	0.01
Limestone, ground	0.75	0.74	0.74	0.87	0.85	0.85
Dicalcium phosphate	0.05	0.04	0.04	0.81	0.81	0.81
Vitamin premix[2]	0.03	0.03	0.03	0.03	0.03	0.03
Mineral premix[3]	0.15	0.15	0.15	0.15	0.15	0.15
Salt	0.22	0.22	0.22	0.22	0.22	0.22
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated composition						
DM, %	90.6	90.6	90.7	90.1	90.1	90.2
ME, kcal/kg	3,402	3,406	3,406	3,400	3,404	3,407
CP, %	23.5	23.6	23.6	21.7	21.8	21.8
SID[4] Lys, %	1.50	1.50	1.50	1.35	1.35	1.35
SID Met+Cys, %	0.82	0.82	0.82	0.74	0.74	0.74
SID Thr, %	0.88	0.88	0.88	0.79	0.79	0.79
SID Trp, %	0.25	0.25	0.25	0.22	0.22	0.22
SID Val, %	0.95	0.95	0.95	0.86	0.86	0.86
Ca, %	0.85	0.85	0.85	0.80	0.80	0.80
STTD[5] P, %	0.45	0.45	0.45	0.40	0.40	0.40
Total P, %	0.66	0.66	0.66	0.64	0.65	0.65

[1]FSO: fermented soy oligopeptides.

[2]The vitamin premix provided the following per kilogram diet: 13,227,513 IU of vitamin A; 3,968,254 IU of vitamin D_3; 66,138 IU of vitamin E; 40 mg of vitamin B_{12}; 13,228 mg of riboflavin; 110,229 mg of niacin; 22,046 mg of d-pantothenic acid; 3,968 mg of menadione; 40 IU of biotin.

Table 2. Continued

[3]The mineral premix provided the following composition: Mineral premix provided the following composition: 1.1 % of Cu; 198.0 mg/kg of I; 7.3 % of Fe; 2.2 % of Mn; 198.0 mg/kg of Se; 7.3 % of Zn.

[4]SID: standardized ileal digestible.

[5]STTD: standardized total tract digestible.

Table 3. Growth performance of nursery pigs fed diets with 0.0, 1.0, or 2.0% fermented soy oligo peptides (FSO) (Exp. 1)[1,2]

Item	Added FSO, %			SEM	*P* value
	0.0	1.0	2.0		
BW, kg					
d 0	5.62	5.62	5.62	0.49	0.964
d 11	7.65	7.48	7.29	0.51	0.157
d 32	16.80^{a}	17.00^{a}	15.30^{b}	1.23	0.049
ADG, g					
d 0 to 11	189	171	150	12	0.107
d 11 to 32	431^{AB}	440^{A}	377^{B}	33	0.077
d 0 to 32	350^{AB}	355^{A}	303^{B}	26	0.054
ADFI, g					
d 0 to 11	243	230	204	22	0.136
d 11 to 32	630^{A}	596^{AB}	535^{B}	57	0.069
d 0 to 32	497^{A}	470^{AB}	421^{B}	44	0.058
G:F					
d 0 to 11	0.755	0.729	0.785	0.033	0.508
d 11 to 32	0.688^{a}	0.746^{b}	0.704^{ac}	0.015	0.006
d 0 to 32	0.709^{a}	0.767^{b}	0.715^{ac}	0.020	0.030

[1]Each least squares mean represents 10 pens of 4 pigs per pen (5 barrow pens and 5 gilt pens). Pigs were fed a phase 1 diet for the first 11 d followed by a phase 2 diet from d 11 to 32.

[2]Means within a row lacking a common superscript small letters are significantly different ($P < 0.05$), and a row lacking a common superscript capital letters indicate means are tended to be different ($0.05 \leq P < 0.10$).

Table 4. Diarrhea incidence and inflammatory parameters of nursery pigs fed diets with fermente d soy oligopeptides (FSO) (Exp. 1)

Item	Added FSO, %			SEM	*P* value
	0.0	1.0	2.0		
Diarrhea incidence[1], %	11.43	4.29	2.86	-	0.027[2]
IgG[3], mg/mL	1.02	1.06	0.92	0.08	0.472
TNF-α[3], pg/mL	99.8	95.3	93.7	6.2	0.668

[1]Each least squares mean represents 10 pens of 4 pigs per pen (5 barrow pens and 5 gilt pens). Pigs were fed a phase 1 diet for the first 11 d followed by a phase 2 diet from d 11 to 32. Fecal scores were 1, normal, firm feces; 1.5, possible slight diarrhea; 2, moderate liquid consistency; 2.5, definitely unformed, and fluid feces; 3, very watery and frothy feces. Diarrhea incidence (%) = number of pigs with diarrhea/(number of pigs × total experimental days) × 100.

[2]Pearson's chi-square test.

[3]IgG: immunoglobulin G; TNF-α: tumor necrosis factor-α.

Table 5. Composition of experimental diets (Exp. 2)

Item	Phase 1 (d 0 to 7 postweaning) FSO[1] (%)				Phase 2 (d 7 to 17 postweaning) FSO (%)				Phase 3 (d 17 to 27 postweaning) FSO (%)			
	0.0	0.5	1.0	1.5	0.0	0.5	1.0	1.5	0.0	0.5	1.0	1.5
Ingredient, %												
Corn, yellow dent	30.40	30.45	30.50	30.55	46.16	46.21	46.26	46.31	64.65	64.70	64.75	64.80
Soybean meal, dehulled	22.80	22.25	21.70	21.15	25.00	24.45	23.90	23.35	30.00	29.45	28.90	28.35
Whey permeate	20.00	20.00	20.00	20.00	12.00	12.00	12.00	12.00	0.00	0.00	0.00	0.00
Cookie meal	10.00	10.00	10.00	10.00	5.00	5.00	5.00	5.00	0.00	0.00	0.00	0.00
Poultry meal	5.00	5.00	5.00	5.00	2.00	2.00	2.00	2.00	0.00	0.00	0.00	0.00
Blood plasma	3.50	3.50	3.50	3.50	2.00	2.00	2.00	2.00	0.00	0.00	0.00	0.00
Fish meal	5.00	5.00	5.00	5.00	3.00	3.00	3.00	3.00	0.00	0.00	0.00	0.00
FSO	0.00	0.50	1.00	1.50	0.00	0.50	1.00	1.50	0.00	0.50	1.00	1.50
Poultry fat	1.00	1.00	1.00	1.00	2.00	2.00	2.00	2.00	1.80	1.80	1.80	1.80
L-Lys HCl	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
DL-Met	0.20	0.20	0.20	0.20	0.17	0.17	0.17	0.17	0.14	0.14	0.14	0.14
L-Thr	0.12	0.12	0.12	0.12	0.11	0.11	0.11	0.11	0.12	0.12	0.12	0.12
Dicalcium phosphate	0.00	0.00	0.00	0.00	0.50	0.50	0.50	0.50	1.20	1.20	1.20	1.20
Limestone, ground	0.60	0.60	0.60	0.60	0.87	0.87	0.87	0.87	0.90	0.90	0.90	0.90
Salt	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22
Vitamin premix[2]	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Mineral premix[3]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Zinc oxide	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Antibiotics[4]	0.33	0.33	0.33	0.33	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Table 5. Continued

ME, kcal/kg	3,400	3,400	3,400	3,400	3,411	3,411	3,411	3,411	3,360	3,360	3,360	3,360
SID[5] Lys, %	1.50	1.50	1.50	1.50	1.35	1.35	1.35	1.35	1.23	1.23	1.23	1.23
SID Met+Cys, %	0.82	0.82	0.82	0.82	0.74	0.74	0.74	0.74	0.68	0.68	0.68	0.68
Ca, %	0.86	0.86	0.86	0.86	0.80	0.80	0.80	0.80	0.70	0.70	0.70	0.70
STTD[6] P, %	0.46	0.46	0.46	0.46	0.43	0.43	0.43	0.43	0.34	0.34	0.34	0.34
Analyzed composition												
CP, %	25.3	25.4	25.3	25.0	21.6	21.0	21.6	21.7	19.0	19.0	19.4	19.6
Calculated composition												
DM, %	90.5	90.5	90.6	90.6	90.1	90.1	90.1	90.1	89.3	89.3	89.3	89.4

[1]FSO: fermented soy oligopeptides.

[2]The vitamin premix provided the following per kilogram diet: 13,227,513 IU of vitamin A; 3,968,254 IU of vitamin D_3; 66,138 IU of vitamin E; 40 mg of vitamin B_{12}; 13,228 mg of riboflavin; 110,229 mg of niacin; 22,046 mg of d-pantothenic acid; 3,968 mg of menadione; 40 IU of biotin.

[3]The mineral premix provided the following composition: Mineral premix provided the following composition: 1.1 % of Cu; 198.0 mg/kg of I; 7.3 % of Fe; 2.2 % of Mn; 198.0 mg/kg of Se; 7.3 % of Zn.

[4]Antibiotics: 0.18% of pennchlor 100g and 0.15% of denagard 10 were added in phase 1; 0.10% of mecadox 10 was added in phase 2 and phase 3, respectively.

[5]SID: standardized ileal digestible.

[6]STTD: standardized total tract digestible.

Table 6. Growth performance of nursery pigs fed diets with increasing levels of fermented soy oli gopeptides (FSO) (Exp. 2)[1]

Item	Added FSO, %				SEM	*P* value	
	0.0	0.5	1.0	1.5		Linear	Quadratic
BW, kg							
d 0	5.35	5.26	5.39	5.31	0.23	0.998	0.969
d 7	5.92	5.90	5.95	5.59	0.32	0.538	0.617
d 17	9.83	10.08	10.3	9.67	0.41	0.698	0.619
d 27	16.14	16.27	16.38	15.55	0.67	0.581	0.482
ADG, g							
d 0 to 7	85	96	98	65	14.9	0.435	0.174
d 7 to 17	380	441	453	413	24.8	0.327	0.056
d 17 to 27	603	604	615	612	27.5	0.763	0.943
d 0 to 27	386	397	406	394	18.8	0.697	0.542
ADFI, g							
d 0 to 7	151	166	167	162	19.7	0.691	0.564
d 7 to 17	522	571	562	512	27.1	0.768	0.084
d 17 to 27	838	884	863	861	37.1	0.792	0.534
d 0 to 27	561	598	601	572	30.2	0.793	0.289
G:F							
d 0 to 7	0.560	0.663	0.596	0.398	0.056	0.052	0.027
d 7 to 17	0.747	0.775	0.798	0.805	0.039	0.288	0.802
d 17 to 27	0.679	0.652	0.665	0.665	0.016	0.718	0.425
d 0 to 27	0.700	0.683	0.710	0.690	0.012	0.954	0.894

[1]Each value in the table represents means of 10 pens (5 barrow pens and 5 gilt pens with 1 pig per pen). Pigs were fed a phase 1 diet for the first 7 d followed by a phase 2 diet from d 7 to 17 and a phase 3 diet from d 17 to 27.

Table 7. Morphology and enterocytes proliferation of small intestine in nursery pigs fed diets with increasing levels of fermented soy oligopeptides (FSO) (Exp. 2) [1]

Item	Added FSO, %				SEM	*P* value	
	0.0	0.5	1.0	1.5		Linear	Quadratic
Duodenum							
Villus height, μm	518	564	573	547	20	0.260	0.083
Villus width, μm	96	99	96	91	4	0.317	0.303
Crypt depth, μm	194	205	183	198	11	0.843	0.898
VH:CD[2]	2.76	2.74	3.16	2.80	0.14	0.240	0.162
Jejunum							
Villus height, μm	443	505	467	463	17	0.737	0.039
Villus width, μm	96	103	102	94	5	0.652	0.098
Crypt depth, μm	160	166	162	161	6	0.960	0.530
VH:CD	2.82	3.03	2.88	2.84	0.13	0.777	0.187
Ki-67, %							
Duodenum	11.89	22.92	19.30	19.27	0.02	0.076	0.018

[1]Each value in the table represents means of 10 pens (5 barrow pens and 5 gilt pens with 1 pig per pen).

[2]VH:CD: villus height to crypt depth ratio.

Table 8. Apparent ileal digestibility of DM and CP of nursery pigs fed diets with increasing levels of fermented soy oligopeptides (FSO) (Exp. 2)[1,2]

Item	Added FSO, %				SEM	*P* value	
	0.0	0.5	1.0	1.5		Linear	Quadratic
DM	72.8	72.9	71.8	79.2	2.4	0.092	0.154
CP	78.1	76.3	79.3	83.9	1.7	0.013	0.079

[1]Each value in the table represents means of 10 pens (5 barrow pens and 5 gilt pens with 1 pig per pen).

[2]DM: dry matter; CP: crude protein.

Table 9. Immune response of nursery pigs fed diets with increasing levels of fermented soy oligopeptides (FSO) (Exp. 2) [1]

Item	Added FSO, %				SEM	*P* value	
	0.0	0.5	1.0	1.5		Linear	Quadratic
IgA[2], mg/g protein							
Duodenum	0.66	0.51	0.62	0.57	0.15	0.798	0.721
Jejunum	0.48	0.43	0.45	0.47	0.04	0.989	0.335
IgG[2]							
Serum, mg/mL	1.07	1.02	1.11	1.03	0.1	0.920	0.872
Tissues, mg/g protein							
Duodenum	0.39	0.35	0.44	0.46	0.04	0.129	0.466
Jejunum	0.54	0.56	0.71	0.54	0.07	0.620	0.208
TNF-α[2]							
Serum, pg/mL	72.8	66.1	52.4	52.6	7.4	0.035	0.640
Tissues, pg/mg							
Duodenum	0.33	0.52	0.32	0.54	0.16	0.553	0.947
Jejunum	1.24	0.46	0.79	0.54	0.22	0.098	0.271
IL-10[2]							
Serum, pg/mL	65.3	67.8	61.6	64.3	7.6	0.474	0.954
Tissues, pg/mg protein							
Duodenum	2.75	3.78	4.25	3.30	1.05	0.379	0.074
Jejunum	8.73	5.06	3.65	4.36	2.32	0.150	0.329

[1]Each value in the table represents means of 10 pens (5 barrow pens and 5 gilt pens with 1 pig per pen).

[2]IgA: immunoglobulin A; IgG: immunoglobulin G; TNF-α: tumor necrosis factor-α; IL-10: interleukin-10.

CHAPTER 3. EFFECTS OF ENZYME-TREATED SOY OLIGOPEPTIDES ON GROWTH PERFORMANCE AND INTESTINAL MORPHOLOGY IN NURSERY PIGS

ABSTRACT: Two experiments were conducted to evaluate effects of an enzyme-treated soy oligopeptides on feed preference, growth performance, diarrhea incidence, intestinal morphology, and immune response of nursery pigs. The ESO was produced by an enzymatic hydrolysis containing 47% CP (87.9% of CP had an average molecular weight of 863 Da). In Exp.1, 24 pigs (12 barrows and 12 gilts at 6.2 ± 0.8 kg of BW) were randomly allotted to 6 pens with 4 pigs (2 barrows and 2 gilts) per pen. Pigs were allowed to choose between two different diets throughout 27-d postweaning. The 2 different diets were provided in separate feeders in each pen: 1) basal diet: a corn-soybean meal based diet, 2) 2.0% ESO diet: 2.0% ESO replaced equal amount of soybean meal of the basal diet. Pigs preferred ($P < 0.05$) 2.0% ESO diet than the basal diet, 71.9% of the total feed intake on d 3 postweaning, whereas it decreased ($P < 0.05$) to 36.1% on d 24 postweaning. In Exp. 2, 128 pigs (64 barrows and 64 gilts at 5.2 ± 0.5 kg of BW) were allotted in a randomized complete block design with sex and initial BW as blocks and randomly assigned to 4 treatments. Pigs were fed a basal diet supplemented with ESO at the level of 0.0, 1.0, 2.0, or 3.0% based on 2 phases (11 and 21 d, respectively). BW and feed consumption were recorded at the end of each feeding phase. Fecal scores were recorded for 14 d to measure diarrhea incidence. On d 32, blood, and duodenal and jejunal mucosa samples were collected for immune response analysis. Duodenum and jejunum segments were collected for morphology evaluation. Data were analyzed using Mixed procedure of SAS. During the entire 32 d, increasing levels of ESO decreased ($P < 0.05$) ADG linearly and a tendency for a quadratic change ($P = 0.077$) was observed. Increasing levels of ESO decreased ADFI linearly ($P < 0.05$), whereas G:F was not affected. Diarrhea incidence of pigs was reduced ($P < 0.05$) by ESO supplementation. A tendency for a quadratic change ($P = 0.062$) was shown in duodenal villus height to crypt depth ratio. A tendency for a quadratic response ($P = 0.059$) was observed in

serum tumor necrosis factor α. In conclusion, 2.0% ESO was preferred by pigs initially which was changed to basal diet as the feeding day increases. Supplementation of ESO up to 3.0% showed negative effects in ADG and ADFI may largely due to depressed feed intake, whereas the maximal ADG was obtained by supplementation of 0.74% in the diets. Supplementation of ESO reduced diarrhea without stimulating immune response and may potentially enhance intestinal morphology.

Key words: enzyme-treated soy oligopeptides, growth performance, nursery pigs

INTRODUCTION

Soybean meal, one of the most commonly used protein supplements, contains various anti-nutritional factors including trypsin inhibitor, lectins, indigestible carbohydrate complexes, and soybean globulins (glycinin and β-conglycinin) that limit its use in nursery pig diets. Although heat treatment and fat extraction can remove most of the trypsin inhibitor, however, antigenic proteins, such as glycinin and β-conglycinin, are still remaining in the soybean meal which can cause hypersensitivity associated abnormal intestinal morphological changes, diarrhea, and depressed growth when fed to young pigs (Li et al., 1990; Dréau et al., 1994; Sun et al., 2008).

Soy protein hydrolysates have been used as a feed source for its high nutritional value with reduced anti-nutritional factors (Hou et al., 2017). It has been shown to improve growth (Zhou et al., 2011) and reduce diarrhea (Song et al., 2010) when fed to newly weaned pigs. Despite the improved nutritional values, the bitter taste of soy hydrolysates resulting from the hydrolysis of soy proteins has been a major problem in food applications (Alder-Nissen, 1986; Kim et al., 2003). The hydrophobic amino acids are shown to be involved in the bitter taste of various peptides (Nishiwaki and Hayashi, 2001). Concealed hydrophobic side chains in the interior of the protein are released with the protein hydrolysis which elucidates bitterness (Matoba and Hata, 1972; Nishiwaki et al., 2002). It is of great interest to test soy hydrolysates supplemented in nursery pig diets to ascertain if it can promote growth of pigs without negatively affecting feed intake of nursery pigs.

In the present study, soy oligopeptides produced by enzymatic hydrolysis was used in the nursery pig diets, and feed preference, intestinal morphology, and immune response were tested to demonstrate its effect on feed intake, intestinal health and growth performance.

MATERIALS AND METHODS

Enzyme-treated Soy Oligopeptides (ESO)

The ESO, soy protein hydrolysate produced using a proprietary mixture of enzymes was obtained from Mytech Biotech Co. Ltd., Chengdu, China. The molecular weight of ESO was analyzed using an analytical ultracentrifuge (ProteomeLab XL-A, Beckman Coulter, Fullerton, CA) according to the method described by Schuck et al. (2002). Briefly, all interference data were collected at a speed of 40,000 rpm in an An-60 Tirotor at 4^{o}C. Homogenized samples were centrifuged at 10,000 rpm for 15 minutes at 4^{o}C (Eppendorf MiniSpin, Eppendorf AG, Hamburg, Germany). A set of 200 scans was collected at 6-min intervals. The proteins were prepared with 2 mL of 20 m*M* Tris-HCl buffer including 0.1% sodium dodecyl sulfate, and 5 m*M* dithiothreitol at pH 7.4. Aliquots (110 uL) of sample solution were loaded into 6 sector sample cells. Absorbance was monitored at 280 nm for the loaded samples. Sedimentation velocity data were analyzed using the software program SEDFIT (version 11.8) (National Institutes of Health, Bethesda, MD) to generate the sedimentation coefficient distribution of protein samples.

Experiment 1

Feed Preference

A total of 24 pigs (12 barrows and 12 gilts at 6.18 ± 0.8 kg of BW) were allotted to 6 pens, with 4 pigs per pen. Two different diets were provided in separate feeders in each pen: 1) basal diet: a corn-soybean meal based diet, 2) 2.0% ESO diet: added 2.0% ESO into the basal diet. Both diets were made to meet or exceed requirement estimates for nursery pigs (NRC, 2012) (Table 2). Pigs were housed in 1.2 × 1.2 m fully slatted, plastic-coated metal floor pens. Each pen was equipped with two stainless steel feeders and a nipple drinker, and pigs had free access to feed and water throughout the experiment. One feeder contained the basal diet and the other

feeder contained the 2.0% ESO diet. The positions of the 2 feeders within the pen were switched every 3 d to minimize positional preference. Feed disappearances and individual BW were recorded every 3 d for 27 d.

Behavior Measurement

Behavioral recording was done on d 3, 6, and 12 as described in Shen et al. (2012). Data was analyzed by classifying eating behavior from others (lying, standing, sitting, and drinking). An instantaneous scan-sampling method with 5-min intervals will be used to determine percentage of time spent on various behaviors during 8 h observation period.

Experiment 2

Experimental Diets and Pigs

A total of 128 pigs (64 barrows and 64 gilts at 5.2 ± 0.5 kg BW) were used. Pigs were grouped by BW and sex into four blocks and randomly assigned to four treatments with four pigs per pen and eight replicate pens per treatment in a randomized complete block design. Pigs were housed in 1.2 × 1.2 m fully slatted, plastic-coated metal floor pens. Pigs had free access to feed and water. ESO was added at the level of 0.0, 1.0, 2.0, or 3.0% into a basal diet to replace the equal amounts of soybean meal in the diets (Table 7). The basal diet and 2.0% ESO diet were same from Exp.1. All diets were made to meet or exceed requirement estimates for nursery pigs (NRC, 2012). The experimental period was divided into 2 phases (11 and 21 d, respectively). Individual BW and feed disappearance of each pen were recorded at the end of each feeding phase.

Sample Collection

On d 32, blood samples (7 mL) were collected from the jugular vein with Vacutainers (BD, Franklin Lakes, NJ). Blood samples were then centrifuged at 3,000 × g for 15 min at 4^{o}C to

obtain serum. On d 32, 32 pigs (8 pigs per treatment) were euthanized to collect duodenum and jejunum sections for histology and immune response analysis. Tissues were rinsed with a 0.9% saline solution and fixed with a 10% buffered formalin solution. Mucosa samples were taken from duodenum (2 cm after the pyloric-duodenal junction until the loop ends) and jejunum (around 100 cm before the ileal-cecal junction) for immune response analysis.

Diarrhea Incidence

The incidence of diarrhea of piglets was recorded every day for the first 14 d of the experimental period. Pig feces were scored according to the modified method of Liu et al., (2010). Fecal scores were: 1 (normal, firm feces); 1.5 (possible slight diarrhea); 2 (moderate liquid consistency); 2.5 (definitely unformed and fluid feces); 3 (very watery and frothy feces). The occurrence of diarrhea was defined as maintenance of feces at Level 2.5 or Level 3 for two continuous days. Diarrhea incidence was calculated according to the formula reported by Sun et al. (2008): diarrhea incidence (%) = number of pigs with diarrhea/(number of pigs × total experimental days) × 100, where "number of pigs with diarrhea" was the total number of pigs with diarrhea observed each day, and "total experimental days" was 14 d.

Intestinal Morphology and Proliferation of Enterocytes

Duodenum and jejunum samples then embedded in paraffin (Paraplast-Sigma) and paraffin sections of 5 µm thick tissue were taken on rotary microtome. The sections were stained with haematoxylin and eosin. Villus height, villus width, and crypt depth of duodenum and jejunum were measured at 40 × magnification as previously described (Shen et al., 2009). Lengths of 10 well-oriented intact villi and their associated crypt were measured in each slide. To count the percentage of Ki-67 antigen forming cells present in the duodenum, immunohistochemistry staining on histological sections was performed using the modified method of Rekiel et al.

(2010). The intact crypt was cropped and Image JS software was used for calculating the ratio of Ki-67 positive cells to total cells in jejunal crypt (Almeida et al., 2012). Crypt cell proliferation, % = Ki-67 positive cells/ Total cells × 100%.

Immune Response

The immunoglobulin subsets immunoglobulin A (IgA) and immunoglobulin G (IgG) were measured according to the method described by the manufacturer using ELISA kits (Bethyl, Montgomery, TX, USA). TNF-α was measured in serum, and duodenum and jejunum mucosa according to the method described by the manufacturer using a Porcine TNF-α Colorimetric ELISA Kit (Pierce Biotechnology, Rock- ford, IL). Briefly, 50 μL of standard plus dilute or 100 mL of sample was added to microplate wells which were already coated with capture antibody in conjunction with biotinylated antibody reagent. Detection occurred by the use of a color reagent substrate and a stop solution of diluted hydrochloric acid, and absorbance was read at 450 nm and 540 nm. Concentrations of TNF-α in the duodenum and jejunum were also analyzed using the ELISA kit. Upon analysis, protein concentrations in tissues were measured using a commercial kit (Thermo Fisher Scientific) according to Smith et al. (1985). The measured protein concentration was used to determine the amounts of immunological subset per gram or milligram of protein of each tissue type. Interleukin-10 concentrations in serum and mucosa were measured according to the method described by the manufacturer using ELISA kit (R&D System, MN).

Statistical Analysis

Data analysis was performed using SAS version 9.3 (SAS Inc, Cary, NC, USA). Data were analyzed by the paired t-test in Exp.1. In Exp. 2, data for diarrhea incidence were analyzed for the effect of supplemental ESO using FREQ procedure with Pearson's chi-square test. Fisher's

exact test was used in place of Pearson's chi-square when at least one cell of the contingency table has an expected frequency less than 5. All other data were analyzed as a randomized complete block design using the Mixed procedure with pen as the experimental unit. Fixed effect was the treatment, and random effects were initial BW and sex blocks. Orthogonal polynomials were used to determine linear and quadratic effects of treatments. One slope broken-line analysis was conducted using the Nonlinear Regression Model (PROC NLIN) of SAS to estimate the breakpoint of ESO supplemental level (Robbins et al., 2006). The 95% confidence interval (CI) of the breakpoint was estimated (Bertolo et al., 1998). The alpha level used for determination of statistical significance was 0.05 and levels between 0.05 and 0.10 were considered as trends.

RESULTS

Molecular Weight (MW) Distribution of Enzyme-treated Soy Oligopeptides (ESO)

The 87.9% of the average MW of soluble protein in FSO was 863 Da, and the rest 12.1% was 32,650 Da (Table 1).

Experiment 1

Feed Preference

The mean value of BW, ADG, and ADFI of pigs from 6 replicate pens are presented in Table 3. On d 3 postweaning, pigs preferred ($P < 0.05$) 2.0% ESO diet to the basal diet, 71.9% and 28.1%, respectively, whereas the preference for the 2.0% ESO decreased ($P < 0.05$) to 36.1% on d 24 postweaning. A positive correlation between feed preference of basal diet and feeding days ($r = 0.737$, $P < 0.05$) (Figure 1) and a negative correlation between the feed preference of 2.0% ESO diet and feeding days ($r = -0.737$, $P < 0.05$) were observed (Figure 2).

Behavior Measurement

The behavioral activities of pigs fed the basal diet and 2.0% ESO diet are presented in Table 5. On d 3, eating behavior of pigs chose the 2.0% ESO diet was higher than that chose the basal diet, whereas on d 6, eating behavior of pigs chose the basal diet was higher than that chose the 2.0% ESO diet (Table 6). No differences were found in eating behavior of choosing the two different diets on d 12.

Experiment 2

Growth Performance

During d 0 to 11 postweaning, increasing levels of ESO tended to decrease G:F (linear, P = 0.059) without affecting ADFI and ADG (Table 8). During d 11 to 32 postweaning, increasing levels of ESO changed ADG (quadratic, $P < 0.05$), ADFI (quadratic, $P < 0.05$), and affected G:F (quadratic, $P < 0.05$). The optimum supplemental level for ADFI was observed at 0.89% (95% CI: -0.27, 2.05; $R^2 = 0.256$) during d 11 to 32 postweaning (Figure 3). Increasing levels of ESO decreased (linear, $P < 0.05$) BW on d 32 postweaning. Increasing levels of ESO decreased ADG (linear $P < 0.05$) and tended to change ADG (quadratic, $P = 0.077$) during the entire 32 d. Maximum ADG was observed at 0.74% (95% CI: 0.61, 0.87; $R^2 = 0.277$) of ESO supplemental level during the entire 32 d (Figure 4). Increasing levels of ESO decreased (linear $P < 0.05$) ADFI without affecting G:F.

Intestinal Morphology and Enterocyte Proliferation

Increasing levels of ESO did not alter villus height, villus width, and crypt depth of duodenum and jejunum (Table 9). A tendency for a quadratic change was shown in duodenal villus height to crypt depth ratio ($P = 0.062$) with increasing levels of ESO. The percentages of

Ki-67 positive cells in the crypts of duodenum and jejunum were not affected by the dietary treatments.

Diarrhea Incidence and Immune Response

During 14-d observation period, diarrhea incidence of pigs fed ESO was lower ($P < 0.05$) than those fed a basal diet (Table 10). No differences were observed in IgA and IgG in serum or in the duodenum and jejunum mucosa among the treatments. Increasing levels of ESO tended to change TNF-α (quadratic, $P = 0.059$) in serum, whereas those in the duodenum and jejunum mucosa were not affected by the treatments. IL-10 levels in serum or in the duodenum and jejunum mucosa were not affected by the dietary treatments.

DISCUSSION

Experiment 1

Feed Preference and Behavior Measurement

It has been demonstrated that the hydrolysis of proteins with proteolytic enzymes is often accompanied by formation of bitter tasting peptides (Guigoz and Solms, 1976; Nishimura and Kato, 1988). The bitterness of protein hydrolysates is caused by the release of peptides containing hydrophobic amino acid at the C- and N-terminal position (Ney, 1971; Matoba and Hata, 1972; Guigoz and Solms, 1976). Degree of hydrolysis is also correlated with bitterness of protein hydrolysates. Lower molecular weight is often associated with higher bitterness of peptides, however, conflicting results on the exact molecular weight range were observed. Hong et al. (2001) reported peptides fraction in the range of 2,000 to 6,000 Da showed the highest bitterness. Kukman et al. (1995) suggested that peptides fraction less than 1,000 Da was the main reason for bitterness of soy protein hydrolysates. In contrast, Cho et al. (2004) showed that the

most bitter fractions from soy protein hydrolysates were in the range of 1,000 to 4,000 Da, whereas, those less than 1,000 Da had the lowest bitterness. The ESO used in the present study was composed of small size peptides, 88% of the soluble protein had average molecular weight of 863 Da. Results from the present study showed that pigs preferred 2.0% ESO diet over the basal diet on d 3 postweaning, however, on d 24 postweaning, pigs preferred the basal diet over the 2.0% ESO diet and those pigs had a normal BW and feed intake. A negative correlation between the feed preference of 2.0% ESO diet and the feeding days was observed indicating that pigs are willing to eat basal diet instead of 2.0% ESO as the time increases. The eating behavior of pigs observed in the present study can partially support the preference result that pigs preferred to eat ESO diet in the beginning of the experiment but preferred to eat the basal diet as the feeding time increases. On the contrary, in a previous study, when enzyme-treated soybean meal (24.6% of soluble protein had MW less than 500 Da) supplemented up to 15%, feed intake of weaned pigs was increased during 28-d experimental period (Zhou et al., 2011). Yang et al. (2007) reported enzyme-treated soybean meal (Hamlet protein, HP300) fed to pigs at the level of 8% increased feed intake of pigs during 14 d postweaning. Most of the MW of HP300 was shown in the range of 20 kDa to 60 kDa (Cervantes-Pahm and Stein, 2010). The negative correlation between the feed preference of 2.0% ESO and the feeding days might be partially related to its high degree of hydrolysis, and hydrophobic side chains, with which fraction attributed to the bitterness are not known. Further research is needed to define the optimum degree of hydrolysis of soy proteins to ameliorate bitter taste of ESO.

Experiment 2

Growth Performance

In the present study, decrease in feed intake with increasing levels of ESO may have contributed to the decreased in ADG of pigs. Based on the result showed in the feed preference study in Exp. 1, the optimum supplemental level of ESO was estimated in Exp. 2. Results from the present study indicate that feed intake was decreased with the increasing levels of ESO supplementation during the 32-d experimental period, however, feed intake during the first 11 d was not affected. During d 11 to 32, optimum supplemental level was observed at 0.89% (95% CI: -0.27, 2.05; $R^2 = 0.256$). ESO supplemental level for the maximum ADG of pigs was at 0.74% (95% CI: 0.61, 0.87; $R^2 = 0.277$) during the entire experimental period. Previous research evaluating enzyme-treated soybean meal on growth performance in nursery pigs showed beneficial effects on feed intake and growth rate. Enzyme-treated soybean meal fed to weaned pigs up to 15% improved feed intake, weight gain, and feed efficiency during 28-d experimental period (Zhou et al., 2011). Yang et al. (2007) reported enzyme-treated soybean at the level of 8% increased weight gain and feed intake of pigs during d 0 to 14 postweaning. Results from the current study indicate that ESO supplementation have a potential to enhance growth performance, but high supplemental level would negatively affect growth performance mainly by reducing feed intake. The supplementation level should be taken into account when applying it into nursery pig diets.

Intestinal Morphology and Enterocyte Proliferation

Antigenic proteins such as conglycinin and β-conglycinin in soybean meal stimulated a hypersensitivity associated with abnormal intestinal morphology including villi atrophy and crypt hyperplasia of the small intestine in weaned pigs (Dréau et al., 1994; Dréau and Lallès,

1999). In the present study, increasing levels of ESO tended to positively change duodenal villus height to crypt depth ratio. ESO was composed of small peptides (with 88% average molecular weight of 863 Da and 12.0% of 32,650 Da) indicating antigenic proteins, including conglycinin and β-conglycinin, were degraded. The MW of acidic and basic subunits of glycinin were reported as 36 kDa (Nielsen, 1985) and 22 kDa (Moreira et al., 1979), and MW of β-conglycinin of α, α' and β subunits were reported as approximately 67, 71, and 50 kDa, respectively (Maruyama et al., 1998). Reduced feed intake is shown to be correlated with shortening in villus height and increase in crypt depth (Pluske et al., 1996). The negative effect observed in feed intake with increased supplemental level of ESO did not negatively affect intestinal architectural structure. Feed intake during the first 11 d after weaning was not affected by the dietary treatments, this might have alleviated potential negative effect on intestinal morphology. Research with lower supplemental level is needed to further demonstrate beneficial effect on intestinal development and growth.

Diarrhea Incidence and Immune Response

Processed soy protein containing low antigenic soy proteins have been shown to alleviate diarrhea in weaned pigs (Song et al., 2010). Reduced diarrhea incidence by supplementation of ESO was observed in the present study. Allergenic proteins were degraded into small peptides that may have helped decrease diarrhea. Song et al. (2010) reported pigs fed fermented soybean meal showed a lower immunoreactivity toward soy antigenic proteins than those fed soybean meal due to partially digested antigenic proteins during fermentation process. Enzyme-treated soybean meal supplementation fed to weaned pigs increased T-cell mediated immunity by increasing T lymphocytes, and its subsets CD4+ and CD8+ concentrations in peripheral blood after feeding 28 d (Zhou et al., 2011). In the current study, increasing levels of ESO tended to

change pro-inflammatory cytokine, TNF-α in serum without affecting immunoglobulin subsets (IgA and IgG), and anti-inflammatory cytokine (IL-10) levels. Results from this study indicate that ESO supplementation levels up to 3% are beneficial in reducing diarrhea without altering immune status after weaning.

CONCLUSION

In conclusion, 2.0% ESO was preferred by pigs initially which was changed to basal diet as the feeding day increases. Supplementation of ESO up to 3% showed negative effects in ADG and ADFI may largely due to depressed feed intake, whereas the maximal ADG was obtained by supplementation of 0.74% in the diets (95% CI: 0.61, 0.87). Supplementation of ESO reduced diarrhea without stimulating immune response and may potentially enhance intestinal morphology.

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Table 1. Molecular weight distribution of enzyme-treated soy oligopeptides (ESO) measured by analytical ultracentrifuge

	Molecular weight, Da	Percentage, %
ESO	32,650	12.1
	863	87.9

Table 2. Composition of experimental diets (Exp. 1)

Item	Phase 1 (d 0 to 11 postweaning) ESO[1] (%)		Phase 2 (d 11 to 32 postweaning) ESO (%)	
	0.0	2.0	0.0	2.0
Ingredient, %				
Corn, yellow dent	43.13	43.29	54.45	54.56
Soybean meal, dehulled	20.00	18.00	23.50	21.50
Whey permeate	20.00	20.00	10.00	10.00
Poultry meal	5.00	5.00	5.00	5.00
Blood plasma	4.00	4.00	2.30	2.30
Fish meal	4.50	4.50	0.00	0.00
ESO[1]	0.00	2.00	0.00	2.00
Poultry fat	1.30	1.25	1.80	1.80
L-Lys HCl	0.47	0.47	0.49	0.49
DL-Met	0.21	0.20	0.19	0.18
L-Thr	0.15	0.16	0.15	0.15
L-Trp	0.01	0.01	0.00	0.00
L-Val	0.03	0.04	0.04	0.04
Limestone, ground	0.75	0.65	0.87	0.77
Dicalcium phosphate	0.05	0.03	0.81	0.81
Vitamin premix[2]	0.03	0.03	0.03	0.03
Mineral premix[3]	0.15	0.15	0.15	0.15
Salt	0.22	0.22	0.22	0.22
Total	100.00	100.00	100.00	100.00
Calculated nutrient composition				
DM, %	90.6	90.6	90.1	90.1
ME, kcal/kg	3,402	3,404	3,400	3,404
CP, %	23.5	23.5	21.7	21.7
SID[4] Lys, %	1.50	1.50	1.35	1.35
SID Met+Cys, %	0.82	0.82	0.74	0.74
SID Thr, %	0.88	0.88	0.79	0.79
SID Trp, %	0.25	0.25	0.22	0.22
SID Val, %	0.95	0.95	0.86	0.86
Ca, %	0.85	0.85	0.8	0.8
STTD[5] P, %	0.45	0.45	0.4	0.4

Table 2. Continued

[1]ESO: enzyme-treated soy oligopeptides.

[2]The vitamin premix provided the following per kilogram diet: 13,227,513 IU of vitamin A; 3,968,254 IU of vitamin D_3; 66,138 IU of vitamin E; 40 mg of vitamin B_{12}; 13,228 mg of riboflavin; 110,229 mg of niacin; 22,046 mg of d-pantothenic acid; 3,968 mg of menadione; 40 IU of biotin.

[3]The mineral premix provided the following composition: Mineral premix provided the following composition: 1.1 % of Cu; 198.0 mg/kg of I; 7.3 % of Fe; 2.2 % of Mn; 198.0 mg/kg of Se; 7.3 % of Zn.

[4]SID: standardized ileal digestible.

[5]STTD: standardized total tract digestible.

Table 3. Growth performance of pigs fed 0.0 and 2.0% enzyme-treated soy oligopeptides (ESO) (Exp. 1)

Item	Mean	SD
BW, kg		
d 0	6.18	0.58
d 3	6.38	0.56
d 6	6.83	0.74
d 9	7.60	0.91
d 12	8.23	1.14
d 15	8.81	1.37
d 18	9.86	1.66
d 21	11.03	1.78
d 24	12.24	1.99
d 27	13.77	2.37
ADG, g		
d 0 to 3	67	84
d 3 to 6	149	71
d 6 to 9	256	71
d 9 to 12	209	79
d 12 to 15	194	87
d 15 to 18	350	135
d 18 to 21	391	71
d 21 to 24	404	156
d 24 to 27	508	125
ADFI, g		
d 0 to 3	136	35
d 3 to 6	271	30
d 6 to 9	324	62
d 9 to 12	358	79
d 12 to 15	389	93
d 15 to 18	445	119
d 18 to 21	535	89
d 21 to 24	621	154
d 24 to 27	707	178

Table 3. Continued

G:F		
d 0 to 3	0.448	0.612
d 3 to 6	0.557	0.263
d 6 to 9	0.784	0.120
d 9 to 12	0.562	0.134
d 12 to 15	0.481	0.133
d 15 to 18	0.770	0.185
d 18 to 21	0.737	0.101
d 21 to 24	0.642	0.131
d 24 to 27	0.723	0.066

Table 4. Feed intake and feed preference of nursery pigs fed 0.0 and 2.0% enzyme-treated soy oligopeptides (ESO) (Exp. 1)

Item	Added ESO, %		SEM	*P* value
	0.0	2.0		
ADFI, g				
d 3	40	96	10	0.001
d 6	132	140	14	0.510
d 9	184	140	18	0.244
d 12	169	189	15	0.542
d 15	219	200	27	0.746
d 18	234	212	31	0.738
d 21	322	213	35	0.129
d 24	418	203	52	0.042
d 27	404	303	67	0.480
Feed preference based on daily feed intake, %				
d 3	28.1	71.9	7.3	<0.0001
d 6	47.4	52.6	5.1	0.628
d 9	55.9	44.1	5.0	0.262
d 12	46.7	53.3	2.9	0.275
d 15	51.6	48.4	5.7	0.795
d 18	51.2	48.8	5.5	0.837
d 21	57.8	42.2	6.6	0.253
d 24	63.9	36.1	7.3	0.048
d 27	54.0	46.0	8.6	0.662

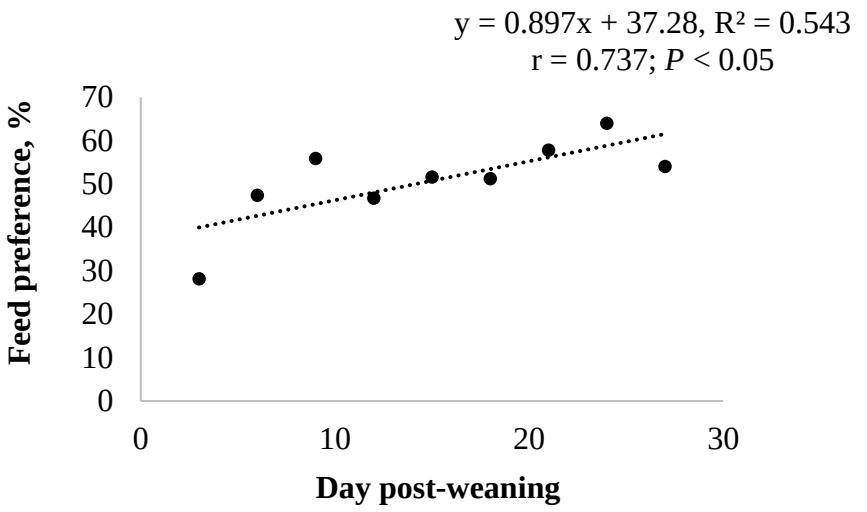

Figure 1. Correlation between feed preference of basal diet and feeding days. The correlation was assessed by Pearson's correlation coefficient (r) (Exp. 1)

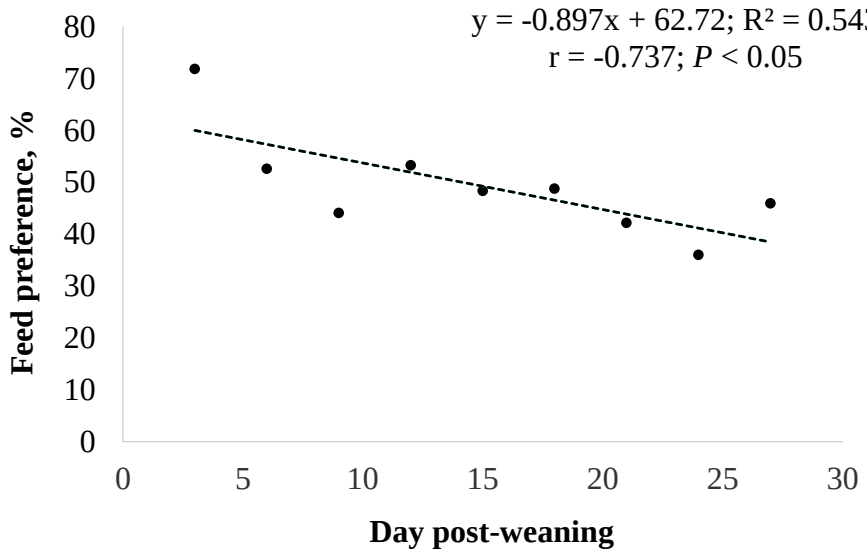

Figure 2. Correlation between feed preference of 2.0% ESO diet and feeding days. The correlation was assessed by Pearson's correlation coefficient (r) (Exp. 1).

Table 5. Behavioral activities of lying, standing and sitting, drinking, and eating of pigs fed 0.0 a nd 2.0% enzyme-treated soy oligopeptides (ESO) (Exp. 1)

Item	Percentage, %
d 3	
Lying	61.3
Standing & Sitting	19.8
Drinking	2.1
Eating	16.9
0.0% ESO	6.0
2.0% ESO	11.0
d 6	
Lying, %	54.1
Standing & Sitting, %	20.9
Drinking, %	1.4
Eating, %	23.6
0.0% ESO	14.3
2.0% ESO	9.3
d 12	
Lying, %	57.6
Standing & Sitting, %	20.3
Drinking, %	1.1
Eating, %	20.9
0.0% ESO	11.6
2.0% ESO	9.4

Table 6. Eating behavior among other behavioral activities of pigs fed 0.0 and 2.0% enzyme-treated soy oligopeptides (ESO) (Exp. 1)[1]

Item	Added ESO, %		SEM	*P* value
	0.0	2.0		
Eating, %				
d 3	6.0	11.0	1.1	0.020
d 6	14.3	9.3	1.2	0.036
d 12	11.6	9.4	1.2	0.395

[1]Percentage of eating behavior among the other behaviors including lying, standing, sitting, and drinking. Behavior of each pig was recorded for 8 h (0080 to 1600) on each day. An instantaneous scan-sampling method with 5-min intervals was used and 97 data points for each pen were generated. Values are presented as the least square of means of 6 replicate pens (Each pen had 4 pigs).

Table 7. Composition of experimental diets (Exp. 2)

Item	Phase 1 (d 0 to 11 postweaning) ESO[1] (%)				Phase 2 (d 11 to 32 postweaning) ESO (%)			
	0.0	1.0	2.0	3.0	0.0	1.0	2.0	3.0
Ingredient, %								
Corn, yellow dent	43.13	43.20	43.29	43.36	54.45	54.50	54.56	54.67
Soybean meal, dehulled	20.00	19.00	18.00	17.00	23.50	22.50	21.50	20.50
Whey permeate	20.00	20.00	20.00	20.00	10.00	10.00	10.00	10.00
Poultry meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Blood plasma	4.00	4.00	4.00	4.00	2.30	2.30	2.30	2.30
Fish meal	4.50	4.50	4.50	4.50	0.00	0.00	0.00	0.00
ESO[1]	0.00	1.00	2.00	3.00	0.00	1.00	2.00	3.00
Poultry fat	1.30	1.30	1.25	1.25	1.80	1.80	1.80	1.75
L-Lys HCl	0.47	0.47	0.47	0.46	0.49	0.49	0.49	0.49
DL-Met	0.21	0.20	0.20	0.20	0.19	0.19	0.18	0.18
L-Thr	0.15	0.15	0.16	0.16	0.15	0.15	0.15	0.16
L-Trp	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00
L-Val	0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.04
Limestone, ground	0.75	0.71	0.65	0.62	0.87	0.82	0.77	0.75
Dicalcium phosphate	0.05	0.03	0.03	0.00	0.81	0.81	0.81	0.76
Vitamin premix[2]	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Mineral premix[3]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Salt	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Calculated nutrient composition								
DM, %	90.6	90.6	90.6	90.6	90.1	90.1	90.1	90.1
ME, kcal/kg	3,402	3,404	3,404	3,406	3,400	3,402	3,404	3,404
CP, %	23.5	23.5	23.5	23.5	21.7	21.7	21.7	21.7
SID[4] Lys, %	1.50	1.50	1.50	1.50	1.35	1.35	1.35	1.35
SID Met+Cys, %	0.82	0.82	0.82	0.82	0.74	0.74	0.74	0.74
SID Thr, %	0.88	0.88	0.88	0.88	0.79	0.79	0.79	0.79
SID Trp, %	0.25	0.25	0.25	0.25	0.22	0.22	0.22	0.22
SID Val, %	0.95	0.95	0.95	0.95	0.86	0.86	0.86	0.86
Ca, %	0.85	0.85	0.85	0.85	0.8	0.8	0.8	0.8
STTD[5] P, %	0.45	0.45	0.45	0.45	0.4	0.4	0.4	0.4
Total P, %	0.66	0.66	0.67	0.66	0.64	0.65	0.65	0.64

Table 7. Continued

[1]ESO: enzyme-treated soy oligopeptides.

[2]The vitamin premix provided the following per kilogram diet: 13,227,513 IU of vitamin A; 3,968,254 IU of vitamin D_3; 66,138 IU of vitamin E; 40 mg of vitamin B_{12}; 13,228 mg of riboflavin; 110,229 mg of niacin; 22,046 mg of d-pantothenic acid; 3,968 mg of menadione; 40 IU of biotin.

[3]The mineral premix provided the following composition: Mineral premix provided the following composition: 1.1 % of Cu; 198.0 mg/kg of I; 7.3 % of Fe; 2.2 % of Mn; 198.0 mg/kg of Se; 7.3 % of Zn.

[4]SID: standardized ileal digestible.

[5]STTD: standardized total tract digestible.

Table 8. Growth performance of nursery pigs fed diets with increasing levels of enzyme-treated soy oligopeptides (ESO) (0 to 3%)[1]

Item	Added ESO, %				SEM	*P* value	
	0.0	1.0	2.0	3.0		Linear	Quadratic
BW, kg							
d 0	5.21	5.20	5.21	5.22	0.30	0.700	0.450
d 11	7.09	6.87	7.10	6.72	0.38	0.210	0.611
d 32	15.79	16.44	15.17	13.80	0.83	0.013	0.108
ADG, g							
d 0 to 11	171	153	164	136	23	0.128	0.706
d 11 to 32	413	451	406	338	31	0.009	0.019
d 0 to 32	336	352	316	273	25	0.004	0.077
ADFI, g							
d 0 to 11	227	239	214	216	27	0.304	0.654
d 11 to 32	588	636	564	484	51	0.008	0.046
d 0 to 32	463	480	444	392	43	0.019	0.140
G:F							
d 0 to 11	0.741	0.644	0.707	0.629	0.050	0.059	0.708
d 11 to 32	0.703	0.739	0.725	0.698	0.014	0.638	0.036
d 0 to 32	0.727	0.731	0.720	0.696	0.029	0.278	0.484

[1]Each least squares mean represents 8 pens of 4 pigs per pen (4 barrow pens and 4 gilt pens). Pigs were fed a phase 1 diet for the first 11 d followed by a phase 2 diet from d 11 to 32.

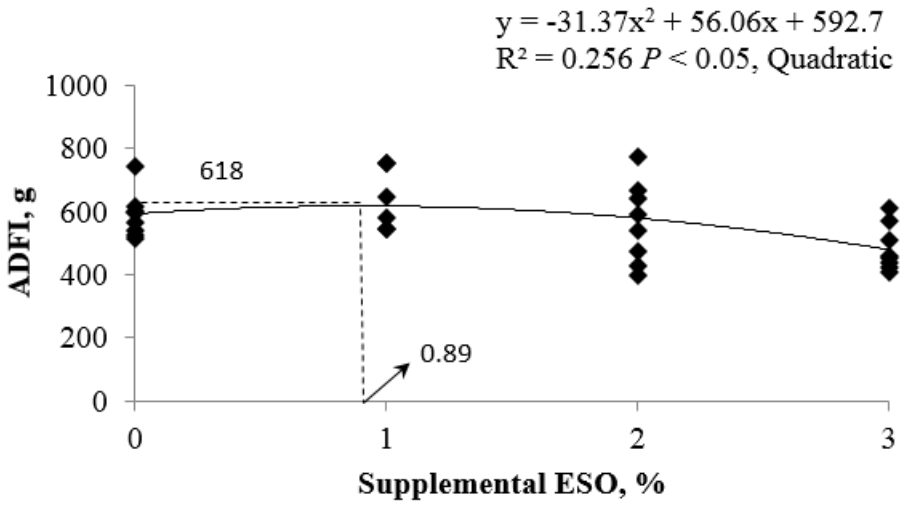

Figure 3. Average daily feed intake of pigs fed increasing levels of enzyme-treated soy oligopeptides (ESO) from d 11 to 32 (phase 2).

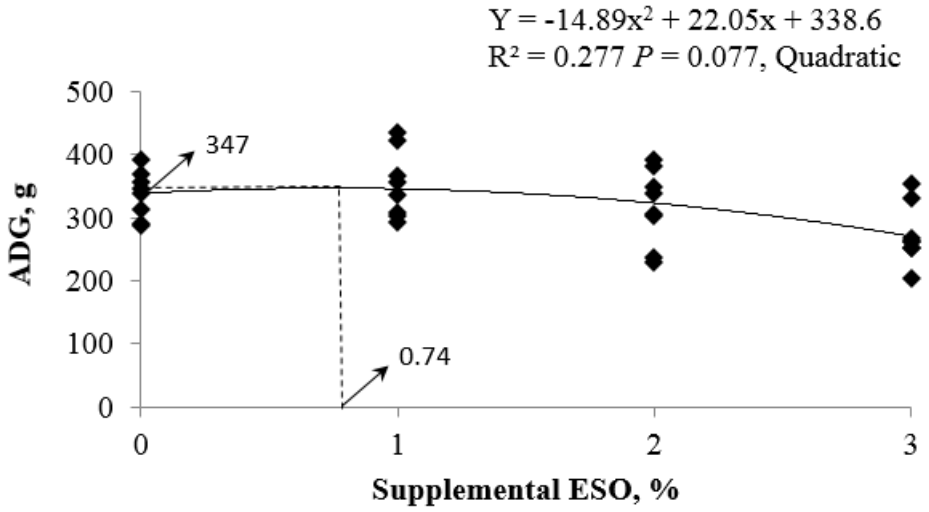

Figure 4. Average daily gain of pigs fed increasing levels of enzyme-treated soy oligopeptides (ESO) from d 0 to 32.

Table 9. Intestinal morphology and enterocytes proliferation of nursery pigs fed diets with increasing levels of enzyme-treated soy oligopeptides (ESO) (0 to 3%)[1]

Item	Added ESO, %				SEM	*P* value	
	0.0	1.0	2.0	3.0		Linear	Quadratic
Duodenum							
Villus height, μm	514	549	512	525	24	0.978	0.666
Villus width, μm	100	94	103	93	7	0.471	0.579
Crypt depth, μm	241	221	214	223	11	0.174	0.135
VH:CD[2]	2.16	2.50	2.42	2.44	0.10	0.252	0.062
Jejunum							
Villus height, μm	437	449	449	424	17	0.612	0.295
Villus width, μm	83	81	82	78	2	0.150	0.407
Crypt depth, μm	186	182	176	182	13	0.586	0.449
VH:CD[2]	2.40	2.48	2.52	2.35	0.20	0.835	0.250
Ki-67, %							
Duodenum	20.18	21.69	20.38	19.70	1.32	0.647	0.415
Jejunum	22.11	22.69	21.05	21.38	1.20	0.276	0.873

[1]Each least squares mean represents 8 pens of 4 pigs per pen (4 barrow pens and 4 gilt pens).

[2]VH:CD: villus height to crypt depth ratio.

Table 10. Diarrhea incidence, and immunological parameters of nursery pigs fed diets with increasing levels of enzyme-treated soy oligopeptides (ESO) (0 to 3%)[1]

Item	Added ESO, %				SEM	*P* value	
	0.0	1.0	2.0	3.0		Linear	Quadratic
Diarrhea incidence[2], %	10.71	1.79	1.79	3.57	-	0.005[3]	-
IgA[4]							
Duodenum, ug/mg protein	3.45	2.93	3.56	3.14	0.60	0.899	0.920
Jejunum, ug/mg protein	1.12	1.26	1.13	1.19	0.16	0.927	0.819
IgG[4]							
Serum, mg/mL	1.08	1.08	1.02	1.16	0.11	0.701	0.529
Tissues, ug/mg protein							
Duodenum	1.09	1.21	1.40	1.35	0.13	0.105	0.525
Jejunum	0.71	0.77	0.77	0.89	0.13	0.258	0.737
TNF-α[4]							
Serum, pg/mL	98.9	91.7	78.2	98.5	7.3	0.652	0.059
Tissues, pg/mg protein							
Duodenum	0.73	0.75	1.43	0.83	0.28	0.271	0.126
Jejunum	0.47	0.95	0.77	0.73	0.33	0.652	0.372
IL-10[4]							
Serum, pg/mL	71.4	72.8	71.9	67.4	4.1	0.441	0.425
Tissues, pg/mg protein							
Duodenum	2.95	2.95	3.25	2.97	0.27	0.721	0.553
Jejunum	2.59	3.09	2.66	2.75	0.27	0.966	0.238

[1]Each least squares mean represents 8 pens of 4 pigs per pen (4 barrow pens and 4 gilt pens).

[2]Pigs were fed a phase 1 diet for the first 11 d followed by a phase 2 diet from d 11 to 32. Fecal scores were 1, normal, firm feces; 1.5, possible slight diarrhea; 2, moderate liquid consistency; 2.5, definitely unformed, and fluid feces; 3, very watery and frothy feces. The occurrence of diarrhea was defined as maintenance of fecal scores of 2.5 or 3 for two continuous days. Diarrhea incidence (%) = number of pigs with diarrhea/(number of pigs × total experimental days) × 100.

[3]Pearson's chi-square test.

[4]IgA: immunoglobulin A; IgG: immunoglobulin G; TNF-α: tumor necrosis factor α; IL-10: interleukin 10.

CHAPTER 4. EFFECTS OF FERMENTED AND ENZYME-TREATED SOYBEAN MEAL ON GROWTH PERFORMANCE, INTESTINAL MORPHOLOGY, AND INTESTINAL MICROBIOTA IN NURSERY PIGS

ABSTRACT: The objective of this experiment was to evaluate the effects of enzyme-treated soybean meal (ESBM), fermented soybean meal (FSBM), and fermented soybean meal containing probiotic microorganisms (PFSBM) when replaced a conventional soybean meal (SBM) on diarrhea incidence, intestinal morphology, intestinal microbiota, and immune response in nursery pigs. A total of 48 pigs (24 barrows and 24 gilts at 7.8 ± 0.7 kg BW) were allotted in a randomized complete block design with sex and initial BW as blocks and randomly assigned to 4 treatments. FSBM, PFSBM, or ESBM were added at the level of 7% into a basal diet and to replace soybean meal in the diets. The experimental period was divided into 3 phases (5, 10, and 12 d, respectively). Fecal scores were determined to measure diarrhea incidence, and jejunal mucosa samples were collected for analysis of intestinal morphology, immune response, and microbiome analysis. Data were analyzed using the Mixed procedure of SAS. G:F of pigs fed ESBM diet was higher ($P < 0.05$) than that of pigs fed SBM diet during d 5 to 15. During d 0 to 27 postweaning, no differences in BW, ADG, and ADFI were found among the treatments. No differences in villus height, villus width, and crypt depth were observed among the treatments. The relative abundance of *Lactobacillaceae* of pigs fed PFSBM was higher ($P < 0.05$) than the other three treatments. At the genus level, the relative abundance of *Lactobacillus* of pigs fed PFSBM was higher ($P < 0.05$) than the other three treatments. The relative abundance of genus *Pseudomonas* of pigs fed PFSBM was tended to be lower ($P = 0.072$) than those fed ESBM diet. The relative abundance of *Stenotrophomonas* of pigs fed PFSBM was lower ($P < 0.05$) than those fed ESBM diet. Mucosa-associated microbiota of pigs fed PFSBM was distinctly different with those fed SBM, ESBM, and FSBM diets. No differences in diarrhea incidence among the treatments were observed. Pigs fed ESBM tended to have a lower ($P = 0.063$) serum TNF-α than

those fed PFSBM diet, whereas no differences in mucosal IgA, mucosal and serum IgG and IL-6 among the treatments were observed.

Key words: enzyme-treated soybean meal, fermented soybean meal, fermented soybean meal containing probiotic microorganisms, growth performance, nursery pigs

INTRODUCTION

Soybean meal (SBM) is the most widely used vegetable protein source in pig diets. However, the presence of a variety of anti-nutritional factors such as trypsin inhibitor, indigestible carbohydrate complexes, and soybean globulins have limited the use of soybean meal in young animal diets (Li et al., 1990). Soybean globulins, such as glycinin and β-conglycinin, can cause hypersensitivity when fed to newly weaned pigs which can induce abnormal intestinal morphology and diarrhea, and thus, reduce growth of pigs (Li et al, 1990; Dréau et al., 1994; Sun et al., 2008).

Microbial fermentation and enzymatic hydrolysis of SBM have been shown to eliminate or reduce the anti-nutritional factors in SBM (Zhu et al., 1998; Hong et al., 2004; Cervantes-Pahm and Stein, 2010). Hong et al. (2004) showed that SBM fermented with *Aspergillus oryzae* have a lower trypsin inhibitor and a higher proportion of small size peptides than the non-fermented SBM. Similarly, Cervantes-Pahm and Stein (2010) reported enzymatic hydrolysis of SBM have improved nutritional value with a lower concentrations of trypsin inhibitor, glycinin, and β-conglycinin than a conventional SBM. Fermented SBM and enzyme-treated SBM when partially replaced a conventional SBM in nursery pig diets reduced diarrhea (Song et al., 2010), enhanced intestinal morphology (Yun et al., 2005), and improved growth performance of pigs (Zhou et al., 2011). In addition, inclusion of fermented or enzyme-treated SBM have been shown to have probiotic effects by the modulation of microbial populations in feces nursery pigs (Jeong and Kim, 2015).

The objective of this study was to test the hypothesis that the use of enzyme-treated SBM (ESBM), fermented SBM (FSBM), and fermented SBM containing probiotic microorganisms (PFSBM) when replaced a conventional SBM may reduce diarrhea, enhance intestinal

morphology, and possess probiotic effects by modulating intestinal microbiota in nursery pigs. To compare the efficacy of the three different types of processed SBM and the conventional SBM in growth and intestinal health of nursery pigs, their effects on growth performance, intestinal morphology, intestinal microbiota, and immune response were evaluated.

MATERIALS AND METHODS

Fermented and Enzyme-treated Soybean Meal

The FSBM and PFSBM were both obtained from a commercial company (CJ CheilJedang Co., Seoul, South Korea). The FSBM was produced by using *Bacillus amyloliquefaciens*, and ultimately sterilized. The PFSBM was produced from the same process with FSBM, but without sterilization step. It contained 1.9×10^7 cfu/g of *Bacillus amyloliquefaciens* in the product. The ESBM (HP300, Hamlet protein, Horsens, Denmark) was produced by an enzymatic hydrolysis using a proprietary mixture of enzymes. The chemical compositions of SBM, ESBM, FSBM, and PFSBM are presented in (Table 1). The molecular weight distribution were analyzed using an analytical ultracentrifuge (ProteomeLab XL-A, Beckman Coulter, Fullerton, CA) according to the method described by Schuck et al. (2002) (Table 2). Briefly, all interference data were collected at a speed of 40,000 rpm in an An-60 Tirotor at 4^{o}C. Homogenized samples were centrifuged at 10,000 rpm for 15 minutes at 4^{o}C (Eppendorf MiniSpin, Eppendorf AG, Hamburg, Germany). A set of 200 scans was collected at 6-min intervals. The proteins were prepared with 2 mL of 20 m*M* Tris-HCl buffer including 0.1% sodium dodecyl sulfate, and 5 m*M* dithiothreitol at pH 7.4. Aliquots (110 uL) of sample solution were loaded into 6 sector sample cells. Absorbance was monitored at 280 nm for the loaded samples. Sedimentation velocity data were

analyzed using the software program SEDFIT (version 11.8) (National Institutes of Health, Bethesda, MD) to generate the sedimentation coefficient distribution of protein samples.

Experimental Diets and Pigs

A total of 48 pigs (PIC 337 x Camborough 22) (24 barrows and 24 gilts at 7.8 ± 0.7 kg BW) were used. Pigs were grouped by BW and sex into 6 blocks and randomly assigned to 4 treatments and 12 replicate pens per treatment in a randomized complete block design. Pigs were housed individually in pens (0.7 × 1.5 m) that were equipped with a feeder and a nipple drinker. Pigs had free access to feed and water. The ESBM, FSBM or PFSBM were added at the level of 7% into a basal diet (Table 3) and replaced 7.7% soybean meal based on evaluated SID AA values (data not shown) in the diets. All diets were made to meet or exceed nutrient requirement estimates for nursery pigs (NRC, 2012). The experimental period was divided into 3 phases (5, 10, and 12 d, respectively). Individual BW and feed disappearance of each pen were recorded at the end of each feeding phase.

Sample Collection

On d 24, blood samples (7 mL) were collected from the jugular vein with Vacutainers (BD, Franklin Lakes, NJ). Blood samples were then centrifuged at 3,000 × g for 15 min at 4^{o}C to obtain serum. On d 27, pigs were euthanized to collect duodenum and jejunum sections for intestinal morphology and intestinal microbiome, and immune response analysis. Tissues for morphology analysis were rinsed with a 0.9% saline solution and fixed with a 10% buffered formalin solution. Mucosa samples were taken from jejunum (around 100 cm before the ileal-cecal junction) for microbiome and immune response analysis.

Diarrhea Incidence

The incidence of diarrhea of piglets was recorded every day for the first 14 d of the experimental period. Pig feces were scored according to the modified method of Liu et al., (2010). Fecal scores were: 1 (normal, firm feces); 1.5 (possible slight diarrhea); 2 (moderate liquid consistency); 2.5 (definitely unformed and fluid feces); 3 (very watery and frothy feces). The occurrence of diarrhea was defined as maintenance of feces at Level 2.5 or Level 3 for two continuous days. Diarrhea incidence was calculated according to the formula reported by Sun et al. (2008): diarrhea incidence (%) = number of pigs with diarrhea/(number of pigs × total experimental days) × 100, where "number of pigs with diarrhea" was the total number of pigs with diarrhea observed each day, and "total experimental days" was 14 d.

Intestinal Morphology

Duodenum and jejunum samples then embedded in paraffin (Paraplast-Sigma) and paraffin sections of 5 µm thick tissue were taken on rotary microtome. The sections were stained with haematoxylin and eosin. Villus height, villus width, and crypt depth of duodenum and jejunum were measured at 40 × magnification as previously described (Shen et al., 2009). Lengths of 10 well-oriented intact villi and their associated crypt were measured in each slide.

Intestinal Microbiota

To sequence mucosa-associated microbiome, DNA were extracted from samples with QIAGEN's QIAamp DNA Stool MiniKit (Qiagen, Crawley, United Kingdom). Samples were sent to Genomics Department of Mako Medical Laboratories (Raleigh, NC) for sequencing. Briefly, samples were prepared for template preparation on the Ion Chef instrument and sequencing on the Ion S5 system (ThermoreFisher). Variable regions V2, V3, V4, V6, V7, V8, and V9 of the 16S rRNA gene were amplified with the Ion 16S Metagenomics Kit

(ThermoFisher Scientific, Inc., Wilmington, DE). Sequences were processed using the Torrent Suite Software (version 5.2.2) (ThermoFisher Scientific, Inc., Wilmington, DE) to produce raw unaligned sequence data files for further analysis. Sequence data analysis, alignment to GreenGenes and MicroSeq databases, alpha and beta diversity plot generation, and OTU table generation were performed by the Ion Reporter Software Suite (version 5.2.2) of bioinformatics analysis tools (ThermoFisher Scientific, Inc., Wilmington, DE). Samples were analyzed using Ion Reporter's Metagenomics 16S workflow powered by Qiime (version w1.1).

Immune Response

The immunoglobulin subsets immunoglobulin A (IgA) and immunoglobulin G (IgG) were measured according to the method described by the manufacturer using ELISA kits (Bethyl, Montgomery, TX, USA). Tumor necrosis factor (TNF)-α was measured in serum, and duodenum and jejunum mucosa according to the method described by the manufacturer using a Porcine TNF-α Colorimetric ELISA Kit (Pierce Biotechnology, Rock- ford, IL). Briefly, 50 μL of standard plus dilute or 100 mL of sample was added to microplate wells which were already coated with capture antibody in conjunction with biotinylated antibody reagent. Detection occurred by the use of a color reagent substrate and a stop solution of diluted hydrochloric acid, and absorbance was read at 450 nm and 540 nm. Concentrations of TNF-α in the duodenum and jejunum were also analyzed using the ELISA kit. Interleukin-6 concentrations in serum and mucosa were measured according to the method described by the manufacturer using ELISA kit (R&D System, MN). Upon analysis, protein concentrations in tissues were measured using a commercial kit (Thermo Fisher Scientific) according to Smith et al. (1985). The measured protein concentration was used to determine the amounts of immunological subsets and cytokines per gram or milligram of protein of each tissue type.

Statistical Analysis

Data analysis was performed using SAS version 9.3 (SAS Inc, Cary, NC, USA). Data were analyzed as a randomized complete block design using the Mixed procedure with pen as the experimental unit. Fixed effect was the treatment, and random effects were initial BW and sex blocks. The LSMEANS procedure was used to calculate mean values for all treatments. When treatment effect was significant or tended to be significant, least squares means among treatments were compared in a pairwise manner using the probability of differences (PDIFF) option with a Tukey adjustment of SAS. The alpha level used for determination of statistical significance was 0.05 and levels between 0.05 and 0.10 were considered as tendency.

RESULTS

Molecular Weight (MW) Distribution of SBM, ESBM, FSBM, and PFSBM

FSBM and PFSBM had much lower molecular weight than that of SBM and ESBM (Table 2). Highest average MW showed in SBM was 134,000 Da (12.9%), and ESBM had 2.1% of relatively high MW, 61,015 Da. The 83.6% of average MW of FSBM was 1,259 Da, and 75.6% of average MW of PFSBM was 7,870 Da.

Growth Performance

During d 5 to 15 (phase 2), G:F of pigs fed ESBM diet was higher ($P < 0.05$) than that of pigs fed SBM diet (Table 4). During d 0 to 27 postweaning, no differences in BW, ADG, and ADFI were found among the treatments.

Intestinal Morphology

No differences in villus height, villus width, crypt depth, and villus height to crypt depth ratio were observed among the treatments (Table 5).

Intestinal Microbiota

Result tables for families and genera operational taxonomic units (OTUs) are shown in Appendix A. In total of 6 phyla were detected (Figure 1). Among them the predominant phyla were Proteobacteria, Firmicutes, and Bacteroidetes. No differences were observed at the phylum level among the treatments. At the family level, the relative abundance of *Lactobacillaceae* of pigs fed PFSBM was higher ($P < 0.05$) than the other three treatments (Figure 2). At the genus level, the relative abundance of *Lactobacillus* of pigs fed PFSBM was higher ($P < 0.05$) the other three treatments. The relative abundance of genus *Pseudomonas* of pigs fed PFSBM was tended to be lower ($P = 0.072$) than those fed ESBM diet. The relative abundance of genus *Stenotrophomonas* of pigs fed PFSBM was lower ($P < 0.05$) than those fed ESBM diet. Alpha diversity estimated with Chao1, Shannon, and Simpson indices were not different among the treatments (Table 6). The principal component analysis (PCA) showed mucosa-associated microbiota of pigs fed PFSBM distinctly different with those fed SBM, ESBM, and FSBM diets (Figure 3).

Diarrhea Incidence and Immune Response

No differences in diarrhea incidence among the treatments were observed (Table 7). Pigs fed ESBM tended to have a lower ($P = 0.063$) serum TNF-α than those fed PFSBM diet. There were no differences among the treatments in mucosal IgA, mucosal and serum IgG and IL-6.

DISCUSSION

Growth Performance

The fermented SBM (FSBM and PFSBM) contained low MW protein. The SBM and ESBM contained high MW of protein similar as reported in a previous study (Cervantes-Pahm

and Stein, 2010). Most of the MW of the two fermented SBM was under 10 kDa. This indicates that antigenic proteins including conglycinin and β-conglycinin were degraded through the fermentation process. The MW of acidic and basic subunits of conglycinin were reported as 36 kDa (Nielsen, 1985) and 22 kDa (Moreira et al., 1979), and MW of β-conglycinin of α, α' and b subunits were reported as approximately 67, 71, and 50 kDa, respectively (Maruyama et al., 1998). The eduction in the antigenic proteins would positively affect growth performance as well as intestinal health of newly weaned pigs. In the present study, the enzyme-treated SBM showed positive effect on growth performance by improving G:F during a part of the feeding period (phase 2). Yang et al. (2007) reported when replaced SBM with an enzyme-treated SBM or two different fermented SBM (fermented with *Aspergillus oryzae* alone or fermented with *Aspergillus oryzae* and *Bacillus subtilis*) in a corn-whey powder based diet fed to pigs (weaned at approximately 23 d), the enzyme-treated SBM and the two different fermented SBM improved ADG during d 0 to 14 postweaning period. Feng et al. (2007) also reported beneficial effects on growth performance when replaced SBM with fermented SBM (fermented with *Aspergillus oryzae*) in a corn-soybean meal based diet fed to pigs (weaned at 35 d). In the current study, except for phase 2 feeding period during which pigs fed ESBM diet had a higher G:F than those fed SBM diet when replaced 7.7% SBM, feeding the four different diets SBM, ESBM, FSBM, and PFSBM did not affect growth performance during 27-d postweaning period. The discrepancy of the results may be related to the different microorganism used to ferment FSBM, inclusion levels, and weaning age. Future study with different inclusion level is recommended to maximize the potential beneficial effects of fermented and enzyme-treated SBM on growth of pigs.

Intestinal Morphology and Intestinal Microbiota

The presence of antigenic proteins glycinin and β-conglycinin in SBM reduced villus height of the small intestine in weaned pigs (Li et al., 1991). These antigenic proteins were degraded via fermentation or enzymatic hydrolysis (Hong et al., 2004; Hrckova et al., 2009). This would improve protein utilization which may in turn positively affect intestinal architectural structure. Previously, fermented SBM when replaced conventional SBM at the level of 6% showed to positively enhance intestinal morphology in weaned pigs when fed for 14 d after weaning (Yun et al., 2005). In the present study, no beneficial effects were found on villus height, villus width, and crypt depth when replaced the conventional SBM with ESBM, FSBM, and PFSBM. This may can partially explain the observation in growth performance that no differences among the treatments were detected during the entire experimental period.

Fermented and enzyme-treated SBM have been shown to exhibit probiotic effect in pigs and broiler chickens. Jeong and Kim (2015) showed when partially replaced fish meal with fermented SBM and enzyme-treated SBM modulated fecal microbial populations in nursery pigs by increasing *Lactobacillus* counts without affecting *Escherichia coli* counts. Similarly, in a broiler chicken study, soybean meal fermented with *Bacillus subtillis* containing probiotic microorganism increased lactic acid bacteria while decreased *Coli*-form bacteria in cecal contents than those fed conventional soybean meal (Kim et al., 2016). Catalán et al. (2017) reported fermented SBM can increase mucin production in proximal intestine when fed to Atlantic salmon. Microbial community within the outer layer of the mucosa is closely connected with host tissues, dietary intervention showed a long lasting effect on ileal mucosa-associated microbiome, but not on that of the ileal digesta during postweaning period (Levesque et al., 2012). In the present study, the potential beneficial effect of different types of processed SBM on

mucosa-associated microbiota in the jejunum was evaluated. Compositional differences among the treatments revealed that mucosa-associated microbiota of pigs fed PFSBM diet distinctly different with those fed SBM, ESBM, and FSBM diets. Pigs fed PFSBM diet had a higher relative abundance of lactic acid-producing bacteria, *Lactobacillus* than those fed SBM, ESBM, and FSBM diets. The relative abundance of genus *Pseudomonas* of pigs fed PFSBM was tended to be lower than those fed ESBM diet, and the relative abundance of *Stenotrophomonas* of pigs fed PFSBM was lower than those fed ESBM diet. Dou et al. (2017) reported that diarrhea-resistant pigs had a higher abundance of *Lactobacillaceae* at postnatal than those developed diarrhea after weaning. Burrough et al. (2017) showed pigs that did not develop swine dysentery had higher abundances of *Lactobacillales* as compared to those of pigs developed that disease. *Lactobacillus* spp. exerted the ability to bind porcine intestinal mucin and exclude against *Salmonella* and *Escherichia* coli in vitro (Li et al., 2008). The increased relative abundance of *Lactobacillus* observed in pigs fed PFSBM compared to the other three diets may be related to the probiotic effects exhibited by *Bacillus amyloliquefaciens* containing in the PFSBM. The *Bacillus amyloliquefaciens* strain showed probiotic effect via inducing autophagy and antibacterial activity against *Escherichia coli* in a murine model (Wu et al., 2017). Additionally, the results of the present study revealed that pigs fed PFSBM diet tended to have a lower relative abundance of *Pseudomonas* and a lower *Stenotrophomonas* than those fed ESBM diet, and this change may have been induced by the probiotic bacteria *Bacillus amyloliquefaciens* in the PFSBM. *Pseudomonas*, a mucin degrading bacteria (Aristoteli and Willcox, 2003), its ability to colonize in the intestine in mice was inhibited by *Lactobacillus casei* (Driessen and De Boer, 1989). Furthermore, the genus *Stenotrophomonas* acted as infectious pathogens of Crohn's disease (Knosel et al., 2009). The reduction in *Stenotrophomonas* may have been induced by the

probiotic effect of *Bacillus amyloliquefaciens* in PFSBM. Results from the current study indicate that fermented SBM containing probiotic microorganisms (*Bacillus amyloliquefaciens*, 1.9 x 10^7 cfu/g) show beneficial effects on community composition of mucosa-associated microbiota and may can be considered as an effective carrier of probiotics. These results may provide insights on how the mucosa-associated bacterial communities of the proximal small intestine change by different processed SBM consumption.

Diarrhea Incidence and Immune Response

In the present study, no differences in diarrhea incidence were observed among the dietary treatments. Previous studies have shown that fermented or enzyme-treated SBM can alleviate allergic reactions induced by conglycinin and β-conglycinin in pigs. Enzyme-treated soybean meal fed to weaned pigs increased T lymphocytes, and its subsets CD4+ and CD8+ concentrations in peripheral blood (Zhou et al., 2011). Zhu et al. (2017) also showed fermented SBM when replaced conventional SBM increased immune function by increasing serum IgG, IgM and IgA levels. In the present study, the concentrations of pro-inflammatory cytokines, TNF-α and IL-6 in the mucosa, as well as the immunoglobulin subsets, mucosal IgA and IgG in the mucosa and in serum were not affected by the dietary treatments. However, pigs fed ESBM tended to have a lower serum TNF-α than those fed PFSBM diet. Daudelin et al. (2011) reported probiotics supplementation reduced attachment of enterotoxigenic *Escherichia coli* to intestinal mucosa and induced a stronger inflammatory reaction with up-regulated IL-6 production in pigs challenged with *E. coli*. Over expression of TNF-α may increase potential pathogen invasion by increasing intestinal permeability (McKay and Baird, 1999; Pié et al., 2004). Considering no differences were found in serum and mucosal immunoglobulin levels among all the treatments, and no differences were found in TNF-α and IL-6 among PFSBM, SBM, and FSBM treatments,

the observation found from the present study that PFSBM inclusion tended to increase serum TNF-α when compared to ESBM may not be related to inflammatory response.

CONCLUSION

Pigs fed ESBM diet positively affected feed efficiency of pigs during a part of the postweaning period, whereas no differences were found in growth performance of pigs fed the SBM, ESBM, FSBM, and PFSBM diets during the entire experimental period. Intestinal morphology and diarrhea incidence were not affected by feeding of the four different types of diets. The PFSBM inclusion showed a greatest impact on mucosa-associated microbiota composition among the different dietary treatments.

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Table 1. Chemical composition of soybean meal (SBM), enzyme-treated soybean meal (ESBM), fermented soybean meal (FSBM), and fermented soybean meal containing probiotic microorganism (PFSBM)

Item	SBM	ESBM	FSBM	PFSBM
DM, %	89.9	92.0	97.1	95.3
CP, %	47.0	56.0	42.2	56.8
Indispensable AA, %				
Arg	2.84	3.62	3.29	3.17
His	1.11	1.43	1.46	1.30
Ile	2.04	2.51	2.52	2.53
Leu	3.38	4.15	4.20	4.12
Lys	2.98	3.39	3.40	3.32
Met	0.66	0.21	0.24	0.24
Phe	2.22	2.71	2.93	2.70
Thr	2.12	3.54	3.11	3.06
Trp	0.53	0.66	0.66	0.69
Val	2.25	2.63	2.63	2.73
Microbial population	-	-	1.1×10^2 cfu/g[1]	1.9×10^7 cfu/g[1]

[1]*Bacillus amyloliquefaciens.*

Table 2. Molecular weight distribution of soybean meal (SBM), enzyme-treated soybean meal (E SBM), fermented soybean meal (FSBM), and fermented soybean meal containing probiotic micr oorganism (PFSBM) measured by analytical ultracentrifuge

Item	Molecular weight, Da	Percentage, %
SBM	134,000	12.9
	30,100	55.6
	883	31.5
ESBM	61,015	2.1
	14,700	83.9
	943	14.0
FSBM	10,750	16.4
	1,259	83.6
PFSBM	7,870	75.6
	440	24.4

Table 3. Composition of experimental diets[1]

Item	Phase 1 (d 0 to 5 postweaning)				Phase 2 (d 5 to 15postweaning)				Phase 3 (d 15 to 27 postweaning)			
	SBM	ESBM	FSBM	PFSBM	SBM	ESBM	FSBM	PFSBM	SBM	ESBM	FSBM	PFSBM
Ingredient, %												
Corn, yellow dent	40.22	41.56	41.57	41.57	55.21	56.39	56.40	56.40	66.78	68.02	68.04	68.04
Soybean meal, dehulled	20.00	12.30	12.30	12.30	23.00	15.30	15.30	15.30	28.00	20.30	20.30	20.30
Whey permeate	22.00	22.00	22.00	22.00	10.00	10.00	10.00	10.00	0.00	0.00	0.00	0.00
Poultry meal	7.00	7.00	7.00	7.00	3.00	3.00	3.00	3.00	0.00	0.00	0.00	0.00
Blood plasma	6.00	6.00	6.00	6.00	3.00	3.00	3.00	3.00	0.00	0.00	0.00	0.00
ESBM	0.00	7.00	0.00	0.00	0.00	7.00	0.00	0.00	0.00	7.00	0.00	0.00
FSBM	0.00	0.00	7.00	0.00	0.00	0.00	7.00	0.00	0.00	0.00	7.00	0.00
PFSBM	0.00	0.00	0.00	7.00	0.00	0.00	0.00	7.00	0.00	0.00	0.00	7.00
Poultry fat	2.00	1.40	1.40	1.40	2.50	2.00	2.00	2.00	2.00	1.50	1.50	1.50
L-Lys HCl	0.50	0.49	0.49	0.49	0.50	0.50	0.50	0.50	0.47	0.46	0.46	0.46
DL-Met	0.23	0.22	0.21	0.21	0.19	0.18	0.17	0.17	0.15	0.14	0.13	0.13
L-Thr	0.15	0.13	0.13	0.13	0.15	0.13	0.13	0.13	0.15	0.13	0.12	0.12
Dicalcium phosphate	0.40	0.40	0.40	0.40	0.90	0.90	0.90	0.90	1.15	1.15	1.15	1.15
Limestone, ground	0.85	0.85	0.85	0.85	0.90	0.95	0.95	0.95	0.90	0.90	0.90	0.90
Vitamin premix[2]	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Mineral premix[3]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Salt	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22
Zinc oxide	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.00	0.00	0.00	0.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 3. Continued

Calculated composition												
DM, %	90.8	90.9	90.9	90.9	90.1	90.2	90.2	90.2	89.4	89.4	89.4	89.4
ME, kcal/kg	3,420	3,421	3,423	3,423	3,421	3,425	3,427	3,427	3,386	3,392	3,394	3,394
CP, %	23.4	23.7	23.7	23.7	20.8	21.1	21.1	21.1	19.5	19.9	19.8	19.8
SID[4] Lys, %	1.50	1.50	1.50	1.50	1.35	1.35	1.35	1.35	1.23	1.23	1.23	1.23
SID Met + Cys, %	0.82	0.82	0.82	0.82	0.74	0.74	0.74	0.74	0.68	0.68	0.68	0.68
SID Trp, %	0.25	0.25	0.25	0.25	0.22	0.22	0.22	0.22	0.20	0.20	0.20	0.20
SID Thr, %	0.88	0.88	0.88	0.88	0.79	0.79	0.79	0.79	0.73	0.73	0.73	0.73
SID Val	0.79	0.82	0.83	0.83	0.76	0.79	0.79	0.79	0.75	0.78	0.79	0.79
Ca, %	0.86	0.85	0.85	0.85	0.80	0.81	0.81	0.81	0.71	0.71	0.71	0.71
STTD[5] P, %	0.45	0.45	0.45	0.45	0.40	0.40	0.40	0.40	0.33	0.33	0.33	0.33
Total P, %	0.67	0.67	0.67	0.67	0.63	0.64	0.64	0.64	0.59	0.59	0.59	0.59
Analyzed value												
CP, %	21.3	21.9	21.5	21.9	19.4	19.5	21.2	19.2	18.6	18.2	18.1	17.9

[1]SBM: Soybean meal; ESBM: enzyme-treated soybean meal; FSBM: fermented soybean meal; PFSBM: fermented soybean meal containing *Bacillus amyloliquefaciens*.

[2]The vitamin premix provided the following per kilogram diet: 13,227,513 IU of vitamin A; 3,968,254 IU of vitamin D_3; 66,138 IU of vitamin E; 40 mg of vitamin B_{12}; 13,228 mg of riboflavin; 110,229 mg of niacin; 22,046 mg of d-pantothenic acid; 3,968 mg of menadione; 40 IU of biotin.

[3]The mineral premix provided the following composition: Mineral premix provided the following composition: 1.1 % of Cu; 198.0 mg/kg of I; 7.3 % of Fe; 2.2 % of Mn; 198.0 mg/kg of Se; 7.3 % of Zn.

[4]SID: standardized ileal digestible.

[5]STTD: standardized total tract digestible.

Table 4. Growth performance of pigs fed diets with enzyme-treated soybean meal (ESBM), fermented soybean meal (FSBM), and fermented soybean meal containing probiotic microorganism (PFSBM) [1,2]

Item	Treatment				SEM	*P* value
	SBM	ESBM	FSBM	PFSBM		
BW, kg						
d 0	7.42	7.47	7.43	7.44	0.72	0.923
d 5	8.73	8.77	8.94	8.73	1.01	0.875
d 15	11.63	12.83	12.58	11.84	1.47	0.300
d 27	17.65	19.91	18.87	18.03	2.02	0.212
ADG, g						
d 0 to 5	263	260	302	257	65	0.838
d 5 to 15	290	406	364	312	51	0.111
d 15 to 27	502	590	524	516	43	0.185
d 0 to 27	379	461	424	392	50	0.227
ADFI, g						
d 0 to 5	325	359	371	308	53	0.657
d 5 to 15	582	603	583	522	63	0.516
d 15 to 27	825	930	852	800	57	0.115
d 0 to 27	632	703	663	606	59	0.262
G:F						
d 0 to 5	0.857	0.686	0.814	0.778	0.065	0.268
d 5 to 15	0.487[a]	0.668[b]	0.596[ab]	0.590[ab]	0.042	0.029
d 15 to 27	0.602	0.633	0.610	0.645	0.024	0.244
d 0 to 27	0.588	0.652	0.627	0.642	0.030	0.108

[1]SBM: Soybean meal; ESBM: enzyme-treated soybean meal; FSBM: fermented soybean meal; PFSBM: fermented soybean meal containing *Bacillus amyloliquefaciens*.

[2]Means within a row lacking a common superscript letters are significantly different ($P < 0.05$).

Table 5. Intestinal morphology of pigs fed enzyme-treated soybean meal (ESBM), fermented soybean meal (FSBM), and fermented soybean meal containing probiotic microorganism (PFSBM)[1]

Item	Treatment				SEM	*P* value
	SBM	ESBM	FSBM	PFSBM		
Villus height, μm	361	362	358	388	18	0.778
Villus width, μm	136	136	148	141	16	0.710
Crypt depth, μm	122	150	137	152	28	0.439
VH:CD[2]	3.38	2.79	2.98	2.97	0.63	0.630

[1]SBM: Soybean meal; ESBM: enzyme-treated soybean meal; FSBM: fermented soybean meal; PFSBM: fermented soybean meal containing *Bacillus amyloliquefaciens*.

[2]VH:CD: villus height to crypt depth ratio.

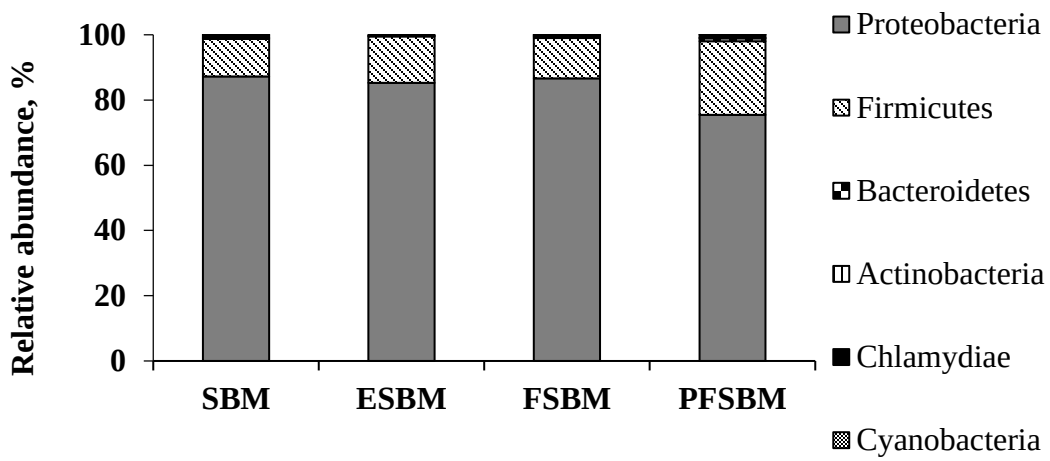

Figure 1. Relative abundance of phyla detected in mucosa-associated microbiota of nursery pigs fed conventional soybean meal (SBM), enzyme-treated soybean meal (ESBM), fermented soybe an meal (FSBM), and fermented soybean meal containing probiotic microorganism (PFSBM).

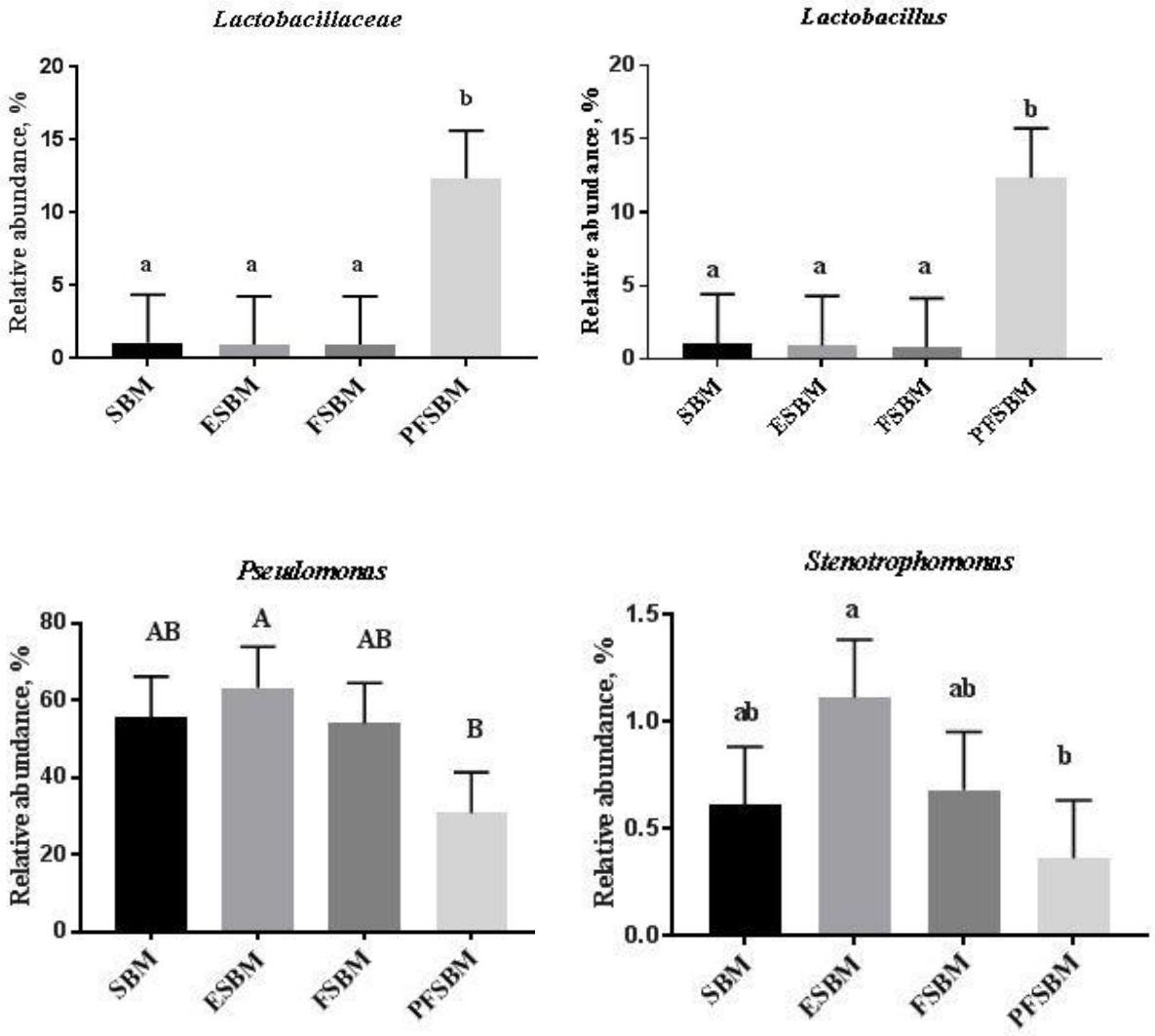

Figure 2. Relative abundance of family *Lactobacillaceae* and genus *Lactobacillus, Pseudomonas and Stenotrophomonas* of mucosa-associated microbiota of nursery pigs fed conventional soybea n meal (SBM), enzyme-treated soybean meal (ESBM), fermented soybean meal (FSBM), and fer mented soybean meal containing probiotic microorganism (PFSBM).
Means lacking a common small letters are significantly different ($P < 0.05$), and those lacking a c ommon capital letters indicate means are tended to be different ($0.05 \leq P < 0.10$).

Table 6. Alpha diversity estimated with Chao1, Shannon, and Simpson indices of jejunal mucosa-associated microbiota of pigs fed enzyme-treated soybean meal (ESBM), fermented soybean meal (FSBM), and fermented soybean meal containing probiotic microorganism (PFSBM)[1]

Item	Treatment				SEM	*P* value
	SBM	ESBM	FSBM	PSBM		
Chao1	18.9	12.0	16.9	15.4	2.5	0.319
Shannon	1.39	0.95	1.28	1.23	0.15	0.215
Simpson	0.52	0.38	0.51	0.48	0.05	0.232

[1]SBM: Soybean meal; ESBM: enzyme-treated soybean meal; FSBM: fermented soybean meal; PFSBM: fermented soybean meal containing *Bacillus amyloliquefaciens*.

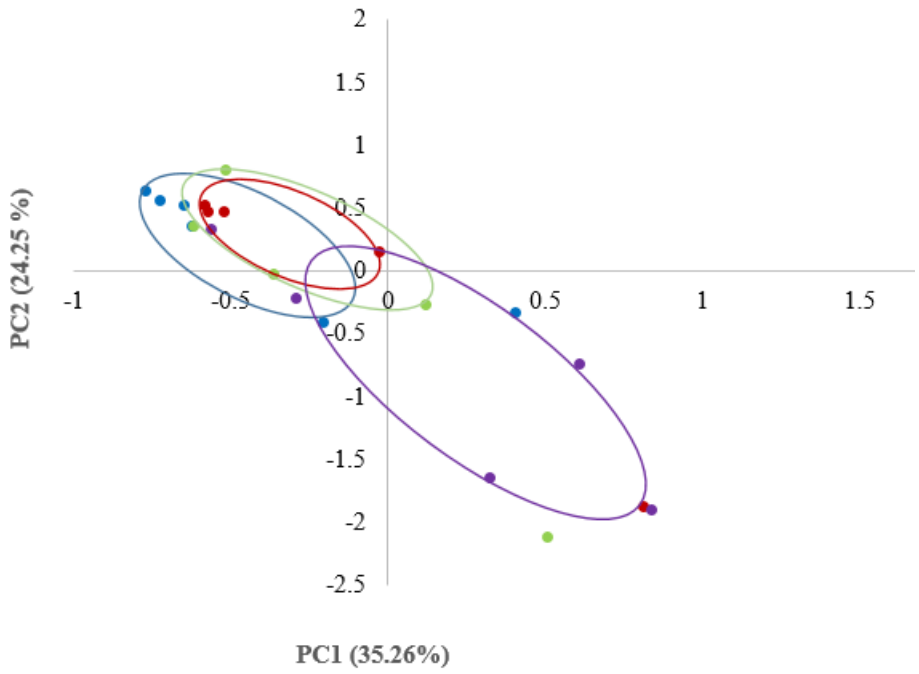

Figure 3. Principal component analysis (PCA) of jejunal mucosal microbiota of nursery pigs. Red circles represent soybean meal (SBM) treatment; blue circles represent enzyme-treated soybean meal (ESBM) treatment; green circles represent fermented soybean meal (FSBM) treatment, and purple circles represent fermented soybean meal containing probiotic microorganism (PFSBM) treatment.

Table 7. Diarrhea incidence and immunological parameters of nursery pigs fed enzyme-treated soybean meal (ESBM), fermented soybean meal (FSBM), and fermented soybean meal containing probiotic microorganism (PFSBM)[1,2]

Item	Treatment				SEM	*P* value
	SBM	ESBM	FSBM	PFSBM		
Diarrhea incidence[3], %	1.79	0.00	0.00	0.00	-	0.145[4]
IgA[5]						
Jejunum, ug/mg protein	0.86	1.28	0.95	1.10	0.16	0.264
IgG[5]						
Serum, mg/mL	2.38	1.94	1.76	2.00	0.22	0.202
Jejunum, ug/mg protein	1.39	1.08	1.19	1.33	0.28	0.729
TNF-α[5]						
Serum, pg/mL	124.9^{AB}	92.5^{A}	113.6^{AB}	141.7^{B}	17.9	0.063
Jejunum, pg/mg protein	1.84	1.70	1.82	2.13	0.19	0.448
IL-6[5]						
Serum, pg/mL	14.4	11.3	11.6	17.3	2.6	0.131
Jejunum, pg/mg protein	1.64	1.30	1.53	1.40	0.17	0.197

[1]SBM: Soybean meal; ESBM: enzyme-treated soybean meal; FSBM: fermented soybean meal; PFSBM: fermented soybean meal containing *Bacillus amyloliquefaciens*.

[2]Means lacking a common superscript capital letters are tended to be different ($0.05 \leq P < 0.10$).

[3]Fecal scores were 1, normal, firm feces; 1.5, possible slight diarrhea; 2, moderate liquid consistency; 2.5, definitely unformed, and fluid feces; 3, very watery and frothy feces. The occurrence of diarrhea was defined as maintenance of fecal scores of 2.5 or 3 for two continuous days. Diarrhea incidence (%) = number of pigs with diarrhea/(number of pigs × total experimental days) × 100.

[4]Pearson's chi-square test.

[5]IgA: immunoglobulin A; IgG: immunoglobulin G; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6.

CHAPTER 5. EFFECTS OF DIETARY LYSOPHOSPHOLIPID COMPLEX ON FAT UTILIZATION, INTESTINAL MORPHOLOGY, BARRIER FUNCTION, AND GROWTH PERFORMANCE OF NURSERY PIGS

ABSTRACT: The objective of this study was to evaluate the supplemental effect of lysophospholipid complex (LPL; Lipidol, Easybio System, Korea) on growth performance, fat utilization, and intestinal health in nursery pigs. The LPL was produced by an enzymatic modification of phospholipids eliminating a fatty acid at the sn-2 position. Twenty-four newly weaned pigs (12 barrows and 12 gilts at 7.2 ± 0.1 kg BW) were randomly allotted to 2 treatments in a randomized complete block design. Sex and initial BW were used as blocks. Pigs were fed a basal diet supplemented with either 0.0 or 0.1 % LPL based on 2 phases (7 and 12 d, respectively). Body weight and feed consumption were recorded on d 7, 14, and 19. Titanium dioxide (0.5%) was added to the diets from d 14 as an indigestible external marker. On d 19, all pigs were euthanized to collect ileal digesta to measure AID of ether extract, fatty acids, DM, CP, and GE. Duodenum and jejunum segments were collected for morphology evaluation. Mucosa samples and serum were collected to measure immune response as well as tight junction proteins. Data were analyzed using the Mixed procedure of SAS. Dietary LPL increased ADG ($P < 0.05$) and ADFI ($P < 0.05$) of nursery pigs from d 14 to 19. Supplementation of LPL tended to increase AID of ether extract ($P = 0.086$) and C18:2 ($P = 0.059$). Supplementation of LPL increased villus height of duodenum ($P < 0.05$) and jejunum ($P < 0.05$) and also tended to increase ($P = 0.086$) villus height to crypt depth ratio in duodenum. Supplementation of LPL increased ($P < 0.05$) the abundance of claudin-1, and tended to increase ($P = 0.056$) zonula occludens-1 in the jejunal mucosa. Dietary LPL tended to decrease ($P = 0.083$) immunoglobulin G in serum. In conclusion, dietary supplementation of LPL positively affected growth of nursery pigs and showed a potential to enhance lipid digestibility, and improved intestinal morphology and barrier function of nursery pigs.

Key words: apparent ileal digestibility, growth performance, intestinal morphology, lysophospholipid, nursery pigs

INTRODUCTION

Animal fats and vegetable oils are commonly supplemented into pig diets to increase growth of pigs, and also to increase diet energy density (Stahly, 1984), palatability (Azain, 2001), and pellet quality (Maxwell and Carter, 2001). Digestion of fat mainly occurs in the small intestine. Coarse emulsion is formed by emulsification process which increases active surface to aid digestion by pancreatic lipase, and the products of lipase hydrolysis are formed into mixed micelles by mixing with emulsifiers (such as bile salts), which facilitate diffusion into enterocytes. Digestibility of fat from solid feed in newly weaned pigs is as low as 73% (Frobish et al., 1967, 1969) and increases gradually to the pre-weaning level ranging from 4 to 6 weeks postweaning (Wiseman, 1984; Cera et al., 1988). Synthesis of hepatic bile acid is low at weaning in pigs (Lewis et al., 2000), therefore, emulsification is a rate-limiting step in the digestion of dietary fat during early period after weaning.

Epithelial cells of the small intestine are the primary site of fatty acids and monoglycerides absorption. Weaning of pigs induces intestinal morphological changes and dysfunction with most evident in villus atrophy and impaired barrier function, which may result in decrease in nutrient absorption and increase the entry of potential pathogenic microorganisms. (Hedemann et al., 2003; Pié et al., 2004; Moeser et al., 2007). Epithelial cells continuously migrate along crypt-villus axis to form mature absorptive enterocytes (Heath, 1996; Yang et al., 2016). The process of epithelial cells resealing and regaining barrier function after injury including weaning-induced intestinal dysfunction is termed restitution (Blikslager et al., 2007).The renewal of epithelial cells via epithelial cell restitution plays key role in maintaining functions of small intestine (Blikslager et al., 2007).

Lysophospholipids are produced from modification of phospholipids (physical, chemical or enzymatic methods) possessing improved emulsifying properties (Van Nieuwenhuyzen, 1981; Joshi et al., 2006). Hydrolysis of phospholipids by phospholipase A_2 at the sn-2 position of fatty acid yields lysophospholipids with different head group including lysophosphatidic acid, lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylinositol, and lysophosphatidylserine (Wendel, 2000; Joshi et al. 2006; Van Nieuwenhuyzen and Tomás, 2008). Soybean lysophospholipids have higher emulsion formation capacity than soybean phospholipids with hydrophilic-lipophilic balance (HLB) value of 19 (Pokorný, 2006), whereas the phospholipids have values of 5 (Estiasih et al., 2013). The HLB values vary from 0 to 20, higher values are assigned to emulsifiers those are more hydrophilic. In addition, lysophospholipids exhibit a wide range of diverse biological activities mediated by specific G-protein coupled receptors, involving in various physiological processes such as cell growth, proliferation and differentiation (Moolenaar 1999; Gardell et al., 2006; Choi and Chun, 2013). Lysophospholipids involve in epithelial cell restitution via cytoskeletal remodeling with activation of actin filament redistribution and stress fiber formation (Hines et al., 2000). It showed to reduce mucosal damage and inflammation by increasing epithelial cell restitution when induced colitis in rats (Sturm et al., 1999). In broiler chickens, lysophospholipids increased crypt cell mitosis (Khonyoung et al., 2015), and enhanced villus morphology (Boontiam et al., 2017).

Lysophospholids as an emulsifier and bioactive mediators would increase fat utilization as well as improve intestinal development and function when fed to nursery pigs. The objective of the present experiment, therefore, was to test the hypothesis that the supplementation of lysophospholipid complex may improve lipid and fatty acid digestibility, enhance intestinal

morphology and enterocyte proliferation, and intestinal barrier function, overall increase growth performance of nursery pigs.

METERIALS AND METHODS

The experimental protocol was approved by the Institutional Animal Care and Use Committee at North Carolina State University (Raleigh, NC).

Experimental Diets and Pigs

A total of 24 pigs (12 barrows and 12 gilts at 7.2 ± 0.1 kg BW) were randomly allotted to 2 treatments in a randomized complete block design. Sex and initial BW were used as blocks. Pigs were housed individually in pens (0.7 × 1.5 m) that were equipped with a feeder and a nipple drinker. Pigs had free access to feed and water. A corn-soybean meal-based basal diet (Table 1) was formulated to meet or exceed requirement estimates for nursery pigs (NRC, 2012). Pigs were fed a basal diet supplemented with either 0.0 or 0.1% LPL in 2 phases (7 and 12 d, respectively). The analyzed fatty acid content of the basal diet fed during phase 2 was mainly a mixture of long-chain fatty acids (Table 2). Individual BW and feed disappearance of each pen were recorded on d 7, 14, and 19. From d 14, titanium dioxide (0.5%) was added to the diets as an external marker for calculation of ileal digestibility.

Sample Collection

Blood samples (7 mL) were collected via puncture of jugular vein of the pig on d 18. Serum samples were then obtained by centrifuging (3,000 × g, for 15 min at 4°C) and stored at -80°C until analyzed for immune responses. Pigs were euthanized on d 19 for sample collection. Immediately after the euthanasia, an ileal portion (a portion of 20 cm prior to ileo-cecal connection) of small intestine was used to obtain digesta in the ileum. Duodenum and jejunum

segments were collected for morphology evaluation. Mucosa samples from duodenum and jejunum were collected for analysis of barrier function and immune response. Digesta from ileum was stored in sterile container and kept frozen at -20°C. Frozen ileal digesta were freeze-dried (24D × 48, Virtis, Gardiner, NY) for storage and chemical analysis.

Apparent Ileal Digestibility of Lipid and Fatty Acids

The titanium dioxide was used as an indigestible marker (Tancharoenrat et al., 2014; Jansen et al., 2015). Titanium concentration in the diets and freeze dried digesta were analyzed according to Myers et al. (2004). Total lipid was extracted using a Soxtec system (Avanti 2055, Foss North America, Eden Prairie, MN) with diethyl ether as a solvent, and fatty acid methylation was performed as previously described (Gatlin et al., 2002; Price et al., 2013). Apparent ileal digestibility (AID, %) of lipid and fatty acids were calculated using the titanium concentration in the diets and digesta by using AID = 100 – [(ND/NF) × (TiF / TiD) × 100], where ND is the nutrient concentration present in the ileal digesta, NF is the concentration of nutrient present in the feed, TiF is the titanium concentration in the feed, and TiD is the titanium concentration in the ileal digesta.

Intestinal Morphology, and Proliferation of Enterocytes

Duodenum and jejunum samples were rinsed with a 0.9% saline solution, fixed with a 10% buffered formalin solution immediately after slaughter. Tissue samples then embedded in paraffin (Paraplast-Sigma) and paraffin sections of 5 µm thick tissue were taken on rotary microtome. The sections were stained with haematoxylin and eosin. Villus height, villus width, and crypt depth of duodenum and jejunum were measured at 40 × magnification as previously described (Shen et al., 2009). Lengths of 10 well-oriented intact villi and their associated crypt were measured in each slide. To count the percentage of Ki-67 antigen forming cells present in

the jejunum, immunohistochemistry staining on histological sections was performed using the modified method of Rekiel et al. (2010).

Tight Junction Proteins

Four samples of jejunal mucosa from each treatment were used to measure tight junction proteins concentration as described by Yang et al. (2015). Briefly, mucosal samples (100 mg) of jejunum were weighed and suspended into 0.5 mL RIPA lysis and extraction buffer containing 5 µL protease inhibitor cocktail. After homogenizing on the ice, the homogenate was centrifuged at 10,000 × *g* at 4 for 10 min to collect supernatant. Protein concentration of the supernatant was adjusted to 8 µg/µL by using a bicinchoninic acid assay (Thermo Fisher Scientific Inc. Rockford, IL). The adjusted supernatant was denatured at 100°C for 5 min in the water bath, and was loaded in each well for SDS-PAGE. After SDS-PAGE, the gel was moved on polyvinylidene difluoride (PVDF) membrane for transferring a target protein to membrane. Protein was electrophoretically transferred at 90 mV for 1 hour. These was then blocked in 5% skim milk, and incubated overnight at 4^{o}C with primary antibodies against claudin-1, occludin, zonula occludens (ZO)-1, and ß-actin. The membrane was subsequently washed and incubated for 1 h at room temperature with horseradish-conjugated secondary antibodies. The immunoblot was developed with the DAB substrate kit (34002; Pierce, Rockford, IL). Density of bands was identified by using image analyzer software (LI-COR Biosciences, Lincoln, NE).

Immunological Parameters

The immunoglobulin subsets immunoglobulin A (IgA) and immunoglobulin G (IgG) were measured according to the method described by the manufacturer using ELISA kits (Bethyl, Montgomery, TX, USA). TNF-α was measured in serum according to the method described by the manufacturer using a Porcine TNF-α Colorimetric ELISA Kit ELISA kit (Pierce

Biotechnology, Rock- ford, IL). Upon analysis, protein concentrations in tissues were measured using a commercial kit (Thermo Fisher Scientific) according to Smith et al. (1985). Serum samples were diluted to 1:8,000 and mucosal samples to 1:400. The measured protein concentration was used to determine the amounts of immunological per gram or milligram of protein of each tissue type.

Statistical Analysis

Data were analyzed as a randomized complete block design using the Mixed procedure of SAS version 9.3 (SAS Inc, Cary, NC, USA), and the pen was the experimental unit. Differences between least squares means were determined using a least significant difference test (PDIFF). The alpha level used for determination of statistical significance was 0.05 and levels between 0.05 and 0.10 were considered as tendency.

RESULTS

Growth Performance

Supplementation of LPL increased ADG ($P < 0.05$), and ADFI ($P < 0.05$) from d 14 to 19 (Table 3). No differences were observed in ADG and ADFI between treatments in other phases. G:F were not affected by the dietary LPL during the entire 19 d.

Apparent Ileal Digestibility of Lipid and Fatty acids

Dietary LPL tended to increase ($P = 0.086$) AID of ether extract (Table 4). Supplementation of LPL tended to increase ($P = 0.059$) AID of C18:2. AID of other fatty acids were not affected by the dietary LPL.

Intestinal Morphology, Proliferation of Enterocytes, Tight junction proteins, and Immune Response

Supplementation of LPL increased villus height ($P < 0.05$), and tended to increase villus height to crypt depth ratio ($P = 0.086$) in the duodenum (Table 5). In the jejunum, supplementation of LPL increased ($P < 0.05$) villus height, and tended to increase ($P = 0.062$) crypt depth. Dietary LPL did not affect percentage of Ki-67 positive cells in the jejunal crypts. Supplementation of LPL increased ($P < 0.05$) abundance of claudin-1, and tended to increase ($P = 0.056$) abundance of zonula occludens-1 in the jejunal epithelial cells (Figure 1). Supplementation of LPL did not affect IgA levels in the duodenum and jejunum (Table 6). Dietary LPL tended to decrease ($P = 0.083$) IgG in serum. Supplementation of LPL did not affect TNF-α concentrations in serum, and in the duodenum and jejunum.

DISCUSSION

Growth Performance, and Lipid Digestibility

In the previous studies with nursery pigs fed diets with emulsifiers, improved growth performance were reported (Xing et al., 2004; Zhao et al., 2015). In the present study, supplementation of LPL improved ADG and ADFI during the last five days of the experiment (from d 14 to d 19 postweaning period). The digestion of fat mainly occurs in the small intestine by pancreatic lipase. To aid the digestion, coarse emulsion is formed by emulsification which provides available surface area for the lipase to interact with. The digestion products are then formed into mixed micelles by emulsifiers, which facilitate diffusion into enterocytes. Emulsification of fat is a rate-limiting step because synthesis of hepatic bile acid is low at weaning in pigs (Lewis et al., 2000). With the hypothesis that LPL supplementation would

increase fat utilization in nursery pigs, AID of ether extract and fatty acid were measured. It showed a potential to increase AID of ether extract and C18:2. Zhao et al. (2015) reported that lysophospholipids supplementation improved apparent total tract digestibility of crude fat when added to a diet containing 3% tallow at d 14 and d 35 postweaning. In a broiler study, Zhang et al. (2011) reported lysophospholipids supplementation increased the apparent total tract digestibility of C16:0 and C18:1 during starter phase, and C18:2 and C18:3 during grower phase when fed diets containing soybean oil, tallow, and poultry fat, respectively, whereas no interactions were found between fat source and lysophospholipids. The fat in the diet fed during the phase 2 was mainly composed of long-chain fatty acids with the UFA to SFA ratio of 2.21. Inconsistent results about the effect of lysophospholipids on lipid digestibility may partly due to different source of fat (Smits et al., 2000).

Intestinal Morphology, Enterocyte Proliferation, and Intestinal Barrier Function

Epithelial cells of the small intestine are important in the absorption of long-chain fatty acids and monoglycerides, where those fatty acids and monoglycerides are reassembled into triglycerides and packaged into chylomicrons for the further absorption into the lymph. Crypts produce epithelial cells which replace older ones migrating from the base to the tip of the villi (Uni et al., 1998). In order to maintain functions of small intestine, renewal of epithelial cells is essential (Yang et al., 2016). Villus atrophy and the reduction in crypt cell production during the post-weaning period result in loss of mature enterocytes, which causes a decrease in nutrient absorption (Hedemann et al., 2003; Pié et al., 2004; Moeser et al., 2007). The most evident changes in intestinal morphology during this period are the reductions in villus height and increases in crypt depth (Pluske et al., 1997; Fan et al., 2004).

Lysophospholipids play important roles in regulating cellular processes as ligands for various G-protein coupled receptors, and the activation initiates signaling cascades involved in cell growth, proliferation, and differentiation (Moolenaar, 1999; Gardell et al., 2006; Choi and Chun, 2013). In the present study, LPL supplementation increased duodenal and jejunal villus height. A tendency for an increase in crypt depth was observed, but this did not affect villus height to crypt depth ratio. These results are in agreement with Boontiam et al. (2017), who reported that the addition of lysophospholipids enhanced villus morphology with improved villus height of the jejunum in broiler chickens. Similarly, increased villus length (height) was observed when fed lysophospholipids to broilers (Brautigan et al., 2017). Khonyoung et al. (2015) showed lysophospholipids can increase duodenal crypts cell mitosis in broiler chickens. In the present study, no differences in the percentage of Ki-67-positive cells, a marker of cell proliferation, were found in the jejunal crypts between the two treatments. Epithelial cell restitution is an important factor to maintain intestinal barrier function following by injury to intestines (Hines et al., 2000; Blikslager et al., 2007; Xiao et al., 2014). Rapid resealing of the epithelial layer is critical in maintenance of the barrier function. Tight junction proteins between epithelial cells form the barriers which seals the paracellular space between epithelial cells regulating the paracellular permeability (Ulluwishewa et al., 2011). These proteins consist of transmembrane proteins occludin and claudins, as well as cytoplasmic proteins such as the zonula occludens (ZO) (Hartsock and Nelson, 2008). The abundances of occludin, claudin-1, and ZO-1 proteins were measured from the jejunal mucosa to evaluate the modulatory effect of LPL on epithelial barrier function in nursery pigs. The LPL supplementation promoted the abundance of claudin-1 protein and tended to increase that of ZO-1 protein, which may in part due to the stimulatory effect of lysophospholipids on epithelial cell restitution (Sturm et al., 1999).

Intestinal barrier function is linked with intestinal inflammation and systemic immune activation (Peterson and Artis, 2014). Supplementation of LPL did not alter pro-inflammatory cytokine, TNF-α, in serum or that in the duodenal and jejunal mucosa. Serum IgG tended to reduce by supplemental LPL, whereas IgG concentrations in the duodenal and jejunal mucosa were not affected, as well as the mucosal IgA was not affected by LPL supplementation. Considering the elevated tight junction proteins by LPL supplementation, it can be assumed that LPL supplementation do not activate mucosal immune response. Taken together, these data indicate that LPL supplementation can enhance villus morphology and epithelial barrier function possibly by increasing epithelial cell restitution.

CONCLUSION

In conclusion, the addition of the LPL to a nursery diet positively affected growth of nursery pigs and showed a potential to improve lipid digestibility, and enhanced intestinal morphology and barrier function of nursery pigs.

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Table 1. Composition of the basal diet (as-fed basis)

Item	Phase 1 (d 0 to 7 postweaning)	Phase 2 (d 7 to 19 postweaning)
Ingredient, %		
Yellow corn	39.10	42.85
Soybean meal	21.50	22.20
Whey permeate	20.00	12.00
Corn distillers grains with soluble	0.00	10.00
Poultry meal	5.00	2.00
Blood plasma	4.00	2.00
Fish meal	5.00	3.00
L-Lys HCl	0.40	0.44
DL-Met	0.19	0.13
L-Thr	0.12	0.11
Dicalcium phosphate	0.00	0.36
Limestone, ground	0.71	0.96
Salt	0.22	0.22
Vitamin premix[1]	0.03	0.03
Mineral premix[2]	0.15	0.15
Zinc oxide	0.25	0.25
Poultry fat	1.00	1.20
Animal-vegetable blend	2.00	2.00
Antibiotics[3]	0.33	0.10
Total	100.00	100.00
Calculated nutrient composition		
DM, %	90.47	90.18
ME, Mcal/kg	3.47	3.47
CP, %	24.11	22.51
SID[4] Lys, %	1.50	1.35
SID Met+Cys, %	0.82	0.74
Ca, %	0.85	0.80
STTD[5] P, %	0.45	0.40
Analyzed composition		
CP, %	22.70	22.90
Ether extract, %	5.53	6.73

[1]The vitamin premix provided the following per kilogram diet: 13,227,513 IU of vitamin A; 3,968,254 IU of vitamin D3; 66,138 IU of vitamin E; 40 mg of vitamin B12; 13,228 mg of riboflavin; 110,229 mg of niacin; 22,046 mg of d-pantothenic acid; 3,968 mg of menadione; 40 IU of biotin.

[2]The mineral premix provided the following composition: Mineral premix provided the following composition: 1.1 % of Cu; 198.0 mg/kg of I; 7.3 % of Fe; 2.2 % of Mn; 198.0 mg/kg of Se; 7.3 % of Zn.

Table 1. Continued

[3]Antibiotics: 0.18% of pennchlor 100g and 0.15% of denagard 10 were added in phase 1; 0.10% of mecadox 10 was added in phase 2, respectively.

[4]SID: standardized ileal digestible.

[5]STTD: standardized total tract digestible.

Table 2. Analyzed fatty acid profiles of the basal diet fed during phase 2

Fatty acid, % of total fatty acids	Basal diet
C8:0	3.50
C10:0	1.46
C14:0	0.82
C16:0	21.38
C16:1	2.41
C18:0	4.03
C18:1	31.40
C18:2	34.97
Total	99.97
Total unsaturated fatty acids	68.79
Total saturated fatty acids	31.19
Total fatty acids	99.97
Ratio unsaturated/saturated	2.21

Table 3. Growth performance of nursery pigs fed diets with either 0.0 or 0.1% LPL[1]

Item	Added LPL, %		SEM	*P* value
	0.0	0.1		
BW, kg				
d 0	7.13	7.23	0.53	0.722
d 7	8.65	8.80	0.46	0.915
d 14	12.27	12.66	0.37	0.461
d 19	15.87	16.39	0.39	0.361
ADG, g				
d 0 to 7	238	239	19	0.989
d 7 to 14	518	545	31	0.427
d 14 to 19	681	774	28	0.037
d 0 to 19	452	490	32	0.206
ADFI, g				
d 0 to 7	242	249	22	0.761
d 7 to 14	649	665	25	0.593
d 14 to 19	1,000	1,089	31	0.014
d 0 to 19	648	678	26	0.310
G:F				
d 0 to 7	0.869	0.933	0.07	0.535
d 7 to 14	0.774	0.797	0.03	0.508
d 14 to 19	0.681	0.715	0.03	0.415
d 0 to 19	0.697	0.72	0.04	0.448

[1]Each value presented as least square of means of 12 pens (6 barrow pens and 6 gilt pens with 1 pig per pen). Pigs were fed a phase 1 diet for the first 7 d followed by a phase 2 diet from d 7 to 19.

Table 4. Apparent ileal digestibility of ether extract and fatty acids of nursery pigs fed diets with either 0.0 or 0.1% LPL[1]

Item	Added LPL, %		SEM	*P* value
	0.0	0.1		
Ether extract	72.7	84.2	4.3	0.086
C8:0	100.0	100.0	0.0	1.000
C10:0	100.0	100.0	0.0	1.000
C14:0	73.8	74.4	5.2	0.851
C16:0	78.7	78.3	2.6	0.915
C16:1	100.0	100.0	0.0	1.000
C18:0	69.7	75.3	6.4	0.327
C18:1	79.0	76.9	3.2	0.623
C18:2	86.7	96.4	2.8	0.059
Total unsaturated fatty acids	83.6	87.5	2.5	0.312
Total saturated fatty acids	80.7	81.1	2.3	0.888
Total fatty acids	78.9	81.4	2.3	0.420

[1]Each value presented as least square of means of 12 pens (6 barrow pens and 6 gilt pens with 1 pig per pen).

Table 5. Morphology of duodenum and jejunum, and proliferation of jejunal crypts of nursery pigs fed diets with either 0.0 or 0.1% LPL[1]

Item	Added LPL, %		SEM	*P* value
	0.0	0.1		
Duodenum				
Villus height, μm	509	589	35	0.022
Villus width, μm	108	121	8	0.103
Crypt depth, μm	262	278	25	0.300
VH:CD[2]	1.9	2.2	0.1	0.086
Jejunum				
Villus height, μm	463	524	18	0.016
Villus width, μm	81	81	4	0.975
Crypt depth, μm	185	201	11	0.062
VH:CD[2]	2.6	2.6	0.2	0.739
Jejunum, Ki-67, %	14.4	14.8	1.3	0.764

[1]Each value presented as least square of means of 12 pens (6 barrow pens and 6 gilt pens with 1 pig per pen).

[2]VH:CD: villus height to crypt depth ratio.

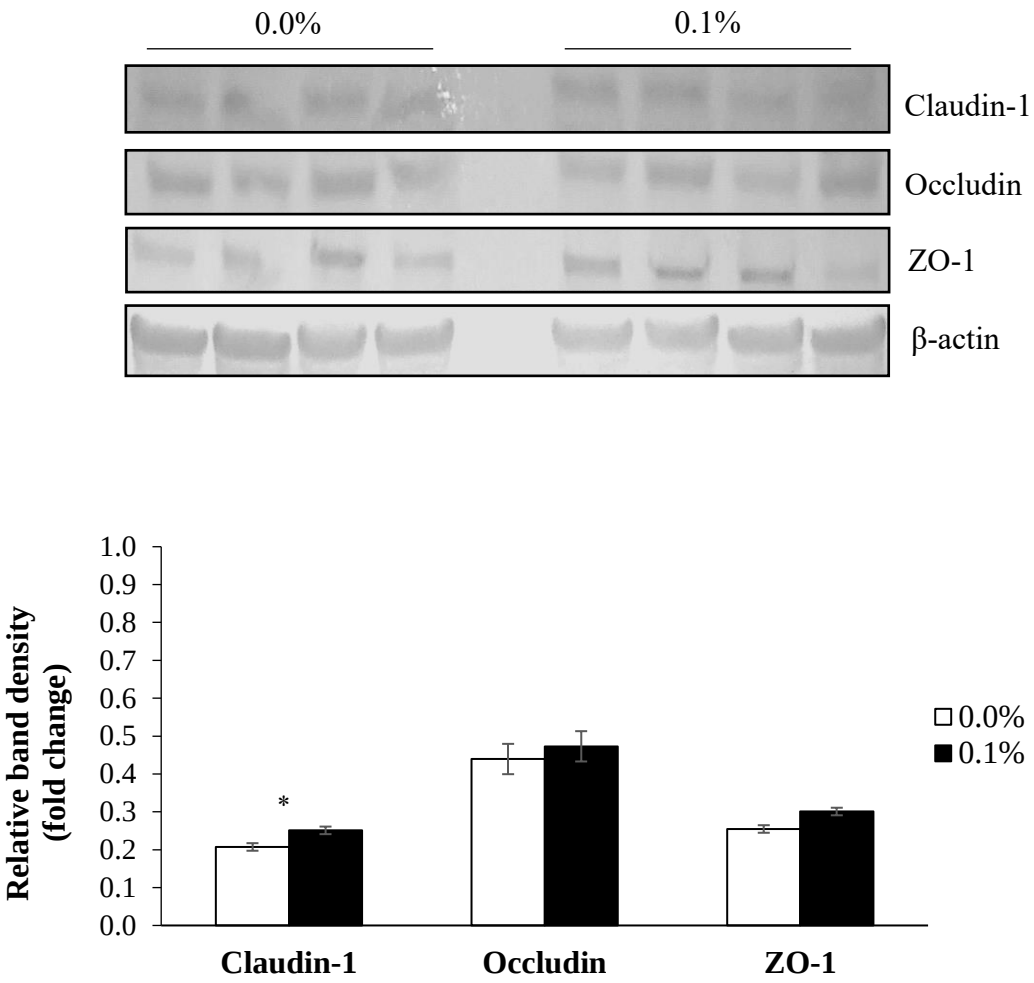

Figure 1. Western blot analysis of tight junction proteins abundance in the jejunal tissue of nursery pigs fed diets with either 0.0 or 0.1% LPL. Fold change for the protein of interest was normalized to the amount of β-actin. Data are represented as least square means of 4 observations. $^{*}P < 0.05$. Claudin-1 ($P < 0.05$); ZO-1 ($P = 0.056$).

Table 6. Immunological parameters of nursery pigs fed diets with either 0.0 or 0.1% LPL[1,2]

Item	Added LPL, %		SEM	*P* value
	0.0	0.1		
IgA, ug/mg protein				
Duodenum	0.64	0.72	0.16	0.655
Jejunum	0.65	0.82	0.17	0.470
IgG				
Serum, mg/mL	1.31	1.16	0.06	0.083
Tissues, ug/mg protein				
Duodenum	1.07	0.90	0.36	0.116
Jejunum	1.43	1.48	0.21	0.868
TNF-α				
Serum, pg/mL	141.9	133.8	34.9	0.788
Tissues, pg/mg protein				
Duodenum	0.78	0.74	0.28	0.533
Jejunum	1.08	1.17	0.15	0.654

[1]Each value presented as least square of means of 12 pens (6 barrow pens and 6 gilt pens with 1 pig per pen).
[2]IgA = immunoglobulin A; IgG = immunoglobulin G; TNF-α= tumor necrosis factor-α.

CHAPTER 6. EFFECTS OF FERMENTED RICE BRAN EXTRACTS ON GROWTH, DIARRHEA INCIDENCE, MUCOSA-ASSOCIATED MICROBIOTA, INTESTINAL MORPHOLOGY, AND IMMUNE RESPONSE IN NURSERY

ABSTRACT: The objective of this experiment was to evaluate the supplemental effects of fermented rice bran extracts (FRBE, Maxcell Co., LA, CA) on growth performance, diarrhea incidence, mucosa-associated microbiota, intestinal morphology, and immune response in nursery pigs. Rice bran was fermented with sucrose by a mixture of probiotics (*Lactobacillus plantarum*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*). Thirty pigs (15 barrows and 15 gilts at 6.6 ± 2.4 kg BW) were allotted in a randomized complete block design with sex and initial BW as blocks and randomly assigned to 3 treatments. Pigs were fed a basal diet supplemented with FRBE at the level of 0.0, 0.5, or 1.0% based on 2 phases (7 and 18 d, respectively). Fecal scores were determined to measure diarrhea incidence, and duodenal and jejunal mucosa samples were collected for analysis of immune response, and microbiome analysis, and jejunum segments were collected for morphology and enterocyte proliferation evaluation. Data were analyzed using the Mixed procedure of SAS. Increasing levels of FRBE tended to change ADG (quadratic, $P = 0.077$) during phase 2, and tended to affect ADG (quadratic, $P = 0.078$) during the entire 25 d. Increasing levels of FRBE tended to affect (quadratic, $P = 0.067$) ADFI during phase 1. Increasing levels of FRBE tended to change (quadratic, $P = 0.058$) the relative abundance of mucosa-associated *Bacteroidetes* phylum. Mucosa-associated microbiota of pigs fed 0.5% FRBE distinctly different with those fed a basal diet. Increasing levels of FRBE increased (linear, $P < 0.05$) villus height to crypt depth ratio of the jejunum. Increasing levels of FRBE changed (quadratic, $P < 0.05$) percentage of Ki-67 positive cells in the jejunal crypts. Increasing levels of FRBE affected IgG in serum (quadratic, $P < 0.05$), and tended to change that (linear, $P = 0.058$) in the duodenal mucosa. In conclusion, dietary supplementation of FRBE may have beneficial effects on growth performance, enhanced

intestinal morphology, and may can modulate mucosa-associated microbiota composition of nursery pigs.

Key words: fermented rice bran extracts, growth performance, intestinal morphology, mucosa-associated microbiota, nursery pigs

INTRODUCTION

Prebiotics has been widely used to improve beneficial microbial populations in the intestines (Castillo et al., 2008). According to a recent definition a prebiotic is "a nondigestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host" (Bindels et al., 2015).

Rice bran is a co-product obtained during rice milling process. Rice bran is rich in cell wall materials such as hemicellulose and cellulose containing neutral detergent fiber in the range of 19 to 34% (Shi et al., 2015). High in fiber is a major limitation of its use in young mono-gastric animal diets. Several methods have been applied to improve rice bran feeding value, such as fermentation (Wizna et al., 2012) and enzymatic treatment (Tangendjaja, 1993; Kompiang et al., 1995). Fermentation of rice bran using lactic acid and yeast showed to reduce fiber contents by utilizing enzymes (such as cellulase) provided by the microbes (Tangendjaja, 1993). The main polysaccharide in the rice bran, hemicellulose, is a complex molecules composed of several kinds of monosaccharides including glucose as backbone constituent, as well as arabinose, galactose and mannose in side chains, among others (Peng et al., 2012). The acid hydrolysis of rice bran soluble non-starch polysaccharide showed they were mainly composed of arabinose, xylose, mannose, galactose, and glucose (Annison et al., 1995). Prebiotic properties of rice bran were reported in the studies with nursery pigs (Herfel et al., 2013). Rice bran oligosaccharides, mainly composed of glucooligosaccharides, was reported to possess prebiotic potential (Kurdi and Hansawasdi, 2015). The glucooligosaccharides were shown to be able to promote the growth of *Lactobacillus* species, which were not hydrolyzed by human intestinal conditions. In addition, Koh et al. (2002) showed fermented rice bran extracts fermented by *Saccharomyces cerevisiae*

and *Bacillus sp.* increased the stimulation of the macrophage activation and the increased intestinal immune modulating activity *in vitro*.

Fermented rice bran extracts used in the present study mainly contained oligosaccharide mixture that composed of glucose as major monomeric constituents. It was hypothesized that the fermented rice bran extracts would improve growth performance by altering intestinal microbiota and may enhance intestinal health as a source of prebiotics. The objective of this study was to evaluate the effect of fermented rice bran extracts on growth performance, diarrhea incidence, intestinal morphology, mucosa-associated microbiota, and immune response in nursery pigs.

METERIALS AND METHODS

The experimental protocol was approved by the Institutional Animal Care and Use Committee at North Carolina State University (Raleigh, NC).

Fermented Rice Bran Extracts (FRBE)

The FRBE was obtained from a commercial company Maxcell Co. (LA, CA). Briefly, rice (*Oryza sativa*) bran medium was inoculated by a mixture of probiotics (*Lactobacillus plantarum, Bacillus subtilis, and Saccharomyces cerevisiae*) with the presence of sucrose. The liquid suspension was centrifuged, and the supernatant was dried and ground into powder. The chemical content of FRBE analyzed by North Carolina Department of Agriculture and Consumer Services Food and Drug Protection Division Laboratory contained 43.0% total carbohydrate, 12.2% crude protein, 16.2% crude fat, 9.1% crude fiber, and 8.0% ash (Table 1). Glycosyl composition and degree of polymerization (DP) of FRBE were analyzed at Complex Carbohydrate Research Center of University of Georgia. It showed 98% of monosaccharide residues was glucose, and the rest was composed of xylose (0.95%), arabinose (0.80%), and

mannose (0.45%). DP of FRBE result showed it had DP from 2 to 6 with main DP as 3 (48.6%) and 4 (27.6%).

Experimental Diets and Pigs

A total of 30 newly weaned pigs (PIC 337 x Camborough 22) (15 barrows and 15 gilts at 6.6 ± 2.4 kg BW) were randomly allotted to 3 dietary treatments in a randomized complete block design. Sex and initial BW were used as blocks. Pigs were housed individually in pens (0.7 × 1.5 m) that were equipped with a feeder and a nipple drinker. Pigs had free access to feed and water. A corn-soybean meal-based basal diet (Table 2) was formulated to meet nutrient requirement estimates (NRC, 2012). Pigs were fed a basal diet supplemented with 0.0, 0.5, or 1.0% FRBE in 2 phases (7 and 18 d, respectively). Individual BW and feed disappearance of each pen were recorded on d 7 and 25.

Diarrhea Incidence

The incidence of diarrhea of piglets were recorded every day for the first 14 d of the experimental period. Pig feces were scored according to the modified method of Liu et al. (2010). Fecal scores were: 1, normal, firm feces; 1.5, possible slight diarrhea; 2, moderate liquid consistency; 2.5, definitely unformed and fluid feces; 3, very watery and frothy feces. The occurrence of diarrhea was defined as maintenance of feces at Level 2.5 or Level 3 for two continuous days. Diarrhea incidence was calculated according to the formula reported by Sun et al. (2008): diarrhea incidence (%) = number of pigs with diarrhea/(number of pigs × total experimental days) × 100, where “number of pigs with diarrhea” was the total number of pigs with diarrhea observed each day, and “total experimental days” was 14 d.

Sample Collection

Blood samples (7 mL) were collected via puncture of jugular vein of the pig on d 25. Serum samples were then obtained by centrifuging (3,000 × g, for 15 min at 4°C) and stored at –80°C until analyzed for immune response. Pigs were euthanized via captive blot on d 25 for sample collection.

Intestinal Morphology and Proliferation of Enterocytes

Duodenum and jejunum samples were rinsed with a 0.9% saline solution, fixed with a 10% buffered formalin solution immediately after slaughter. The duodenum samples then embedded in paraffin (Paraplast-Sigma) and paraffin sections of 5 µm thick tissue were taken on rotary microtome. The sections were stained with haematoxylin and eosin. Villus height, villus width, and crypt depth of duodenum and jejunum were measured at 40 × magnification as previously described (Shen et al., 2009). Lengths of 10 well-oriented intact villi and their associated crypt were measured in each slide. Immunohistochemistry staining were carried out to measure enterocytes proliferation. The jejunum samples were embedded in paraffin and epitope retrieval were performed using 10 mM citrate buffer, pH 6.0 in a pressure cooker (Dako, Carpinteria, CA). Endougenous peroxidase were quenched with 3% hydrogen peroxide and sections were blocked using protein block reagent (Dako, Carpinteria, CA). Primary monoclonal antibody of Ki-67 (Dako, Carpinteria, CA) was used after 1:500 dilutions. Secondary antibody were attached using Vector ImmPRESS anti-mouse polymer reagent (Vector Laboratories, Burlingame, CA) after 1:2 dilutions. Diaminobenzamine (DAB) reagent (Vector Laboratories, Burlingame, CA) were used as the chromogen. Image JS software were used for calculating Ki-67 positive cells. Crypt cell proliferation, % = Ki-67 positive cells/ Total cells × 100%.

Microbiome Sequencing

To sequence mucosa-associated microbiome, DNA were extracted from samples with QIAGEN's QIAamp DNA Stool MiniKit (Qiagen, Crawley, United Kingdom). Samples were sent to Genomics Department of Mako Medical Laboratories (Raleigh, NC) for sequencing. Briefly, samples were prepared for template preparation on the Ion Chef instrument and sequencing on the Ion S5 system (ThermoreFisher). Variable regions V2, V3, V4, V6, V7, V8, and V9 of the 16S rRNA gene were amplified with the Ion 16S Metagenomics Kit (ThermoFisher Scientific, Inc., Wilmington, DE). Sequences were processed using the Torrent Suite Software (version 5.2.2) (ThermoFisher Scientific, Inc., Wilmington, DE) to produce raw unaligned sequence for further analysis. Sequence data analysis, alignment to GreenGenes and MicroSeq databases, alpha and beta diversity plot generation, and OTU table generation were performed by the Ion Reporter Software Suite (version 5.2.2) of bioinformatics analysis tools (ThermoFisher Scientific, Inc., Wilmington, DE). Samples were analyzed using Ion Reporter's Metagenomics 16S workflow powered by Qiime (version w1.1).

Intestinal Mucosal Immunity

Tumor necrosis factor (TNF)-α was measured in serum according to the method described by the manufacturer using a Porcine TNF-α Colorimetric ELISA Kit (Pierce Biotechnology, Rock- ford, IL). Briefly, 50 μL of standard plus dilute or 100 mL of sample was added to microplate wells which were already coated with capture antibody in conjunction with biotinylated antibody reagent. Detection occurred by the use of a color reagent substrate and a stop solution of diluted hydrochloric acid, and absorbance was read at 450 nm and 540 nm. Concentrations of TNF-α in duodenum and jejunum were also analyzed using the ELISA kit. Upon analysis, protein concentrations in tissues were measured using a commercial kit (Thermo

Fisher Scientific) according to Smith et al. (1985). The measured protein concentration was used to determine the amounts of immunological subset per gram or milligram of protein of each tissue type. The immunoglobulin subsets immunoglobulin A and immunoglobulin G were measured according to the method described by the manufacturer using ELISA kits (Bethyl, Montgomery, TX, USA).

Statistical Analysis

Data were analyzed as a randomized complete block design using the Mixed procedure of SAS 9.3 (SAS Inc., Cary, NC, USA), and the pen was the experimental unit. Fixed effect was the treatment, and random effect was the blocks. Orthogonal contrasts were used to test for linear and quadratic effects of FRBE supplementation. Data for diarrhea incidence were analyzed using FREQ procedure with Pearson's chi-square test. Data for microbial diversity were analyzed using Princomp procedure. The alpha level used for determination of statistical significance was 0.05 and levels between 0.05 and 0.10 were considered as tendency.

RESULTS

Growth Performance

Increasing levels of FRBE tended to affect (quadratic, $P = 0.064$) BW on d 25 (Table 3). Increasing levels of FRBE tended to change ADG during d 7 to d 25 postweaning (quadratic, $P = 0.077$) as well as during the entire 25 d (quadratic, $P = 0.078$). Increasing levels of FRBE tended to change (quadratic, $P = 0.067$) ADFI during d 0 to d 7 postweaning. G:F was not affected by the dietary treatment during the whole experimental period.

Mucosa-associated Microbiota

Result tables for families and genera operational taxonomic units (OTUs) are shown in Appendix B. The predominant phylum detected in all 3 treatments were *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* (Figure 1). No differences were observed in *Proteobacteria* and *Firmicutes* phyla by FRBE supplementation, whereas a tendency for a quadratic response (P = 0.058) was observed in *Bacteroidetes* phylum (Figure 2). Family level of microbial communities in the jejunal mucosa shows the majority of the community was composed of *Helicobacteraceae, Clostridiaceae, Bacillaceae, Lactobacillaceae, Enterobacteriaceae, Streptococcaceae, Prevotellaceae, Succinivibrionaceae, Xanthomonadaceae, Veillonellaceae, Pseudomonadaceae, Lachnospiraceae, and Eubacteriaceae* (Figure 3). Increasing levels of FRBE tended to change the abundance of family *Streptococcaceae* (quadratic, P = 0.092), and the abundance of genus *Streptococcus* (quadratic, P = 0.090), respectively (Figure 4). Alpha diversity estimated with Chao1, Shannon, and Simpson indices were not different among the treatments (Table 4). Principal component analysis showed mucosal microbiota of pigs fed 0.5% FRBE distinctly different with those fed a basal diet (Figure 5 and 6), whereas, mucosal microbiota of pigs fed 1.0% FRBE were not different with those fed a basal diet.

Intestinal Morphology and Proliferation of Enterocytes

Increasing levels of FRBE did not affect villus height, villus width, crypt depth, and villus height to crypt depth ratio of the duodenum (Table 5). Increasing levels of FRBE increased (linear, $P < 0.05$) villus height to crypt depth ratio of the jejunum. Increasing levels of FRBE affected (quadratic, $P < 0.05$) percentage of Ki-67 positive cells in the jejunal crypts.

Diarrhea Incidence and Intestinal Mucosal Immunity

Supplementation of FRBE did not affect diarrhea incidence during 14-d postweaning (Table 6). Increasing levels of FRBE did not change IgA in the duodenum and jejunum mucosa. Increasing levels of FRBE affected (quadratic, $P < 0.05$) serum IgG. Increasing levels of FRBE tended to change (linear, $P = 0.058$) IgG in the duodenal mucosa. Increasing levels of FRBE did not affect TNF-α levels in serum and in the duodenal and jejunal mucosa.

DISCUSSION

Growth Performance

Studies have been shown the positive effect of rice bran products in promoting growth of pigs (Herfel et al., 2013; Hossain et al., 2016) and broilers (Lokaewmanee et al., 2012; Supriyati et al., 2015). In the present study, supplementation of FRBE showed a potential to positively affect growth performance. A tendency for a quadratic response was observed in ADFI during the first 7 d after weaning, and a tendency for a quadratic change was observed in ADG during the 25 d experimental period, with the greatest value observed in 0.5% FRBE treatment. The tendency for the quadratic response showed in ADFI and ADG may due to the potential negative effect on feed intake when fed 1.0% FRBE during the first 7 d of experimental period. Reduced feed intake in pigs was observed by Hong et al. (2016), who reported a mixture product of fermented rice bran and cassava added into grower and finisher diets at the level of 15% decreased feed intake when compared to that of non-fermented treatment. On the contrary, Herfel et al. (2013) reported rice bran supplemented into a diet up to 10% did not affect feed intake of pigs (weaned at 21-d-old) during 28-d feeding period. Inclusion of rice bran oligosaccharides (mainly composed of arabino- and xylo-oligosaccharides) up to 6% in the diet

did not affect feed intake of broiler chickens (Annison et al., 1995). Also, glucooligosaccharides fed to pigs (weaned at 28-d-old) with inclusion level of 2.0% in the diet for 77 days did not alter feed intake (Rossi et al., 2008). It is interesting to note that FRBE supplementation did not affect feed intake except for the tendency for the quadratic response during the first 7 days and it did not affect G:F during the whole experimental period. It was speculated that FRBE supplemented into diets above 1.0% would reduce feed intake during early postweaning period and it may not be associated with the oligosaccharide mixtures, rather, it may be related with metabolites produced during fermentation process but further research is needed to elucidate it.

Mucosa-associated Microbiota

In comparison to microbial population in the hindgut which is the major site of fermentation in pigs, in the small intestine it is less diverse (Kelly et al., 2017). However, the small intestine is a major place for nutrient absorption, the microbial populations in the small intestine are more susceptible to dietary impact (Levesque et al., 2012). The small intestinal mucosa is often exposed to exogenous antigens and microbial components from feed ingredients. Changes in mucosa-associated microbiota may have enormous effect on host growth and development. Rice bran consumption increased lactobacilli in a murine model (Henderson et al., 2012) and in nursery pigs (Herfel et al., 2013). Oligosaccharide mixtures (composed of glucose, galactose, and mannose) extracted from rice bran showed to possess prebiotic potential with promoting growth of *Lactobacillus and Bifidobacterium* species (Kurdi and Hansawasdi, 2015). Results from the current study showed increasing levels of FRBE supplementation tended to change the relative abundance of *Bacteroidetes* phylum, where 0.5% FRBE treatment had the lowest value. Substrate availability is a major factor for dynamics of mucosa-associated microbiota populations. Many species within the *Bacteroidetes* phylum proliferate by degrading

dietary fiber and complex carbohydrates (Thomas et al., 2011). These species produce numerous carbohydrate-active enzymes that allowing them to utilize a wide variety of substrates including dietary polysaccharides. FRBE used in the present study had a major DP (degree of polymerization) from 3 to 4. The tendency for a quadratic change showed in *Bacteroidetes* phylum with increasing levels of FRBE might due to the less complex oligosaccharide mixtures provided by FRBE. *Streptococcus sp.* are facultative anaerobes that ferment simple carbohydrates at a high rate to produce mainly lactate (Holt, 1994). The abundances of *Streptococcaceae* and *Streptococcus* belonging to *Firmicutes* phylum had a tendency for a quadratic change with increasing levels of FRBE, which indicate that it may alter lactate production in the small intestine. In addition, FRBE supplementation on microbiome diversity was measured by both alpha (within samples) and beta (between samples) diversities. Alpha diversity was not different among the treatments, however, beta diversity which shows compositional differences among the treatments revealed that mucosa-associated microbiota of pigs fed 0.5% FRBE distinctly different from those fed a basal diet. The major difference was a potential shift in the population abundance of lactic acid producing bacteria, the *Streptococcaceae* family. These results suggest that supplementation of FRBE can alter mucosa-associated microbiota, but the effect depends on the supplemental level of FRBE.

Intestinal Morphology and Proliferation of Enterocytes

Weaning induces both structural and functional changes in the small intestine among which, villus atrophy and increases in crypt depth are the most evident (Pié et al., 2004). Maintaining intestinal architecture after weaning is critical in nutrient absorption during post-weaning period of pigs (Hedemann et al., 2003; Pié et al., 2004). Stem cells in the crypts undergo cell division and differentiation to form mature absorptive enterocytes (Wright and Alison,

1984), and those enterocytes continuously migrate and mature towards along crypt-villus axis. To replace exfoliated enterocytes, continual cell production in the crypts is critical in minimizing weaning induced villus atrophy. Intestinal morphological measurement is an important index for assessing intestinal health. An increase in crypt depth is an indicator of increased cell production in the crypts (Hedemann et al., 2003), and decreased villus height is associated with cell loss (Pluske et al., 1996). In the present study, no differences were observed in villus height, villus width, and crypt depth of the duodenum and jejunum, whereas villus height to crypt depth ratio of the jejunum was increased with increasing levels of FRBE. Furthermore, a quadratic change was observed in the enterocyte proliferation in the jejunal crypts, with the same manner showed in the growth performance. Due to the fact that no difference was observed in crypt depth among the treatments, it can be assumed that the change in enterocyte proliferation did not concur with crypt hyperplasia. In previous studies with prebiotic oligosaccharides supplemented into diets showed to increase villus height of pigs (Rossi et al., 2008). The potential reason of the enhanced morphology would be related to the production of short chain fatty acid that stimulates enterocyte proliferation (Blottiere et al., 2003). Alterations in intestinal microbiota showed in the present study might be connected with the changes showed in villus morphology and crypt cell proliferation.

Diarrhea Incidence and Intestinal Mucosal Immunity

The current results showed no evidence of illness with FRBE supplementation. No difference in diarrhea incidence was observed among the treatments. A quadratic response in serum IgG was observed and that in the duodenal mucosa tended to increase with increasing levels of FRBE, however, no differences were found in mucosal IgA or pro-inflammatory cytokine (TNF-α) levels. These results indicate that FRBE supplementation had no adverse effect

on immune status. Mucus layer that overlies enterocytes containing IgA provides protection against bacterial invasion (Kelly et al., 2015). Mucus barrier is the first anatomical site of intestinal barrier, preventing pathogens from penetrating enterocytes (Sun et al., 2016). Prebiotics are shown to influence the mucus barrier by increases in mucus production through microbial colonization (Ten Bruggencate et al., 2004). Yang et al. (2015) reported rice bran supplementation showed to protect enteric pathogen infections via prebiotic effect, where it promoted the growth of *Lactobacillus rhamnosus GG* and *Escherichia coli Nissle* in gnotobiotic pigs, and stimulated intestinal mucosal immunity by increasing mucosal IgA and interferon-gamma during human rotavirus infection. Future research under pathogen challenge condition is recommended to further explore effect of FRBE on mucosal immunity.

CONCLUSION

In summary, supplementation of FRBE showed a potential to promote growth performance during 25 d postweaning with tendencies for a quadratic response in ADG and BW as the FRBE supplemental level increased. In addition, supplementation of FRBE showed to enhance intestinal morphology and changed enterocyte proliferation as the FRBE supplemental level increased. Furthermore, supplementation of FRBE altered mucosa-associated microbiota composition, in which 0.5% FRBE treatment was distinctly different from that of 0.0% treatment. These results suggest that supplemental level is an important factor to consider when applying fermented rice bran extracts to nursery pig diets.

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Table 1. Chemical and glycosyl residue composition, and degree of polymerization (DP) of fermented rice bran extracts (FRBE)

Item	FRBE
Dry matter	97.64
Total carbohydrate	43.00
Crude protein	12.20
Crude fat	16.20
Crude fiber	9.10
Ash	8.00
Glycosyl composition, %	
Glucose	98.00
Xylose	0.95
Arabinose	0.80
Mannose	0.45
Degree of polymerization, %	
2	19.3
3	48.6
4	27.6
5	3.5
6	0.9

Table 2. Composition of experimental diets

Item	Phase 1 (d 0 to 7 postweaning) FRBE[1] (%)			Phase 2 (d 7 to 25 postweaning) FRBE (%)		
	0.0	0.5	1.0	0.0	0.5	1.0
Ingredient, %						
Corn, yellow dent	41.50	41.18	40.86	52.92	52.60	52.28
Soybean meal, dehulled	20.00	20.00	20.00	24.50	24.50	24.50
Whey permeate	20.00	20.00	20.00	10.00	10.00	10.00
Poultry meal	6.00	6.00	6.00	3.00	3.00	3.00
Blood plasma	7.00	7.00	7.00	3.00	3.00	3.00
FRBE	0.00	0.50	1.00	0.00	0.50	1.00
Poultry fat	2.50	2.36	2.23	3.30	3.16	3.03
L-Lys HCl	0.45	0.45	0.45	0.46	0.46	0.46
DL-Met	0.22	0.22	0.22	0.18	0.18	0.18
L-Thr	0.15	0.15	0.15	0.13	0.13	0.13
Limestone	1.30	1.30	1.30	0.94	0.94	0.94
Dicalcium phosphate	0.20	0.20	0.20	0.92	0.92	0.92
Zinc oxide	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin premix[2]	0.03	0.03	0.03	0.03	0.03	0.03
Mineral premix[3]	0.15	0.15	0.15	0.15	0.15	0.15
Salt	0.25	0.25	0.25	0.22	0.22	0.22
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated composition						
DM, %	91.5	91.1	90.7	90.2	89.8	89.4
ME, kcal/kg	3,440	3,435	3,430	3,456	3,434	3,447
CP, %	23.7	23.7	23.6	21.2	21.2	21.2
SID[4] Lys, %	1.51	1.51	1.51	1.35	1.35	1.35

Table 2. Continued

SID Met+Cys, %	0.83	0.83	0.83	0.74	0.74	0.74
SID Trp, %	0.26	0.26	0.26	0.22	0.22	0.22
SID Thr, %	0.89	0.89	0.89	0.79	0.79	0.78
Ca, %	0.86	0.86	0.86	0.80	0.80	0.80
STTD[5] P, %	0.46	0.46	0.46	0.40	0.40	0.40

[1]FRBE: fermented rice bran extracts.

[2]The vitamin premix provided the following per kilogram diet: 13,227,513 IU of vitamin A; 3,968,254 IU of vitamin D_3; 66,138 IU of vitamin E; 40 mg of vitamin B_{12}; 13,228 mg of riboflavin; 110,229 mg of niacin; 22,046 mg of d-pantothenic acid; 3,968 mg of menadione; 40 IU of biotin.

[3]The mineral premix provided the following composition: Mineral premix provided the following composition: 1.1 % of Cu; 198.0 mg/kg of I; 7.3 % of Fe; 2.2 % of Mn; 198.0 mg/kg of Se; 7.3 % of Zn.

[4]SID: standardized ileal digestible.

[5]STTD: standardized total tract digestible.

Table 3. Growth performance of nursery pigs fed diets with fermented rice bran extracts (FRBE)

Item	Added FRBE, %			SEM	*P* value	
	0.0	0.5	1.0		Linear	Quadratic
BW, kg						
d 0	6.77	6.72	6.48	0.35	0.170	0.556
d 7	6.78	6.86	6.44	0.37	0.250	0.245
d 25	13.50	14.69	12.67	1.05	0.419	0.064
ADG, g						
d 0 to d 7	1	22	-5	18	0.800	0.241
d 7 to d 25	375	436	348	43	0.569	0.077
d 0 to d 25	271	320	249	33	0.582	0.078
ADFI, g						
d 0 to d 7	105	133	86	17	0.414	0.067
d 7 to d 25	899	972	838	79	0.477	0.142
d 0 to d 25	424	464	393	38	0.449	0.107
G:F						
d 0 to d 7	-0.154	-0.065	-0.316	0.292	0.691	0.628
d 7 to d 25	0.598	0.601	0.656	0.054	0.464	0.581
d 0 to d 25	0.581	0.697	0.642	0.048	0.365	0.165

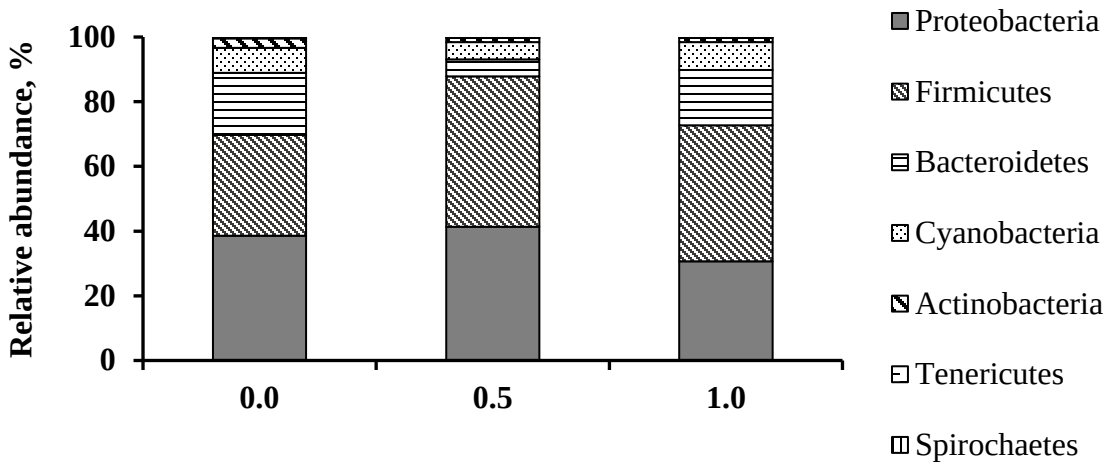

Figure 1. Relative abundance of phyla detected in mucosa-associated microbiota of nursery pigs fed diets with increasing levels of fermented rice bran extracts (FRBE).

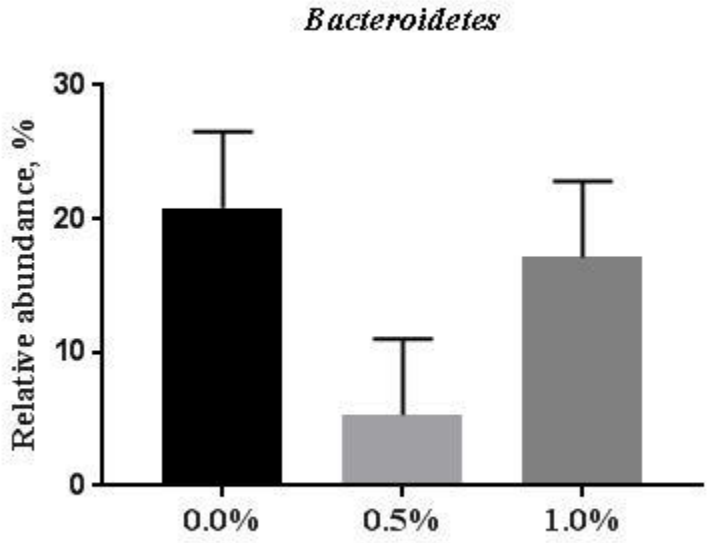

Figure 2. Relative abundance of phylum *Bacteroidetes* of pigs fed diets with increasing levels of fermented rice bran extracts (FRBE). 0.0% = basal diet; 0.5% = supplementation of FRBE at the level of 0.5%; 1.0% = supplementation of FRBE at the level of 1.0% (quadratic, $P = 0.058$).

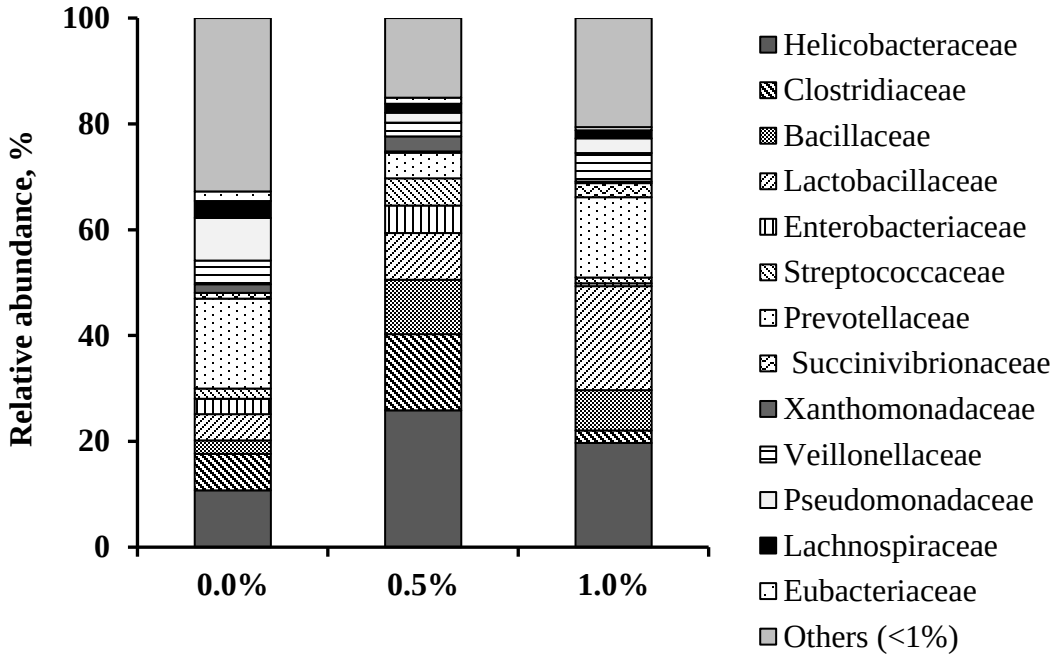

Figure 3. Relative abundance of the family sequences of jejunal mucosa-associated microbiota of nursery pigs fed diets with fermented rice bran extracts (FRBE). Others represents individual sequences totaling <1.0% of total population combined together. 0.0% = basal diet; 0.5% = supplementation of FRBE at the level of 0.5%; 1.0% = supplementation of FRBE at the level of 1.0%.

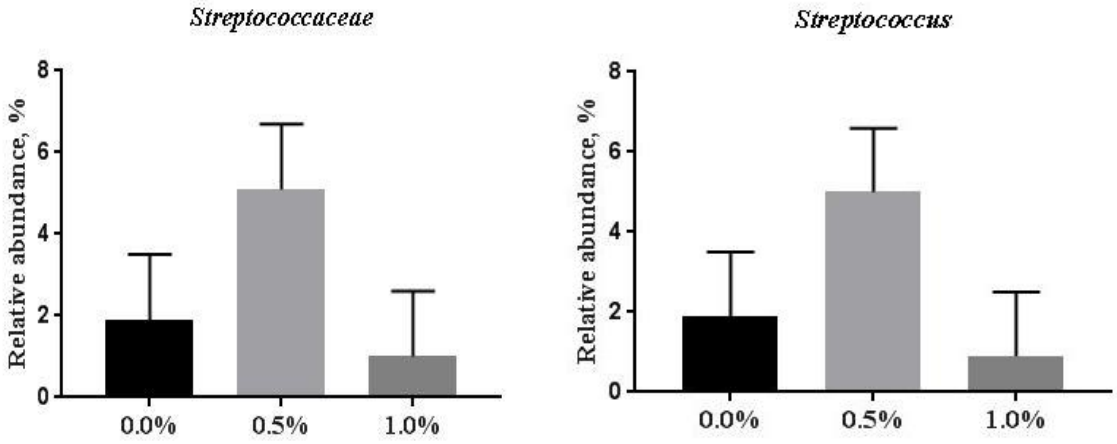

Figure 4. Relative abundance of the family *Streptococcaceae* and genus *Streptococcus* of jejunal mucosa-associated microbiota of nursery pigs fed diets with fermented rice bran extracts (FRBE). 0.0% = basal diet; 0.5% = supplementation of FRBE at the level of 0.5%; 1.0% = supplementation of FRBE at the level of 1.0%. *Streptococcaceae* (quadratic, $P = 0.092$); *Streptococcus* (quadratic, $P = 0.090$).

Table 4. Alpha diversity estimated with Chao1, Shannon, and Simpson indices of jejunal mucosa-associated microbiota of pigs fed with fermented rice bran extracts (FRBE)

Item	Added FRBE, %			SEM	*P* value	
	0.0	0.5	1.0		Linear	Quadratic
Chao1	67.4	69.3	70.9	10.0	0.366	0.823
Shannon	2.91	3.29	3.21	0.26	0.520	0.583
Simpson	0.74	0.81	0.80	0.03	0.400	0.524

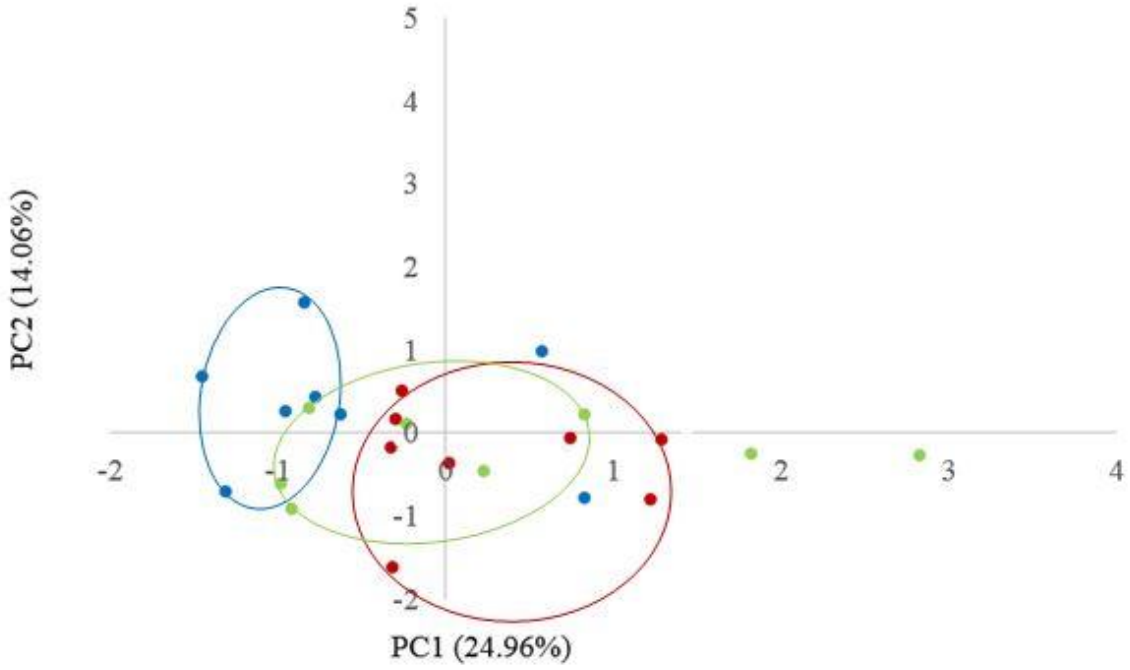

Figure 5. Principal component analysis (PCA) of jejunal mucosa-associated microbiota of nursery pigs fed diets with increasing levels of FRBE.
Red circles represent basal diet; blue circles represent 0.5% FRBE; and green circles represent 1.0% FRBE.

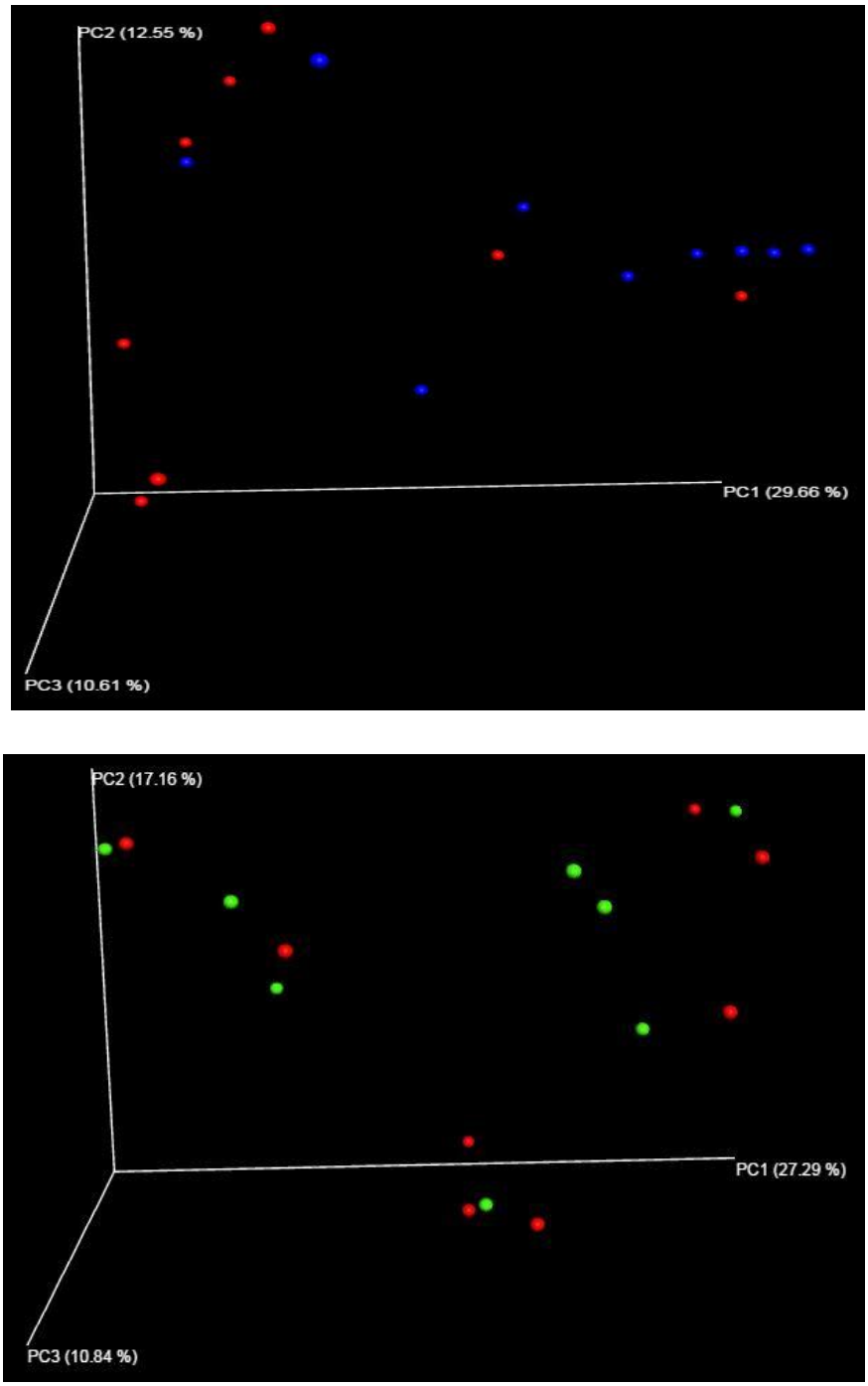

Figure 6. 3-dimentional analysis using three components (PC1 to PC3).
Red circles represent basal diet; blue circles represent supplementation of FRBE at the level of 0.5%; green circles represent supplementation of FRBE at the level of 1.0%.

Table 5. Morphology of duodenum and jejunum, and enterocytes proliferation of jejunum of nurs ery pigs fed diets with fermented rice bran extracts (FRBE)

Item	Added FRBE, %			SEM	*P* value	
	0.0	0.5	1.0		Linear	Quadratic
Duodenum						
Villus height, μm	551	556.5	526.2	24	0.503	0.592
Villus width, μm	94	94.6	101.2	5	0.341	0.402
Crypt depth, μm	305	295.4	280.5	16	0.286	0.827
VH:CD[1]	1.8	1.9	1.9	0.1	0.520	0.715
Jejunum						
Villus height, μm	452	445	468	19	0.496	0.456
Villus width, μm	74	73	72	2	0.659	0.954
Crypt depth, μm	214	205	197	8	0.193	0.915
VH:CD	2.1	2.2	2.4	0.1	0.018	0.501
Jejunum Ki-67, %	15.4	18.4	15.8	1.16	0.775	0.043

[1]VH:CD: villus height to crypt depth ratio.

Table 6. Diarrhea incidence and immunological parameters of nursery pigs fed diets with fermented rice bran extracts (FRBE)

Item	Added FRBE, %			SEM	*P* value	
	0.0	0.5	1.0		Linear	Quadratic
Diarrhea incidence[1], %	8.57	6.43	3.57	-	0.338[2]	
IgA[3], ug/mg protein						
Duodenum	2.40	3.48	2.29	0.57	0.974	0.194
Jejunum	1.13	1.30	1.35	0.11	0.208	0.689
IgG[3]						
Serum, mg/ml	1.04	1.42	1.12	0.14	0.669	0.036
Tissues, ug/mg protein						
Duodenum	1.64	1.91	2.1	0.18	0.058	0.864
Jejunum	1.71	1.68	1.69	0.18	0.964	0.955
TNF-α[3]						
Serum, pg/mL	15.68	15.91	17.10	0.96	0.351	0.714
Tissues, pg/mg						
Duodenum	1.19	0.80	0.95	0.17	0.258	0.185
Jejunum	1.15	1.00	0.88	0.13	0.180	0.903

[1] Fecal scores were 1, normal, firm feces; 1.5, possible slight diarrhea; 2, moderate liquid consistency; 2.5, definitely unformed, and fluid feces; 3, very watery and frothy feces. Diarrhea incidence (%) = number of pigs with diarrhea/(number of pigs × total experimental days) × 100.

[2]Pearson's chi-square test.

[3]IgA: immunoglobulin A; IgG: immunoglobulin G; TNF-α: tumor necrosis factor-α.

CHAPTER 7. GENERAL CONCLUSIONS

The primary goal of nursery pig management is making a smooth weaning transition to minimize weaning associated severe depressed growth and diseases. While various stressors induce post-weaning growth depression, the abrupt change from milk to solid feed is one of the most apparent challenges to pigs. In order to manage the harsh weaning transition, investigating the roles of functional feed additives which can promote intestinal health and growth of pigs is necessary. The maintenance of normal intestinal architecture and well-regulated integrity of the small intestine is important to improve nutrient digestion and absorption. Among many of potential functional feed additives, soy protein hydrolysates produced from fermented or enzymatic hydrolysis (fermented or enzyme-treated soy oligopeptides and soybean meal), lysophospholipids, and fermented rice bran extracts were evaluated their functional roles in enhancing intestinal health and growth of nursery pigs.

Fermented soybean meal and enzyme-treated soybean meal have been used in nursery pig feeds to partially replace the conventional soybean meal because extensive use of conventional soybean meal in nursery pig feeds can cause hypersensitivity and diarrhea resulting in growth depression. During the fermented or enzymatic hydrolysis of soybean meal, proteins are cleaved to smaller molecules such as free amino acids and small size peptides. Few studies were conducted to investigate the effects of soy protein hydrolysates that containing high proportion of small size peptides on intestinal health and growth of newly weaned pigs. Those soy protein hydrolysates possessing high nutritional value with increased contents of small size peptides and eliminated allergenic proteins would require less digestion before absorption compared to intact soy proteins, and have a potential to be absorbed more efficiently without inducing allergenic response. Thus, when supplemented to newly weaned pig diets may positively affect intestinal function and growth performance of pigs.

The study in Chapter 2 concluded that fermented soy oligopeptides (FSO, 93% average MW was 859 Da; 7% was 27.7 kDa) supplementation at 1.0% improved feed efficiency without affecting feed intake, whereas, when supplemented at 2.0%, it decreased body weight, in large part may due to depressed feed intake. Although, negative effect was shown in growth performance, diarrhea incidence was reduced by supplementation of FSO up to 2.0%. When the maximum supplementation level was adjusted to 1.5%, it showed a potential to promote growth performance, enhanced intestinal morphology, and improved crude protein digestibility in a dose-dependent manner.

Based on the result observed in Chapter 2, which showed that 2.0% FSO supplementation may negatively affect feed intake. One of the major interest in the study carried out in Chapter 3 was to see how the enzyme-treated soy oligopeptides (ESO, 88% average MW was 863 Da; 12.0% was 32.7 kDa) affects feed intake. As described in Chapter 3, pigs preferred 2.0% ESO diet initially but they preferred a basal diet (0.0% ESO) over the 2.0% ESO diet as the feeding day increased. Supplementation of ESO up to 3% showed negative effects in ADG and ADFI may largely due to depressed feed intake. It was concluded that ESO supplementation level of 0.74% may be the most effective level in improving growth performance of nursery pigs. Furthermore, the supplementation of ESO showed to reduce diarrhea incidence without altering immune status, and showed a potential to enhance intestinal morphology.

Despite the beneficial effects shown on crude protein digestibility and the potential beneficial effects on intestinal morphology, depressed feed intake and growth performance were noticed in the studies that used soy protein hydrolysates containing high proportion of small peptides produced via fermentation or enzymatic hydrolysis. The potential reason for this observation may be related to that the bitter tasting hydrophobic amino acids concealed in the

interior of the protein that are released with the protein hydrolysis. The bitterness may also be related with the molecular weight of peptides. Future research to investigate the range of molecular weight of peptides in FSO that may contribute bitterness, and to elucidate which fraction of the peptides are needed to enhance the nutritional value of FSO.

Improvements in growth of nursery pigs when soybean meal is partially replaced by fermented or enzyme-treated soybean meal have been observed in previous studies. What is less understood is how the fermented and enzyme-treated soybean meal modulates intestinal microbiota. Fermented soybean meal containing probiotic microorganisms would exhibit a probiotic effect by modulating microbial composition. With a particular interest in how fermented soybean meal, fermented soybean meal that containing probiotic microorganisms, and enzyme-treated soybean meal affect mucosa-associated microbiota, 16S rRNA Pyrosequencing was conducted to find out if there is any difference among the different types of processed soybean meal. As described in Chapter 4, when conventional soybean meal is partially replaced with the fermented soybean meal, fermented soybean meal containing probiotic microorganisms *(Bacillus amyloliquefaciens*, 1.9×10^7 cfu/g), and enzyme-treated soybean meal, the fermented soybean meal containing probiotic microorganisms showed a greatest impact on mucosa-associated microbiota composition among the different dietary treatments. However, when evaluating their effects on growth performance, except the improved feed efficiency observed by the inclusion of enzyme-treated soybean meal during a part of the feeding phase, no differences in growth performance was observed among the treatments during the entire experimental period. This observation indicates that the shift in the microbiota composition by the fermented soybean meal containing probiotic microorganisms was not associated with the growth of pigs. Although no positive effects were observed in pig growth, dietary inclusion of fermented

soybean meal would be beneficial in maintaining intestinal health by increasing colonization of beneficial bacteria during the postweaning period. The findings from this study provide insights on how the mucosa-associated bacterial communities of the proximal small intestine change by different processed SBM consumption. Future studies under pathogenic bacteria challenged condition will be needed to further understand the beneficial effects on intestinal health by modulating intestinal microbiota.

At weaning, the synthesis of hepatic bile acid is low in pigs, fat emulsification is a rate-limiting step in the digestion of dietary fat in weaned pigs. An exogenous emulsifier, lysophospholipid complex (LPL) was evaluated for its effect on fat digestibility and growth performance. Besides the emulsifying ability, in previous studies, lysophospholipids showed to involve epithelial cell restitution via cytoskeletal remodeling. Therefore, LPL effects on epithelial proliferation, intestinal morphology, and intestinal barrier function were evaluated for its potential beneficial effects on intestinal health. The study in Chapter 5 concluded that the addition of the LPL at 0.1% to a nursery diet positively affected growth performance and showed a potential to improve lipid digestibility, and enhanced intestinal morphology and barrier function of nursery pigs.

Previous research suggested rice bran can be used as a source of prebiotics in nursery pig diets. However, high in fiber is a major limitation of its use in young pigs. Fermentation is a way to reduce fiber in the rice bran and the fermented rice bran have been used as an alternative feed ingredient in pigs. However, few studies were conducted on fermented rice bran extracts that containing high proportion of glucooligosaccharides. As described in Chapter 6, fermented rice bran extracts (FRBE) was evaluated its effects on growth performance and intestinal health as a source of prebiotics. Results indicated that FRBE supplementation up to 1.0% can potentially

increase growth performance and can enhance intestinal morphology in a dose-dependent manner. Furthermore, supplementation of 0.5% FRBE altered mucosa-associated microbiota composition. These results suggest that FRBE has a potential to use in a nursery pig diets as prebiotics, but the effect is depended on its supplemental level.

In summary, the use of fermented and enzyme-treated soy oligopeptides, fermented and enzyme-treated soybean meal, lysophospholipids, and fermented rice bran extracts showed a potential as a source of functional feed additives on promoting intestinal health which may in turn enhance growth performance of nursery pigs. The palatability of the ingredient and the optimum supplemental level are important factors to be considered when applying into nursery pig diets.

APPENDICES

Appendix A

Supplemental Table S1. Relative abundance of families detected in mucosa-associated microbiota of nursery pigs fed conventional soybean meal (SBM), enzyme-treated soybean meal (ESBM), fermented soybean meal (FSBM), and fermented soybean meal containing probiotic microorganism (PFSBM) from Chapter 4[1]

Item	Treatment				SEM	*P* value
	SBM	ESBM	FSBM	PFSBM		
Acetobacteraceae	0.01	0.00	0.01	0.01	0.00	0.611
Acidaminococcaceae	0.02	0.00	0.12	0.00	0.06	0.463
Aeromonadaceae	0.00	0.00	0.00	0.00	0.00	0.582
Bacillaceae	0.06	0.08	0.05	0.07	0.06	0.965
Bifidobacteriaceae	0.07	0.05	0.17	0.09	0.08	0.713
Brachyspiraceae	0.12	0.00	0.00	0.03	0.06	0.447
Campylobacteraceae	0.53	0.05	0.26	0.44	0.17	0.222
Carnobacteriaceae	0.02	0.01	0.02	0.00	0.01	0.334
Caulobacteraceae	0.10	0.00	0.02	0.02	0.05	0.518
Chlamydiaceae	0.74	0.00	0.27	1.00	0.60	0.579
Clostridiaceae	1.08	3.87	0.81	2.08	2.18	0.799
Coriobacteriaceae	0.00	0.00	0.01	0.00	0.00	0.288
Corynebacteriaceae	0.00	0.07	0.10	0.09	0.10	0.943
Enterobacteriaceae	0.00	0.02	0.18	0.03	0.09	0.468
Erysipelotrichaceae	0.57	0.00	0.00	0.00	0.29	0.415
Eubacteriaceae	0.04	0.00	0.01	0.03	0.03	0.675
Helicobacteraceae	22.98	8.76	22.80	32.96	12.15	0.524
Lachnospiraceae	0.04	0.09	0.00	0.13	0.08	0.648
Lactobacillaceae	1.07[a]	0.96[a]	0.96[a]	12.35[b]	3.31	0.033
Moraxellaceae	0.01	0.02	0.09	0.07	0.06	0.613
Nostocaceae	0.03	0.03	0.05	0.02	0.03	0.860
Oxalobacteraceae	0.02	0.00	0.00	0.12	0.06	0.439
Pasteurellaceae	2.16	0.11	0.00	0.03	1.10	0.430
Peptostreptococcaceae	0.15	0.02	0.00	1.26	0.65	0.442
Porphyromonadaceae	0.00	0.00	0.02	0.12	0.06	0.447
Prevotellaceae	0.21	0.18	0.40	0.71	0.37	0.712
Propionibacteriaceae	0.10	0.02	0.01	0.00	0.03	0.167
Pseudomonadaceae	53.28	61.43	51.96	33.90	8.80	0.135
Rhodobacteraceae	0.00	0.01	0.02	0.00	0.01	0.238
Rikenellaceae	0.00	0.09	0.00	0.00	0.04	0.413

Supplemental Table S1. Continued

Ruminococcaceae	0.00	0.29	1.61	0.01	0.81	0.455
Staphylococcaceae	6.81	8.62	7.65	4.57	1.19	0.120
Streptococcaceae	0.07	0.00	0.06	0.04	0.03	0.353
Succinivibrionaceae	0.10	0.06	0.04	0.32	0.17	0.613
Veillonellaceae	0.68	0.31	1.02	1.49	0.83	0.774
Xanthomonadaceae	8.51	14.84	11.28	7.91	2.67	0.105

[1]Means lacking a common small letters are significantly different ($P < 0.05$).

Supplemental Table S2. Relative abundance of genera detected in mucosa-associated microbiota of nursery pigs fed conventional soybean meal (SBM), enzyme-treated soybean meal (ESBM), fe rmented soybean meal (FSBM), and fermented soybean meal containing probiotic microorganism (PFSBM) from Chapter 4[1]

Item	Treatment				SEM	*P* value
	SBM	ESBM	FSBM	PFSBM		
Acidaminococcus	0.00	0.00	0.12	0.00	0.06	0.422
Acinetobacter	0.00	0.01	0.07	0.06	0.05	0.504
Actinobacillus	1.84	0.12	0.00	0.03	0.93	0.431
Bacillus	2.09	0.04	0.05	0.57	1.11	0.472
Bifidobacterium	0.07	0.06	0.17	0.09	0.08	0.717
Brachyspira	0.12	0.00	0.00	0.00	0.06	0.417
Brevundimonas	0.00	0.00	0.00	0.01	0.00	0.445
Campylobacter	0.51	0.04	0.26	0.42	0.17	0.232
Candidatus Arthromitus	0.24	4.48	0.08	0.09	1.75	0.400
Carnobacterium	0.02	0.02	0.02	0.00	0.01	0.244
Chlamydia	0.74	0.03	0.27	1.00	0.60	0.579
Cloacibacterium	0.01	0.00	0.05	0.00	0.03	0.471
Clostridium	1.74	0.02	0.18	1.90	1.27	0.525
Corynebacterium	0.11	0.07	0.03	0.09	0.09	0.617
Dialister	0.01	0.02	0.47	0.15	0.24	0.493
Enterobacter	0.00	0.00	0.00	0.01	0.00	0.464
Enterococcus	0.00	0.03	0.00	0.00	0.01	0.417
Escherichia	0.00	0.00	0.06	0.00	0.03	0.417
Faecalibacterium	0.00	0.15	0.00	0.01	0.06	0.424
Gibbsiella	0.00	0.00	0.01	0.01	0.00	0.582
Haemophilus	0.19	0.00	0.00	0.00	0.09	0.419
Helicobacter	19.30	8.64	22.78	32.83	11.58	0.520
Herbaspirillum	0.00	0.00	0.16	0.01	0.08	0.423
Lachnospira	0.00	0.03	0.00	0.00	0.01	0.417
Lactobacillus	1.07[a]	0.55[a]	0.80[a]	12.35[b]	3.35	0.031
Leclercia	0.01	0.00	0.02	0.00	0.01	0.497
Massilia	0.00	0.00	0.00	0.07	0.04	0.417
Megasphaera	0.04	0.06	0.16	0.21	0.10	0.603
Mitsuokella	0.26	0.14	0.25	0.56	0.27	0.757
Pasteurella	0.00	0.00	0.00	0.00	0.00	0.417
Pedobacter	0.00	0.00	0.00	0.01	0.00	0.417
Phascolarctobacterium	0.02	0.00	0.00	0.00	0.01	0.417
Prevotella	0.18	0.16	0.33	0.62	0.32	0.707

Supplemental Table S2. Continued

Propionibacterium	0.10	0.02	0.01	0.00	0.03	0.167
Pseudochrobactrum	0.00	0.00	0.00	0.00	0.00	0.417
Pseudomonadaceae	0.00	14.45	0.00	4.93	2.46	0.417
Pseudomonas	55.61^{AB}	47.58^{A}	54.00^{AB}	30.84^{B}	10.56	0.072
Psychrobacter	0.01	0.01	0.01	0.00	0.01	0.524
Roseburia	0.00	0.00	0.00	0.13	0.06	0.417
Sarcina	0.00	0.00	0.00	0.04	0.02	0.417
Sphingobacterium	0.00	1.79	0.01	0.02	0.01	0.484
Staphylococcus	6.53	7.21	7.53	4.53	1.18	0.126
Stenotrophomonas	0.61^{ab}	0.82^{a}	0.68^{ab}	0.36^{b}	0.27	0.037
Streptococcus	0.07	0.00	0.06	0.04	0.03	0.343
Succinivibrio	0.05	0.04	0.03	0.32	0.16	0.527
Terrisporobacter	0.00	0.00	0.00	0.01	0.00	0.417
Turicibacter	0.57	0.00	0.00	0.00	0.28	0.415
Xanthomonadaceae	0.00	12.06	0.00	0.00	0.04	0.417

[1]Means lacking a common small letters are significantly different ($P < 0.05$), and those lacking a common capital letters indicate means are tended to be different ($0.05 \leq P < 0.10$).

Appendix B

Supplemental Table S3. Relative abundance of families detected in mucosa-associated microbiota of nursery pigs fed diets with fermented rice bran extracts (FRBE) from Chapter 6

Item	Added FRBE, %			SEM	*P* value	
	0.0	0.5	1.0		Linear	Quadratic
Acetobacteraceae	2.41	0.96	1.54	1.05	0.564	0.439
Acidaminococcaceae	0.34	0.26	0.44	0.18	0.591	0.407
Aerococcaceae	0.25	0.04	0.00	0.15	0.239	0.605
Bacillaceae	2.51	10.28	7.63	7.57	0.529	0.461
Bifidobacteriaceae	0.17	0.13	0.24	0.09	0.524	0.395
Brachyspiraceae	0.37	0.05	0.02	0.20	0.219	0.552
Campylobacteraceae	1.38	0.73	0.04	0.71	0.192	0.979
Caulobacteraceae	0.73	0.16	0.31	0.19	0.132	0.135
Clostridiaceae	6.89	14.38	2.36	4.81	0.513	0.113
Corynebacteriaceae	0.38	0.20	0.20	0.14	0.347	0.601
Enterobacteriaceae	2.86	5.20	0.53	2.16	0.456	0.199
Enterococcaceae	0.22	0.14	0.75	0.37	0.331	0.467
Eubacteriaceae	2.79	1.12	0.64	0.78	0.308	0.925
Flavobacteriaceae	2.94	0.27	0.17	1.50	0.192	0.479
Helicobacteraceae	10.77	25.89	19.71	8.26	0.453	0.305
Lachnospiraceae	3.20	1.70	1.51	0.91	0.204	0.561
Lactobacillaceae	13.72	17.79	21.72	11.1	0.196	0.795
Microbacteriaceae	0.63	0.04	0.11	0.18	0.160	0.145
Moraxellaceae	0.84	0.29	0.21	0.34	0.204	0.572
Oxalobacteraceae	0.82	0.09	0.62	0.34	0.643	0.109
Paenibacillaceae	0.54	0.26	0.50	0.19	0.887	0.284
Phormidiaceae	0.91	0.80	1.42	0.41	0.390	0.466
Porphyromonadaceae	0.52	0.24	1.01	0.47	0.409	0.313
Prevotellaceae	16.99	4.83	15.14	6.13	0.817	0.117
Propionibacteriaceae	0.97	0.47	0.56	0.33	0.380	0.456
Pseudanabaenaceae	0.18	0.25	0.08	0.16	0.618	0.497
Pseudomonadaceae	8.05	1.87	2.78	2.88	0.167	0.279
Pseudonocardiaceae	0.64	0.01	0.01	0.36	0.223	0.486
Rhodobacteraceae	0.22	0.20	0.28	0.10	0.593	0.604
Ruminococcaceae	0.85	0.76	0.93	0.37	0.788	0.626
Sphingobacteriaceae	0.23	0.02	0.44	0.19	0.453	0.201
Sphingomonadaceae	0.35	0.21	0.30	0.21	0.813	0.570
Staphylococcaceae	0.34	0.19	0.20	0.15	0.511	0.657
Streptococcaceae	1.95	5.13	1.13	1.63	0.725	0.092
Succinivibrionaceae	1.12	0.22	2.69	1.20	0.207	0.124
Veillonellaceae	4.47	1.64	5.35	1.46	0.624	0.152
Xanthomonadaceae	1.64	2.84	0.32	1.56	0.558	0.343

Supplemental Table S4. Relative abundance of genra detected in mucosa-associated microbiota o f nursery pigs fed diets with fermented rice bran extracts (FRBE) from Chapter 6

Item	Added FRBE, %			SEM	*P* value	
	0.0	0.5	1.0		Linear	Quadratic
Acidaminococcus	0.26	0.20	0.21	0.11	0.785	0.795
Acinetobacter	0.96	0.30	0.23	0.48	0.253	0.601
Actinoplanes	0.00	0.12	0.00	0.07	0.990	0.204
Anoxybacillus	1.80	0.67	0.52	0.54	0.108	0.405
Bacillus	1.52	9.74	7.11	5.34	0.488	0.460
Bifidobacterium	0.26	0.17	0.31	0.12	0.762	0.449
Blautia	0.45	0.17	0.25	0.22	0.409	0.414
Bulleidia	0.06	0.11	0.08	0.05	0.732	0.461
Burkholderia	0.05	1.09	0.03	0.68	0.984	0.206
Campylobacter	2.24	0.99	0.29	1.31	0.225	0.846
Catenibacterium	0.08	0.18	0.09	0.07	0.896	0.232
Clostridium	7.21	10.54	2.82	6.54	0.528	0.261
Corynebacterium	0.62	0.30	0.29	0.22	0.301	0.572
Delftia	0.23	0.11	0.12	0.09	0.363	0.516
Dialister	0.35	0.21	0.36	0.13	0.933	0.331
Dyella	0.00	0.11	0.00	0.07	0.990	0.204
Enterobacter	0.02	0.00	0.00	0.01	0.125	0.267
Enterococcus	0.41	0.20	1.41	0.78	0.295	0.403
Eubacterium	0.46	0.20	0.08	0.05	0.179	0.516
Eubacterium	0.04	0.09	0.08	0.05	0.496	0.533
Faecalibacterium	0.54	0.41	0.67	0.20	0.621	0.380
Frateuria	0.02	1.86	0.04	1.17	0.990	0.204
Gemmiger	0.18	0.19	0.40	0.14	0.253	0.579
Gordonia	0.11	0.10	0.02	0.06	0.328	0.637
Helicobacter	10.77	25.89	19.71	6.88	0.372	0.224
Lactobacillus	13.62	17.71	21.62	11.22	0.183	0.825
Lactococcus	0.05	0.02	0.01	0.03	0.170	0.723
Luteibacter	0.01	1.22	0.02	0.77	0.990	0.204
Megasphaera	0.32	0.28	0.57	0.15	0.274	0.133
Mitsuokella	0.48	0.22	0.41	0.20	0.722	0.204
Myroides	0.01	0.19	0.02	0.13	0.926	0.244
Nostocaceae	5.71	7.10	10.86	3.43	0.347	0.806
Phascolarctobacterium	0.26	0.16	0.45	0.16	0.292	0.243
Prevotella	13.92	3.01	11.63	4.41	0.737	0.105
Propionibacterium	1.57	0.70	0.81	0.52	0.317	0.460
Pseudomonas	7.86	2.40	2.25	2.30	0.151	0.283

Supplemental Table S4. Continued

Roseburia	1.55	0.66	0.84	0.62	0.275	0.346
Ruminococcus	0.14	0.10	0.12	0.05	0.756	0.481
Ruminococcus	0.14	0.10	0.12	0.05	0.756	0.481
Sarcina	2.69	1.34	1.38	1.08	0.108	0.842
Selenomonas	0.94	0.84	0.95	0.39	0.994	0.797
Sphingomonas	0.25	0.16	0.16	0.14	0.573	0.742
Staphylococcus	0.54	0.31	0.26	0.22	0.378	0.732
Stenotrophomonas	0.19	0.10	0.10	0.16	0.278	0.712
Streptococcus	1.95	5.13	1.13	1.63	0.725	0.090
Succinivibrio	0.94	0.22	2.31	0.73	0.247	0.140
Turicibacter	0.03	0.21	0.12	0.07	0.422	0.155
Xanthomonadaceae	1.62	2.84	0.27	1.42	0.550	0.342