DIETARY EFFECTS ON PIG HEALTH

BY

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DISSERTATION

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ABSTRACT

Although practical health management practices have been used to control disease problems in the swine industry, they cannot guarantee freedom from diseases. Moreover, use of antibiotics as a powerful health management practice is being restricted because of health safety concerns. Therefore, the swine industry has been looking for all kinds of alternatives for antibiotics and increasingly considers use of dietary factors like feed ingredients, feed additives, feed formulation practices, or feeding methods because they provide physiological activities to pigs to improve their health and performance by modulation of microbial populations in the digestive tract and/or immune system. The 6 experiments described in this dissertation were conducted to evaluate whether specific dietary factors can be important components of health management programs.

The first experiment evaluated whether and how dietary antibiotics modulate microbial populations in the digestive tract of pigs. Virginiamycin treatments reduced the number of total bacterial cells (wk 2: 11.1 vs. 11.5, log/g ileal digesta; wk 3: 11.2 vs. 11.5, log/g ileal digesta; wk 4: 11.3 vs. 12.00, log/g feces; $P < 0.05$) during the virginiamycin feeding (wk 2 to 4) compared with control treatment (CON). Carbadox treatments made pigs more similar to each other in ileal microbiota during the carbadox feeding (wk 2 to 4) after an initial disruption (wk 2: intratreatment similarity coefficients (Cs) 76 vs. 93%; wk 4: intratreatment Cs 92 vs. 80%; $P < 0.05$) compared with the CON. However, intertreatment Cs values did not show effects of the antibiotics. Some specific bands (1 or more species of microbes) were present in most pigs fed the CON, but absent from most pigs fed either antibiotics. In conclusion, both virginiamycin and

carbadox modified microbial populations in digestive tract of pigs by eliminating some species of microbes.

The second experiment evaluated whether dietary spray-dried plasma (SDP) improves pregnancy rate after transport stress using mated female mice as a model for stressed sows. The SDP markedly improved ($P < 0.05$) pregnancy rate (49 vs. 11%) regardless of initial BW of mice (BW < 16 g: 36 vs. 4%; BW ≥ 16 g: 57 vs. 16%; no interactions between SDP and initial BW of mice) compared with the CON. In conclusion, SDP improved pregnancy rate of the mated female mice after transportation stress.

The third experiment evaluated whether dietary SDP moderates inflammation and ameliorates impairment of reproduction caused by lipopolysaccharide (LPS) using pregnant mice as a model for inflammation in sows. The SDP increased ($P < 0.05$) ADG (0.712 vs. 0.638 g/d) before the LPS challenge (gestation day (GD) 3 to 17) compared with the CON. The LPS challenge on GD 17 increased ($P < 0.10$) pregnancy loss, fetal death, spleen weight (WT), and pro-inflammatory cytokines (PRO) in uterus (U) and placenta (P), and reduced growth performance and anti-inflammatory cytokines (ANTI) in the U only compared with the PBS challenge. The SDP increased BW gain (6 h after the LPS challenge (6H): 0.13 vs. -0.14 g, $P = 0.06$; 24 h after the LPS challenge (24H): 0.81 vs. 0.30 g, $P < 0.05$) and avg live fetal WT (6H: 0.65 vs. 0.56 g, $P < 0.05$; 24H: 0.76 vs. 0.71 g; $P = 0.09$), and reduced spleen WT (6H: 0.29 vs. 0.35% of BW, $P = 0.08$; interaction, $P = 0.09$) compared with the CON. In addition, the SDP reduced ($P < 0.05$) PRO (pg/mg TP) in both U (TNF-α: 3.83 vs. 6.93; IFN-γ: 0.97 vs. 2.37) and P (TNF-α: 4.15 vs. 5.71; IFN-γ: 0.19 vs. 0.46) and ANTI (ng/mg TP) in the U only (IL-10: 0.039 vs. 0.050; TGF-β1: 0.28 vs. 0.50) compared with the CON, and attenuated the LPS effect on PRO (interactions: TNF-α in the P ($P = 0.09$), IFN-γ in both U ($P = 0.08$) and P ($P < 0.05$)). In

conclusion, SDP improved growth performance of pregnant mice before and after acute inflammation caused by the LPS, and their fetal WT after the acute inflammation, and attenuated the acute inflammation, but did not affect pregnancy loss and fetal death after the acute inflammation.

The fourth experiment evaluated whether dietary clays reduce diarrhea of weaned pigs experimentally infected with a pathogenic *Escherichia coli*. In the *E. coli* challenged group of the first study, smectite treatments (with different levels and timing of introduction) reduced diarrhea score (DS) for the overall period (1.77 vs. 2.01; $P < 0.05$) and ratio between β-hemolytic coliforms to total coliforms (RHT) on d 6 (0.60 vs. 0.87; $P < 0.05$) and d 9 (0.14 vs. 0.28; $P = 0.08$), and altered differential white blood cells (WBC) on d 6 (neutrophils, 48 vs. 39%, $P = 0.09$; lymphocytes, 49 vs. 58%, $P = 0.08$) compared with the CON. In the *E. coli* challenged group of the second study, clay treatments (smectite, kaolinite, and zeolite individually and all possible combinations) reduced DS for the overall period (1.63 vs. 3.00; $P < 0.05$), RHT on d 9 (0.32 vs. 0.76; $P < 0.05$) and d 12 (0.13 vs. 0.39; $P = 0.09$), and total WBC on d 6 (15.2 vs. 17.7 $x10^3/\mu L$; $P = 0.07$) compared with the CON. However, no clay effects were found on growth performance in either study. In conclusion, clays alleviated diarrhea of weaned pigs experimentally infected by a pathogenic *E. coli*, but did not affect their growth performance.

The fifth experiment evaluated whether dietary spray-dried egg (SDE) can improve growth performance or health of weaned pigs. In the first two studies, SDE improved ($P < 0.05$) ADG (Study 1: 243 vs. 204 g/d; Study 2: 204 vs. 181 g/d) and ADFI (Study 1: 236 vs. 204 g/d; Study 2: 263 vs. 253 g/d) compared with control diet, but did not affect G:F. In the last two studies, there were no differences on growth performance between SDE treatments and treatments without the SDE. However, in the third study as a commercial farm trial, the SDE

treatments reduced frequency of medical treatments (per pen and day) during the first wk after weaning (0.73 vs. 1.33%; $P < 0.05$) and overall 6 wk period (0.83 vs. 1.00%; $P = 0.06$) compared with the treatments without the SDE, but did not affect removal rate. In conclusion, SDE can be an efficacious protein source in nursery pig diets by its nutrient contributions to improve growth performance and perhaps physiological benefits to improve health of weaned pigs.

The sixth experiment evaluated whether dietary enzymes modulate ileal microbial populations of pigs fed diets containing distillers grains with solubles (DDGS). Xylanase treatments made pigs less similar to each other in ileal microbiota (intratreatment Cs 45.4 vs. 51.3%; $P < 0.05$) compared with treatments without the xylanase, but this pattern was not found in pigs fed phytase treatments. There were no differences on the number of bands and intertreatment Cs values between treatments. In a few cases, specific bands were present or absent in most pigs fed the CON, but absent or present from most pigs fed either phytase or xylanase treatments. The sequences of the specific bands matched *Lactobacillus avarius* and *Burkholderia cepacia* with 99% and 100% similarities, respectively, in pigs fed the phytase treatments, members of the genus *Serratia* and *Burkholderia* with 100% similarities in pigs fed the xylanase treatments, and members of the genus *Pseudomonas and Serratia* with 99% similarity in pigs fed the CON. In conclusion, both phytase and xylanase enzymes may modify ileal microbial populations of pigs fed DDGS.

Overall, swine nutrition needs to consider strongly the present concerns about the restricted use of antibiotics or perhaps a total ban of antibiotics use in the near future along with the role of practical health management practices. Based on the evidence of above potential benefits, some dietary factors (dietary SDP, clays, SDE, and enzymes in this dissertation, but

other dietary factors as well) are believed to be potential solutions because they are able to provide physiological activities to pigs to improve their health and performance by modulation of microbial populations in the digestive tract and/or immune system. Therefore, it is suggested that some dietary factors may be important components in pig health management programs.

DEDICATION

This dissertation is dedicated with my deepest gratitude to God,

my parents, Emeritus Professor Samhong Song & Mrs. Cheonghee Son,

my parents-in-law, Mr. Whichang Lee and Mrs. Myungsook Han,

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CHAPTER 1

INTRODUCTION

The main goals of swine production are maximization of pig productivity and profits. In swine production, nutrition, genetics, and management have been most important to meet the goals. In the other side, the swine industry has also emphasized health improvement to keep pigs healthy from diseases by most practical management factors such as all-in /all-out pig flow, age segregation, intense biosecurity practices, sanitation, new vaccinations, and depopulation /repopulation (Hardy, 2002; Adjiri-Awere and van Lunen, 2005; NAHMS, 2008). All of these technologies are powerful, but they cannot guarantee freedom from diseases for pigs, as the Swine Survey 2006 (NAHMS, 2008) shows mortality of pigs from pre-weaning to market is increased by various causes compared with previous surveys, the Swine Surveys 2000, 1995, and 1990. Moreover, the use of antibiotics in swine production has been changing (Hardy, 2002; Pettigrew, 2006; Stein and Kil, 2006) to more restricted use of in-feed antibiotics that are growth promoters and are powerful in disease control as well (Cromwell, 2002; Gaskins et al., 2002), due to potential safety issues of use of antibiotics for livestock animals (Hardy, 2002; Pluske et al., 2002; Adjiri-Awere and van Lunen, 2005).

Due to the above two main issues, swine production increasingly considers use of dietary factors like feed ingredients, feed additives, feed formulation practices, or feeding methods to improve pig health (Pluske et al., 2002; Pettigrew, 2006; Stein and Kil, 2006) as all kinds of alternatives for antibiotics. Especially, the post-weaning period has been emphasized not only to

maintain or improve the health of weaning pigs because of potential stresses by weaning and their immature immune system, but also to provide adequate nutrients to pigs because of their immature digestive tracts (Pluske et al., 2002; Lallès et al., 2007; van Heugten, 2007). Therefore, use of dietary factors for the weaning pigs has been more emphasized and tested to maximize productive performance and to minimize disease problems (Hardy, 2002; Adjiri-Awere and van Lunen, 2005; Lalles et al., 2007), and it is going further for pigs in any other stages.

It has been known that some dietary factors can improve pig health as well as productive performance (Pluske et al., 2002; Pettigrew, 2006; Stein and Kil, 2006). For instance, pig health and performance can be improved by provision of bioavailable nutrients as well as physiological activities from some feed ingredients (spray-dried plasma (Coffey and Cromwell, 2001; van Dijk et al., 2001), spray-dried egg (DeRouchey et al., 2003; Harmon et al. 2007), milk products (Grinstead et al., 2000; Severin and Wenshui, 2005), rice (Pluske et al., 2003; Vicente et al., 2008), etc.), by provisions of physiological activities from feed additives (clay (Carretero, 2002; Trckova et al., 2009), enzymes (Partridge and Tucker, 2000; Kiarie et al., 2007), etc.), by feed formulating practices such as low protein diets (Nyachoti et al., 2006; Heo et al., 2008, 2009), or by feeding methods such as fermented or liquid feeding (van Winsen et al., 2001; Lawlor et al., 2002).

In general, they are suggested to provide potential physiological benefits through modulation of microbial populations in the pig digestive tract and/or modulation of the immune system directly or indirectly, resulting in improvement of gut health and/or immunity of pigs and thereby growth performance as the energy to maintain gut health and immunity may be conserved to be used for pig growth. For example, spray-dried plasma provides bioavailable nutrients as an excellent protein source (van Dijk et al., 2001) and physiological activities such

as immune competence (antibacterial activity), modulation of microbiota and/or immune system, and integrity of intestinal barrier function, etc. (Pettigrew et al., 2006), perhaps resulting in enhancement of their intestinal health, immunity, and further growth performance. Besides spray-dried plasma, several other feed ingredients and additives are proposed to improve pig health and/or growth performance. Increasing experimental evidence shows that such ingredients improve pig health and/or growth performance (Pluske et al., 2002; Pettigrew, 2006; Stein and Kil, 2006).

Based on the evidence of these potential benefits, some dietary factors are believed to be able to improve pig health as well as productive performance. Therefore, it is suggested that some dietary factors may be important components in health management programs.

The overall objective of this dissertation was to evaluate whether some dietary factors can be alternatives for antibiotics as they potentially improve pig health and/or growth performance by modulating microbial populations in the digestive tract and/or immune system of pigs. The first specific objective was to evaluate effects of dietary antibiotics on ileal and fecal microbial ecology of pigs. The second specific objective was to evaluate the effect of dietary spray-dried plasma on pregnancy rate of mated female mice after transport as a model for stressed sows. The third specific objective was to evaluate effects of dietary spray-dried plasma on growth, reproductive, and immune responses of pregnant mice to lipopolysaccharide as a model for inflammation in sows. The fourth specific objective was to evaluate effects of dietary clays on diarrhea of newly weaned pigs experimentally infected with a pathogenic *Escherichia coli*. The fifth specific objective was to evaluate effects of dietary spray-dried egg on growth performance and health of weaned pigs. The sixth specific objective was to evaluate effects of

dietary enzymes on ileal microbial ecology of pigs fed diets containing distillers dried grains with solubles.

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CHAPTER 2

LITERATURE REVIEW

2.1. Changes in Swine Health

In the swine industry, the practical management practices to keep pigs healthy are all-in /all-out pig flow, age segregation, intense biosecurity practices, sanitation, new vaccinations, and depopulation /repopulation (Hardy, 2002; Adjiri-Awere and van Lunen, 2005; NAHMS, 2008). All of these technologies are now common and powerful, but they cannot guarantee freedom from disease for pigs, as shown in the recent national swine survey, Swine 2006 (NAHMS, USDA, 2008).

The USDA's National Animal Health Monitoring System (NAHMS) monitors changes and trends in national swine health and management, and has reported an overview of changes in U.S. swine management and health from 1990. The Swine 2006 was the fourth report. It surveyed 17 participating states accounting for 94% of swine operations by randomly selecting 5,000 swine producers with 100 or more pigs and by up to two visits to each farm by veterinary medical officers. The Swine 2006 (NAHMS, 2008; Figures 2.1 to 2.10) shows an increased annual culling rate of sows (48.8 vs. 37.7%) mainly because of reproductive failure, injury, or performance (Figures 2.1 and 2.2), an increased mortality of pre-weaning pigs (13.2 vs. 11.8%) because of mainly diarrhea, starvation, or respiratory problems (Figures 2.3 and 2.4), an increased mortality of post-weaning pigs (2.9 vs. 2.6%) because of mainly diarrhea, respiratory problems, or CNS/meningitis by diarrheic *Escherichia coli* (*E.coli*), porcine reproductive and

respiratory syndrome virus (PRRSV), *Streptococcus*, *Haemophilus*, or others (Figures 2.5, 2.6, and 2.7), and an increased mortality of growing-finishing pigs (3.9 vs. 2.9%) because of mainly respiratory problems or diarrhea by PRRSV, *Lawsonia*, swine flu, *Mycoplasma*, or others (Figures 2.8, 2.9, and 2.10) compared with the previous Swine 2000 survey (NAHMS, 2008). These results also show increases of about 11.1% units in sow culling rates and about 2.7 % units in mortality from birth to market.

In summary, productivity of swine production has been improved by many efforts. Despite of the efforts, about 20% pigs have been still died from born to market because of disease problems. Although practical management practices have been used to control disease problems, they cannot guarantee freedom from diseases. Therefore, other approaches are needed to improve pig health, along with the practical management practices.

2.2. Dietary Factors and Importance of Post-Weaning Period

Use of Antibiotics. The challenge to keep pigs healthy is not easy. The U.S. swine industry has widely used antibiotics as antimicrobials to prevent or control diseases to suppress or inhibit the growth of certain microorganisms, or as growth promoters via their inhibition in the normal microbiota, which result in improvement of growth rate, efficiency, and reproductive performance, and in reduction of mortality and morbidity (Cromwell, 2002; Gaskins et al., 2002). However, safety issues of use of antibiotics in livestock have been raised, such as their potential threat to consumer (human) health and mainly potential antibiotic-resistant enteric bacteria which causes a potential animal and human health risk (Cromwell, 2002; Hardy, 2002; Pluske et al., 2002). These issues have made use of antibiotics for livestock animals more restricted or may make them completely banned in the U.S. as in the European Union. Therefore,

the swine industry has considered all kinds of alternatives for antibiotics to improve pig health without the potential safety issues as antibiotics have.

Dietary Factors. It is imperative that the swine industry prepares for such restriction in case it occurs. Recently, the swine industry increasingly considers use of dietary factors like feed ingredients, feed additives, feed formulation practices, or feeding methods to improve pig health and performance (Pluske et al., 2002; Pettigrew, 2006; Stein and Kil, 2006). There are now remarkably rich supplies of products and practices available to the swine industry and they are proposed to improve pig health as well as productive performance (Table 2.1).

Characteristics of Dietary Factors. Characteristics of these dietary factors are different from those of antibiotics (Pettigrew, 2006). First, antibiotics are non-nutritive feed additives, whereas some dietary factors are important nutrient sources. Second, antibiotics are powerful and used for preventing pigs from specific enteric diseases, targeting specific microbes that cause the enteric disease as well as non-specific microbes. However, dietary factors are less powerful and used mainly for physiological benefits. Some dietary factors provide physiological components such as immunoglobulins, glycoproteins, peptides, etc. which are directly active against pathogenic microbes or viruses. In addition, some dietary factors provide physiological activities such as modulation of the intestinal environment, adsorption property, etc. which are active indirectly or directly against pathogenic microbes. Third, inclusion rates of antibiotics are low in diets, whereas those of some dietary factors are much higher in diets than those of antibiotics.

General Proposed Mechanisms of Dietary Factors. Most of these dietary factors appear to act either by changing the microbial populations in the digestive tract and/or by modifying the action of the immune system of pigs, although some have other mechanisms (Pluske et al., 2002;

Hardy, 2002; Lalles et al., 2007). However, some dietary factors may be effective either with antibiotics or without antibiotics (Pettigrew, 2006; Stein and Kil, 2006).

Importance of Post-Weaning Period. The post-weaning period has been especially emphasized to maintain or improve pig health as well as to provide adequate nutrients to pigs because of several reasons (Lalles et al., 2007; van Heugten, 2007). First, piglets are moved from a known to an unknown environment and are mixed with other piglets, which create social and behavioral changes. Second, piglets are removed from the sows, which previously provided protection from diseases through antibodies in her milk. The immune system of the newly weaned pig is still relatively immature and thus it is very susceptible to diseases. Third, the weaned piglet is switched from a liquid diet of sow milk to a solid feed of nursery diet. Pigs need to learn how to consume the feed and have to develop the digestive capacity to break down the feed into nutrients that can be absorbed. Therefore, weaning is a stressful event for nursery pigs. Because of these stresses by environmental changes as well as by immature immune system and digestive tract, various nutritional and health management practices for nursery pigs have been emphasized and tested to maximize productive performance and to minimize disease problems (Pluske et al., 2002; Lalles et al., 2007).

In summary, one of the powerful health management practices is use of antibiotics, but their use is being restricted because of health safety issues. The swine industry has been looking for all kinds of alternatives for antibiotics and increasingly considers use of dietary factors like feed ingredients, feed additives, feed formulation practices, or feeding methods instead of antibiotics to improve pig health and performance. These dietary factors provide physiological activities to pigs to improve their health and performance by modulation of microbial populations in the intestinal digestive tract and/or of immune system. Especially weaning is a

stressful event for nursery pigs because of stresses from a new environment, new solid feed, and immature immune system and digestive tract. That's why various nutritional and health management practices for nursery pigs have been emphasized and tested to maximize productive performance and to minimize disease problems. Therefore, dietary factors may be one solution to improve health and performance of weaning pigs.

2.3. Dietary Factors on Pig Health

As Table 2.1 shows, there are several potential dietary factors to improve pig health and performance. This list is not complete and more potential dietary factors will come out in the future. In this review, only four different dietary factors (and antibiotics) are addressed. It does not mean other dietary factors are not powerful. Other dietary factors also have potential effects to improve pig health and performance by modulation of microbiota and/or immune system of pigs.

2.3.1. Antibiotics

Definition. The Association of American Feed Control Officials (AAFCO, 2008) mentions antibiotics are "a class of drug which are usually synthesized by a living microorganism and in proper concentration inhibit the growth of other microorganisms" and are non-nutritive feed additives (Jacela et al., 2009).

General Effects. It has been known that antibiotics mainly improve growth rate and efficiency of feed utilization as well as reproductive performance and thus they are called "growth promoters" (Gaskins et al., 2002; Hardy, 2002; Dibner and Richards, 2005), and that antibiotics improve animal health as they reduce mortality and morbidity by preventing or

treating diseases (Cromwell, 2002; Dibner and Richards, 2005). In addition, antibiotics have many effects physiologically, nutritionally, and metabolically (Table 2.2; Gaskins et al., 2002).

General Proposed Mechanisms. Antibiotics suppress or inhibit the growth of certain microorganisms or subclinical infection by damaging their cell wall formation, disrupting their nucleic acid synthesis, etc. (Gaskins et al., 2002; Hardy, 2002; Niewold, 2007). The result is reduced competition between host and microbes for nutrients and reduced microbial metabolites that depress host growth (Gaskins et al., 2002; Dibner and Richards, 2005; Jacela et al., 2009). In addition, antibiotics reduce intestinal density (thinner intestinal villi and total intestinal wall) by the loss of mucosal cell proliferation in the absence of luminal short chain fatty acids from microbial fermentation (Hardy, 2002; Gaskins et al., 2002).

Pig Performance and Health. Antibiotics improve growth rate and the efficiency of feed utilization for young pigs under research station environments (Dritz et al., 2002; Manzanilla et al., 2006; Walsh et al., 2007), but more powerfully under commercial farm conditions (Cromwell, 2002; Gaskins et al., 2002), maybe by disease control effects or reduction in microbial loading in the intestinal tract. In addition, antibiotics reduce mortality and morbidity under normal conditions as well as more strongly under high-disease conditions (Cromwell, 2002). Antibiotics also improve digestibility of specific nutrients such as calcium and phosphorus (Agudelo et al., 2007), maybe by reduction of competition between host and microbes for nutrients.

Antibiotics modulate ileal microbiota (Castillo et al., 2006; Gong et al., 2008; Rettedal et al., 2009), make microbial populations in the ileum more homogenous (Collier et al., 2003), and reduce the total number of bacteria (Collier et al., 2003) by eliminating certain bacterial groups or changing the ecological diversity.

Antibiotics modulate immune responses. They reduce intraepithelial lymphocytes and lymphocytes in the lamina propria in the small intestine (Manzanilla et al., 2006) and reduce serum TNF-α concentration and ileal mRNA TNF-α expression (Weber and Kerr, 2008). In addition, Niewold (2007) proposed most antibiotics have a non-antibiotic anti-inflammatory effect. Generally, the antibiotics accumulate in phagocytic inflammatory cells and inhibit phagocyte function, resulting in attenuation of inflammatory responses such as reduction of pro-inflammatory cytokines.

Safety Issues. The safety issues have been concerned because their potential threat to human health such as the drug-resistant bacteria which can transfer their resistance to pathogenic bacteria and can cause a potential public health risk (Hardy, 2002; Adjiri-Awere and van Lunen, 2005; Dibner and Richards, 2005). A long term study at the University of Kentucky showed antibiotic resistance seems to decrease after withdrawal of antibiotics in the pig diets, but the shedding of resistant bacteria is present even without antibiotics in the diets (Cromwell, 2002). This indicates a total ban of antibiotics will not completely eliminate the antibiotic resistant bacteria. In addition, despite the reduction or total ban of antibiotics, there may be a risk of negative effects such as animal welfare, nutrient utilization, manure production, and economic loss (Cromwell, 2002; Adjiri-Awere and van Lunen, 2005).

In summary, antibiotics are non-nutritive feed additives and one of most powerful practical management practices for pig health and performance. There are several beneficial physiological activities such as suppression or inhibition of the growth of certain microorganisms or subclinical infection, reduction of competition between host and microbes for nutrients, reduction of microbial metabolites that depress host growth, reduction of intestinal density, etc. Those beneficial effects can contribute to improvement of pig performance and

health by modulation of microbiota and immune system. However, the health safety issues against antibiotic resistant microbes are of concern.

2.3.2. Spray-Dried Plasma

Definition. Spray-dried plasma (SDP) is made from blood collected at slaughter plants. An anticoagulant, sodium citrate, is added to the blood and the plasma is separated by centrifugation and subsequently spray-dried (van Dijk et al., 2001; Pettigrew et al., 2006).

Nutritional and Physiological Components. SDP is an excellent nutrient source for nursery pigs because of excellent balance of essential amino acids with high digestibility, over 85% of digestibility of essential amino acids, and contains high metabolizable energy, about 4 mcal/kg, compared with soybean meal which is one of the main protein and energy sources in pig diets (Table 2.3; NRC, 1998; Gottlob et al., 2006). SDP is also a complex mixture of many physiological components including immunoglobulins, glycoproteins, albumin, growth factors, peptides, and other physiologically active components (Coffey and Cromwell, 2001; Markowska-Daniel and Pejsak, 2006; Moreto and Perez-Bosque, 2009). Based on established benefits, the swine industry has used SDP commercially to improve growth rate, feed intake, and feed efficiency and to reduce mortality and morbidity of early-weaned pigs.

General Proposed Mechanisms. SDP has several potential effects when it is included in pig diets. Firstly, SDP improves growth performance such as increasing feed intake, average daily gain, and gain:feed ratio (efficiency) (Coffey and Cromwell, 2001; van Dijk et al., 2001; Pettigrew, 2006) because of immune-competence or high palatability (Ermer et al., 1994), but the mechanism is not clear. Secondly, SDP improves pig health by protective effects against diseases including post-weaning diarrhea (Coffey and Cromwell, 2001; van Dijk et al., 2001;

Pettigrew et al., 2006), as SDP provides the physiological components such as immunoglobulins, glycoproteins, or others that adhere to some pathogens and prevent the colonization of enterocytes by the pathogens. Thirdly, SDP improves the intestinal barrier function (Perez-Bosque et al., 2006; Moreto and Perez-Bosque, 2009) by modulation of intestinal immune systems or by some physiologically active components in SDP, but the mechanism is not clear.

Pig Performance and Health. SDP has been used as one of main protein sources in nursery pig diets because of provision of bioavailable nutrients as well as physiologically active components in the SDP. As three previous review papers showed, SDP improves the growth rate of weaned pigs by increasing feed intake through immune-competence or high palatability, about 25% (Coffey and Cromwell, 2001), 27% (van Dijk et al., 2001), and 23% (Pettigrew, 2006), compared with control diets. In addition, those benefits are more pronounced in a conventional or non-sanitary environment (Coffey and Cromwell, 1995; Zhao et al., 2007). Based on the evidence, it is clear that SDP is beneficial for nursery pigs against limited growth rate and disease susceptibility during the weaning transition (Pettigrew et al., 2006).

SDP also improves intestinal morphology (Owusu-Asiedu et al., 2003a,b; Carlson et al., 2005; Bhandari et al., 2008) measured an increase of villous height, decrease of crypt depth, or increase of villous height:crypt depth ratio, but it is not consistent (Jiang et al., 2000; Touchette et al., 2002; Nofrarias et al., 2006). The improvement of intestinal morphology can improve nutrient absorption, resulting in improvement of growth performance.

One of the main potential effects of SDP is an antigen-antibody interaction or anti-bacterial effect in the digestive tract. With pathogenic *E. coli* challenges which generally cause diarrhea problems of weaning pigs and further mortality or morbidity, SDP improves growth performance (Nollet et al., 1999; van Dijk et al. 2002; Bosi et al., 2004), reduces diarrhea score

(Niewold et al., 2007; Bhandari et al., 2008), reduces the pathogenic *E. coli* counts in feces (Nollet et al., 1999; Owusu-Asiedu et al., 2003a,b; Niewold et al., 2007), and reduces mortality (Nollet et al., 1999; Owusu-Asiedu et al., 2003a,b; Bhandari et al., 2008) by inhibition of binding of pathogens to the intestinal epithelial cells by immunoglobulins or glycoproteins in SDP (Nollet et al., 1999; Pettigrew et al., 2006).

SDP modulates inflammatory responses. Some reports showed SDP reduces intestinal wall thickness, villous width, and lamina propria area (Jiang et al., 2000; Carlson et al., 2005; Nofrarias et al., 2006), maybe by suppressing inflammation. Nofrarias et al. (2006) also showed SDP modulates the intestinal immune system by reducing immune cell subsets (monocytes, macrophages, B lymphocytes, γδ+ T cells, etc.) in blood and ileal Peyer's patches, but Zhao et al. (2008) did not show SDP effects on pro- and anti-inflammatory cytokine mRNA expressions in the small intestine of nursery pigs after weaning.

With challenges, several reports showed SDP modulates the intestinal immune system by reducing tissue pro-inflammatory cytokine mRNA expressions against a pathogenic *E. coli* (Bosi et al., 2004) and LPS challenge (Touchette et al., 2002), and by reducing acute phase proteins and TNF-α mRNA expression (Frank et al., 2003) in environmental stress (low temperature), as SDP may inhibit pathogenic microbial growth or colonization of pathogens in the intestine and improve mucosal integrity. Thus, energy can be diverted from activation of the immune system to growth (Touchette et al., 2002; Nofrarias et al., 2006). In addition, SDP modulates stress responses by reducing mRNA expression on the hypothalamic-pituitary-adrenal axis (Carroll et al., 2002) against LPS challenge, but it's not consistent in cold stress (Frank et al., 2003). In the other side, SDP makes pigs more susceptible to overstimulation of serum pro-inflammatory cytokines against LPS challenge (Touchette et al., 2002) and cold stress with LPS challenge

(Frank et al., 2003), resulting in major damage of the mucosa of the gastrointestinal tract, and to activation of stress responses in serum on hypothalamic-pituitary-adrenal axis (Carroll et al., 2002) against LPS challenge.

The intestinal barrier is formed by enterocyte membranes and tight junctions between enterocytes in the intestinal epithelium, secreted mucus, and immunologic factors (Lambert, 2009). It is a selective barrier to allow the uptake of nutrients and to prevent or not to allow biological and chemical agents (e.g., food antigens, endotoxins, hydrolytic enzymes, intestinal microbes, etc.) across the epithelium (Lambert, 2009; Moreto and Perez-Bosque, 2009). The integrity of this barrier can be made dysfunctional by physiological, pathological, psychological, or pharmacological stress (Lambert, 2009). It leads to increased intestinal permeability to the biological and chemical agents by reducing the interlocking proteins related to the tight junctions and causes local and/or systemic inflammatory reactions (Moreto and Perez-Bosque, 2009). For the inflammatory reactions, pro-inflammatory cytokines are produced and then inflammatory cells are recruited. The inflammatory cells release reactive oxygen species to eliminate the pathogens, but the reactive oxygen species also cause tissue damages (Perez-Bosque et al., 2006; Moreto and Perez-Bosque, 2009). Thus, it is beneficial for the integrity of intestinal barrier against the stresses if the reduction of the tight junction proteins is prevented and/or the pro-inflammatory cytokines are suppressed during inflammation.

Perez-Bosque et al. (2006) showed SDP improves the intestinal barrier functions during intestinal inflammation, using rats challenged with *Staphylococcus aureus* enterotoxin B. SDP prevents reductions of the proteins such as ZO-1 (tight junction protein) and β-catenin (adherent junction protein) and reduces intestinal permeability which is measured by the passage of high

molecular weight probes (Lambert, 2009; Moreto and Perez-Bosque, 2009) across the intestinal barrier.

Using rats challenged with *Staphylococcus aureus* enterotoxin B, SDP also reduces pro-inflammatory cytokines (IFN-γ and IL-6) in the intestinal mucosa and Peyer's patches (Moreto et al., 2008) and the activation of T-helper lymphocytes and γδ-T lymphocytes in the gut-associated lymphocyte tissues (Peyer's patches, lamina propria, and intraepithelial compartments) (Perez-Bosque et al., 2004, 2008) and increases anti-inflammatory cytokine (IL-10) in the intestinal mucosa (Moreto et al., 2008).

*In summary, **s**pray-dried plasma is a blood product and provides bioavailable nutrients as an excellent protein source with balanced and highly digestible amino acids and provides physiologically active components such as immunoglobulins, glycoproteins, growth factors, peptides, etc. Based on those components of spray-dried plasma, there are several beneficial physiological activities such as immune competence (antibacterial activity), modulation of microbiota and/or immune system, and integrity of intestinal barrier function, etc. Those beneficial effects can contribute to improvement of pig performance and health by modulation of microbiota in the digestive tract and/or immune system.*

2.3.3. Clays

Definition. "The term 'clay' refers to a naturally occurring material composed primarily of fine-grained minerals (< 2.0 μm in diameter), which is generally plastic at appropriate water contents and will harden when dried or fired" (Guggenheim and Martin, 1995). Clay deposits are mostly composed of "clay minerals refers to phyllosilicate minerals and to minerals which

impart plasticity to clay and which harden upon drying and firing" and variable amounts of water is trapped in the mineral structure by polar attraction (Williams et al., 2009).

Structure. Natural clay deposits are rarely pure and most contain mixtures of a variety of minerals from the various clay mineral groups such as kaolinite, montmorillonite-smectite, illite, chlorite, or others (Williams et al., 2009). There are three different structures: 1) 1:1 layer structure formed between a single octahedral sheet ((Al, Mg, Fe)O_6 and a single tetrahedral sheet ((Al, Si)O_4), 2) 2:1 layer structure formed from sandwiching a single octahedral sheet ((Al, Mg, Fe)O_6) between two tetrahedral sheets ((Al, Si)O_4), and 3) framework structure, three dimensional frameworks of SiO_4^{4-} and AlO_4^{5-} tetrahedra linked through the shared oxygen atoms) (Papaioannou et al., 2005; Williams and Haydel, 2010; Figure 2.11).

General Effects. Clays have several potential effects when they are administered orally or topically (Carretero, 2002; Gomes and Silva, 2007; Tateo and Summa, 2007). As oral applications, first, clays are used as gastrointestinal protectors, especially palygorskites or kaolinites. The gastric and intestinal mucous membrane can be protected as clays adhere to them and absorb toxins, bacteria, or even viruses, but they also eliminate enzymes or other nutritive elements. Second, clays are used as osmotic laxatives to encourage defecation, especially sodium smectites. This is not a function of the clay itself, but of the interlayered Na^+ as it spreads and produces the osmotic pressure in the intestines. Third, clays are used as antidiarrheics, especially clays with absorbent minerals such as kaolinites, palygorskites, or calcium smectites which have high capacity of water absorption. They work by reducing the quantity of liquid and the speed of passage in the intestines as clays absorb excess water as well as gases in the digestive tract. Fourth, clays have potential antibacterial (bacteriostatic or bactericidal) effects by penetration of the cell wall or inhibiting metabolism of bacteria.

General Proposed Mechanisms. There are two types of proposed mechanisms, physical and chemical means (Papaioannou et al., 2005; Williams et al., 2009). For an example of physical means, clays are hydrophilic or organophilic. Organophilic smectities (modified clays) made by inserting alkylammonium compounds into the clay interlayer can attract the bacterial cell to the surface of the clay with enough physical force that the cell membrane is torn (adsorption property), causing bacterial cell death (Papaioannou et al., 2005; Williams et al., 2009). Natural clays also have the same effect of bacterial cell lysis by physical force. This adsorption property with physical force of clays may be beneficial for killing bacteria. However, clays may harm host tissues because they can also adhere to gastrointestinal walls and modify or reinforce the mucus lining of intestines (Tateo and Summa, 2007).

For an example of chemical means, French green clays used for treating Buruli ulcer caused by *mycobacterium ulcerans* are dominated by illite and Fe-smectite mineralogically, which are hydrophilic (Papaioannou et al., 2005; Williams et al., 2008). These natural clays have potential effects that may kill bacteria by chemical exchange in aqueous media through providing a toxin to bacteria, depriving bacteria of essential nutrients for their metabolism, or changing pH and oxidation state in the intestines.

There are also other clay effects such as dermatological protectors, excipients for drug, pelotherapy, etc., but only oral application cases are considered in this review.

Pig Performance and Health. Mycotoxins (aflatoxin, ergot alkaloids, fumonisin, orchartoxin, vomitoxin, or zearalenone) are the toxic secondary metabolites produced by fungi (*Aspergillus, Fusarium, Penicillium,* and *Claviceps* species) on cereal grains during storage, growth, harvest, transportation, or processing (Lindemann et al., 1993; Ledoux and Rottinghaus, 2000). These mycotoxins are detrimental to animal growth, production, and health when animals

consume diets contaminated with the mycotoxins. A practical approach has been the addition of adsorbents to contaminated feed to bind the mycotoxins and to reduce the detrimental effects by mycotoxins (Ledoux and Rottinghaus, 2000). One solution may be the addition of clays in the livestock diets. A hydrated sodium calcium aluminosilicate (HSCAS, clay) has been known to bind some of these mycotoxins when added to the livestock diets (Phillips et al., 1988; Papaioannou et al., 2005). *In vitro* studies showed the adsorption of mycotoxins by clays (Lemke et al., 1998, 2001) and *in vivo* studies showed the addition of clays in the pig diets reduce the adverse effects of aflatoxin in the diets on growth rate and serum indicators of protein synthetic capabilities and of liver damage of pigs (Lindemann et al. 1993; Schell et al., 1993a,b).

Field observations suggest clays in the pig diets also have anti-toxic or -diarrheic effects. Without challenge, clays may improve (Pond et al., 1988; Papaioannou et al., 2004; Alexopoulos et al., 2007) or may not improve (Ward et al., 1991; Poulsen and Oksbjerg, 1995; Parisini et al., 1999) growth rate of pigs because ion exchange, adsorption, and catalytic properties of clays may reduce passage rate, reduce hydrolysis of diets by enzymes, and reduce absorption of nutrients (Shurson et al., 1984; Pond et al., 1988). Clays may not affect serum minerals (Papaioannou et al., 2002; Alexopoulos et al., 2007) or may affect them because of ion exchange properties of clays or interference of mineral ions (e.g. Al) from degradation of clays in acidic environment (Shurson et al., 1984; Ward et al., 1991). Clays may reduce serum urea nitrogen (Shurson et al., 1984; Poulsen and Oksbjerg, 1995; Alexopoulos et al., 2007) or toxic compounds (Shurson et al., 1984; Ramu et al., 1997) because of high affinity of clays for ammonium ions from the deamination of proteins and for toxic compounds from microbial degradation. Clays may affect or may not affect (Alexopoulos et al., 2007) hematological parameters such as

hematocrit, white blood cell count, and hemoglobin concentrations because of intestinal irritation or inflammation by clays.

Clays reduce piglets' diarrhea (Stojic et al., 1998; Papaioannou et al., 2004) after weaning, maybe because of antibacterial effects by clays' adsorption properties. With a pathogenic *E. coli* challenge, Trckova et al. (2009) reported the clay treatment improves body weight gain but not growth efficiency, reduces the colonization and shedding of a pathogenic *E. coli*, and do not change haematological parameters of serum of pigs or histopathological features of mucosa in small and large intestines of pigs compared with pigs fed the control diet.

In addition, some *in vitro* studies and human research support those antibacterial and mycotoxin binding effects of clays. Ramu et al. (1997) showed clays adsorb and inactivate the heat-labile (LT) enterotoxins of *E. coli* and the cholera enterotoxins (CT) of *Vibrio cholerae*. Some reports showed clays eliminate or inhibit growth of pathogenic *E. coli* (Tong et al., 2005; Hu and Xia, 2006; Haydel et al., 2008), *Salmonella choleraesuis* (Tong et al., 2005), and other antibiotic-susceptible and antibiotic–resistant bacteria (Haydel et al., 2008) by injury of bacterial cell wall, leakage of bacterial enzymes, inhibition of bacterial respiratory metabolism, or changing chemical conditions such as pH and oxidation state. There is also some evidence for those benefits by *in vivo* studies of human health. Some reports showed clays attenuate overall disorder of diarrhea-predominant irritable bowel syndrome and abdominal pain and discomfort intensity (Chang et al., 2007) and severity of acute diarrhea of children (Madkour et al., 1993; Dupont et al., 2009). In addition, clay may reduce exposure and adverse effects of mycotoxin-contaminated food for human (Wang et al., 2005).

Even, some *in vitro* studies show antiviral effects of clays. Some reports showed clays also adsorb rotavirus and coronavirus (Clark et al., 1998), which generally causes gastroenteritis

(acute diarrheal disease), and reovirus (Lipson and Stotzky, 1983), which causes gastrointestinal and respiratory problems, with high affinity by physical forces such as van der Waals forces and hydrogen bonding and by formation of a cation bridge between clays and viruses, although the clay-virus complex retained infectivity.

In summary, clay is a naturally occurring material, is composed primarily of fine-grained minerals (phyllosilicate minerals), and has a specific structure. This specific structure has the ability to lose and gain water reversibly, to adsorb molecules, and to exchange ions. Based on these properties, there are several beneficial physiological activities such as protection of the intestinal tract, anti-diarrheic and antibacterial effects, etc. Those beneficial effects can contribute to improvement of pig performance and health by reducing pathogenic bacteria in the intestinal digestive tract (modulation of microbiota), especially a pathogenic E. coli that cause piglets' diarrhea after weaning.

2.3.4. Spray-Dried Egg

Definition. The Association of American Feed Control Officials (AAFCO, 2008) mentions about spray-dried egg (SDE) that "Egg product is the product obtained from egg graders, egg breakers, and/or hatchery operations that is dehydrated, handled as liquid, or frozen. This product shall be free of shells or other non-egg materials except in such amounts which might occur unavoidably in good processing practices, and contains a maximum ash content of 6% on a dry matter basis". There are two types of SDE in marketing now and both of them are called spray-dried egg and are approved by AAFCO as the egg product. One is produced by only eggs without shell and the other one is produced by eggs without shell including hatchery wastes, not only eggs. This review considers SDE produced from only eggs without shell.

Norberg et al. (2004) also described “SDE is an egg by-product that is produced by only eggs without shell that are below the USDA Grade B standards which have thinner whites and wider and flatter yolks than higher grades eggs and have intact shells”. In addition, “SDE is prepared by removing the shell and mixing the yolk and albumen together. The mixture of yolk and albumen is pasteurized and then sprayed into an oven that is heated to approximately 70ºC, producing a powder with approximately 4.25% moisture. The finished product must test negative for *Salmonella* before inclusion into a diet”.

Nutritional and Physiological Components. SDE is an excellent nutrient source for nursery pigs because of excellent balance of amino acids from egg white (albumen) (Rose et al., 1974; Schmidt et al., 2003) with high digestibility, and contains rich content of fat (about 30%), and high metabolizable energy (about 5 mcal/kg) compared with other general protein ingredients for nursery pigs (Table 2.3; DeRouchey et al., 2003, Figueiredo et al., 2003, and Harmon and Richert, 2007).

SDE contains physiological components such as immunoglobulins, lysozyme, etc. Rose et al. (1974) reported that the concentration of immunoglobulin Y (IgY; generally, most IgG in hen serum is transferred to egg yolk, which is called IgY (IgG in egg yolk)) in SDE is about 30,000 mg/kg. However, Akita and Nakai (1992) and Harmon et al. (2002) reported the concentration of IgY in SDE is about 12,000 mg/kg. Lysozyme is one of the antimicrobial proteins in egg whites. Lysozyme is a relatively low-molecular-weight protein composed of 129 amino acid residues and is an enzyme, hydrolase, which catalyze hydrolysis of 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan (Cunningham et al., 1991) which is a component of bacterial cell walls.

General Proposed Mechanisms. Immunoglobulins and other physiologically active agents are transmitted from the hen to her chick through the egg, so it may be reasonable to postulate that adding SDE to the pig diet may impact pig health and performance. Chicken IgY antibodies can be easily obtained by conventional immunization methods compared with IgG antibodies. They do not interfere with mammalian IgG and do not activate mammalian complement (Tini et al., 2002). The concentration of IgY in egg yolk is much higher than that of IgG in hen serum (Rose et al., 1974; Akita and Nakai, 1992; Harmon et al., 2002). These characteristics are advantageous to the application of IgY as antibiotic-alternative therapy (Tini et al., 2002). The catalytic activity of lysozyme of SDE can damage bacterial cell walls, but it is only effective against gram positive bacteria. However, some research showed enhancement of this catalytic activity of lysozyme by heating or irradiation can reduce the survival rate of gram negative bacteria as well as gram positive bacteria (Ibrahim et al., 1996; Schmidt et al., 2007).

Pig Performance and Health. Several experiments were conducted to evaluate effects of SDE on growth performance of pigs. For example, some researchers replaced SDP with SDE and showed SDE may not improve growth rate (Norberg et al., 2001; Figueiredo et al., 2003; Schmidt et al., 2003) or some researchers replaced soybean meal with SDE and showed SDE may improve growth rate (DeRouchey et al., 2003; Shao et al., 2003). Therefore, the results on growth rate have not been conclusive.

There is little information about direct SDE effects on pig health. However, there is some research about immune egg products. Hens can be immunized against pig pathogens and then they produce antibodies against those pathogens and deposit them in the yolk of the eggs they produce. Feeding the yolk to pigs provides passive immunity to the target disease (Kim et al., 1999; Marquardt et al., 1999). Based on this concept, some research was focused on IgY to

prevent enterotoxigenic *E. coli* (ETEC) strains expressing K88, K99 and 987P fimbrial antigens (Jin et al., 1998) which cause post-weaning diarrhea. Several studies showed immune egg yolk reduces adherence and colonization of ETEC strains, mortality, clinical signs, or diarrhea score in neonatal pigs (Imberechts et al., 1997; Yokoyama et al., 1997; Zuniga et al., 1997; Owusu-Asiedu et al., 2002). In addition, there is one research about extracted albumen egg products containing lysozyme. Schmidt et al. (2003) showed *Enterobacteriaceae* counts in pigs fed the spray-dried technical albumen (SDTA) and SDTA-ht (stored in a hot room (70ºC) for 3 days to enhance catalytic activity of lysozyme) are not different from pigs fed spray-dried porcine plasma. Those results indicate components (IgY and lysozyme) of SDE may be beneficial for pig health. Therefore, more research is needed to support direct SDE effect on pig health.

In summary, spray-dried egg is a by-product from egg without shell and is an excellent nutrient source for nursery pigs because of excellent balance of amino acids with high digestibility and contains rich content of fat and high metabolizable energy, and provides physiologically active components such as immunoglobulins, lysozymes, etc. Based on those components of spray-dried egg, there is a beneficial effect such as an antibacterial effect. This beneficial effect may contribute to improvement of pig performance and health by modulation of microbiota in the intestinal digestive tract of pigs. However, there is little information for spray-dried egg effect on pig health.

2.3.5. Enzymes

Definition. The Association of American Feed Control Officials (AAFCO, 2008) mentions enzymes are “a protein made up of amino acids or their derivatives, which catalyzes a

defined chemical reaction and required cofactors should be considered an integral part of the enzyme".

General Effects. Pigs are not able to utilize some nutrients because they cannot produce some enzymes to break down the structure of the nutrients. Therefore, supplementation of some enzymes in pig diets has been used to improve efficiency of feed utilization. Generally, there are two main types of enzymes which have been used in the swine industry. The first one is carbohydrases (Gdala et al., 1997; Kim et al., 2003; Barrera et al., 2004). Some carbohydrate ingredients such as wheat, barley, sorghum, distillers dried grain with soluble (DDGS), etc. include non-starch polysaccharides, such as cellulose, pectins, β-glucans, and arabino-xylans. They cannot be broken down by pig enzymes and can only be fermented by microbes. Thus, there are commercially available enzymes such as α-galactosidase, xylanase, β-glucanase, cellulase, α-amylase, etc. to break down the carbohydrates to be utilized by pigs. The second one is phytase (Omogbenigun et al., 2004; Olukosi et al., 2007; Kim et al., 2008). Some ingredients include phytate. However, pigs cannot produce the enzyme, phytase, to break down phytate to utilize phosphorus, and thus it is commercially available.

General Proposed Mechanisms. Enzymes can be used to break down the structures of nutrients which pigs cannot digest and/or absorb and to help pigs' digestion and/or absorption of the substrates from the nutrients. The substrates produced by the enzyme property (breaking down the structure of nutrients) may modulate microbial populations in the digestive tract (Pluske et al., 2002) and immune responses of pigs. Those also change factors in the intestinal environment such as pH, passage rate, viscosity, etc. (Kiarie et al., 2007; Vahjen et al., 2007; Emiola et al., 2009). Thus, enzymes may improve pig health indirectly by providing the substrates which can modulate intestinal microbiota and it may affect immune response as well.

Enzymes decrease the viscosity in the digestive tract and increase the passage rate. It causes improvement of digestion and absorption for host, resulting in decreasing substrates for some bacterial growth (Durmic et al., 2000; Hardy, 2002; Vahjen et al., 2007). Enzymes also provide substrates to beneficial microbes such as lactic acid bacteria, causing them to dominate (Pan et al., 2002; Kiarie et al., 2007; Vahjen et al., 2007).

Enzymes, especially proteases (proteolytic enzymes), may inhibit activation of pathogenic *E. coli* receptors in the mucosal and epithelial cells of the digestive tract and may prevent diarrheic diseases (Chandler et al., 1994; Jin and Zhao, 2000).

Pig Performance and Health. It has been known that enzymes improve growth rate (Mavromichalis et al., 2000; Kim et al., 2008; Emiola et al., 2009), feed efficiency (Pan et al., 2002; Kim et al., 2003; Barrera et al., 2004), digestibility (Gdala et al., 1997; Kiarie et al., 2007; Vahjen et al., 2007), and villus height (Kim et al., 2003) when enzymes are supplemented in the pig diets at least under some conditions, because pigs utilize substrates as enzymes break down the structure of nutrients which pigs cannot digest.

Enzymes also modulate microbial populations in the digestive tract (Pan et al., 2002; Kiarie et al., 2007; Vahjen et al., 2007), maybe by providing substrates which some specific microbes can utilize, causing more production of lactic acid and volatile fatty acids (Pan et al., 2002; Kiarie et al., 2007). With a pathogenic *E. coli* (K88) challenge, enzymes, especially proteases, reduce the incidence of diarrhea and improve growth rate as they inhibit the activity of *E. coli* receptors (Mynott et al., 1996; Chandler and Mynott, 1998).

In summary, enzymes are proteins and non-nutritive additives to help pigs' digestion and/or absorption of the substrates from the nutrients by breaking down the structure of nutrients. Based on this physiological activity, enzymes indirectly provide substrates for pigs'

absorption and for microbes' fermentation. In the other side, some enzymes can directly inhibit the activity of E. coli receptors. These beneficial effects may contribute to improvement of pig performance and health by modulation of microbiota in the digestive tract of pigs. However, there is little information for enzyme effect on pig health.

SUMMARY

The main goals of swine production are maximization of pig productivity and profits. For these goals, the swine industry has focused on nutrition, genetics, and management practices as well as health. From those many efforts, productivity of swine production has been tremendously improved. However, many pigs still die before they reach market weight because of disease problems. For those problems, most practical management practices such as all-in /all-out pig flow, age segregation, intense biosecurity practices, sanitations, new vaccinations, and depopulation /repopulation have been used, but they cannot guarantee freedom from disease for pigs. Moreover, the use of antibiotics in swine production has been restricted because of safety issues. Thus, recently the swine industry increasingly considers use of dietary factors like feed ingredients, feed additives, feed formulation practices, or feeding methods to improve pig health and/or performance, especially during the post-weaning period. Use of dietary factors for nursery pigs has been tested to maximize productive performance and to minimize disease problems. A lot of research has shown improvement of pig health as well as productive performance by modulating microbial populations and/or the immune system directly or indirectly as dietary factors provide physiological activities. Based on this evidence, some dietary factors, e.g. spray-dried plasma, clay, spray-dried egg, enzymes, etc., are believed to be able to improve pig health as well as productive performance. Therefore, it is suggested that dietary factors may be

important components of strong swine health management programs along with the practical management practices.

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Table 2.1. An incomplete list of dietary factors that may help in prevention and control of swine diseases (Adapted from Pettigrew, 2006)

Energy & AA sources	**Additives**	**Formulating/Feeding practices**
Spray-dried plasma	Zinc and copper products	Low-protein diets
Milk products	Acids	Limit feeding
Fiber sources	Immune egg products	Fermented liquid feeds
Yeast products	Mannan oligosaccharide	etc.
Rice	Probiotics	
Fish meal	Prebiotics	
Spray-dried eggs	Essential oils	
etc.	Omega-3 fatty acids	
	Enzymes	
	Adsorbent clays	
	etc.	

Table 2.2. Summary of reported physiological, nutritional, and metabolic effects of antibiotics (Gaskins et al., 2002)

Physiological	**Nutritional**	**Metabolic**
Increase		
Nutrient absorption	Energy retention	Liver protein synthesis
Feed intake	Nitrogen retention	Gut alkaline phosphatase
	Vitamin absorption	
	Trace element absorption	
	Fatty acid absorption	
	Glucose absorption	
	Calcium absorption	
	Plasma nutrients	
Decrease		
Food transit time	Gut energy loss	Ammonia production
Gut wall diameter	Vitamin synthesis	Toxic amine production
Gut wall length		Aromatic phenols
Gut wall weight		Bile degradation products
Fecal moisture		Fatty acid oxidation
Mucosal cell turnover		Fecal fat excretion
		Gut microbial urease

Table 2.3. Chemical and amino acid composition of protein sources for nursery diets (data on as-fed basis; amino acids as % of crude protein)

	Ingredients		
Item	**SBM[1]**	**SDP[2]**	**SDE[3]**
Crude protein, %	47.50	78.00	50.91
Crude fat, %	3.00	2.00	26.58
Calcium, %	0.34	0.15	0.26
Phosphorus, %	0.69	1.71	0.54
ME, mcal/kg	3.38	3.98	4.75
Arginine, %	7.33	5.83	6.13
Histidine, %	2.69	3.27	2.49
Isoleucine, %	4.55	3.47	5.37
Leucine, %	7.71	9.76	8.69
Lysine, %	6.36	8.77	7.37
Methionine, %	1.41	0.96	3.69
Phenylalanine, %	5.03	5.67	5.51
Threonine, %	3.89	6.05	4.55
Tryptophan, %	1.37	1.74	2.05
Valine, %	4.78	6.33	6.50

[1]Soybean meal without hulls; NRC, 1998.

[2]Spray-dried plasma; NRC, 1998 and Gottlob et al., 2006.

[3]Spray-dried egg; DeRouchey et al., 2003, Figueiredo et al., 2003, Norberg et al., 2004, and Harmon and Richert, 2007.

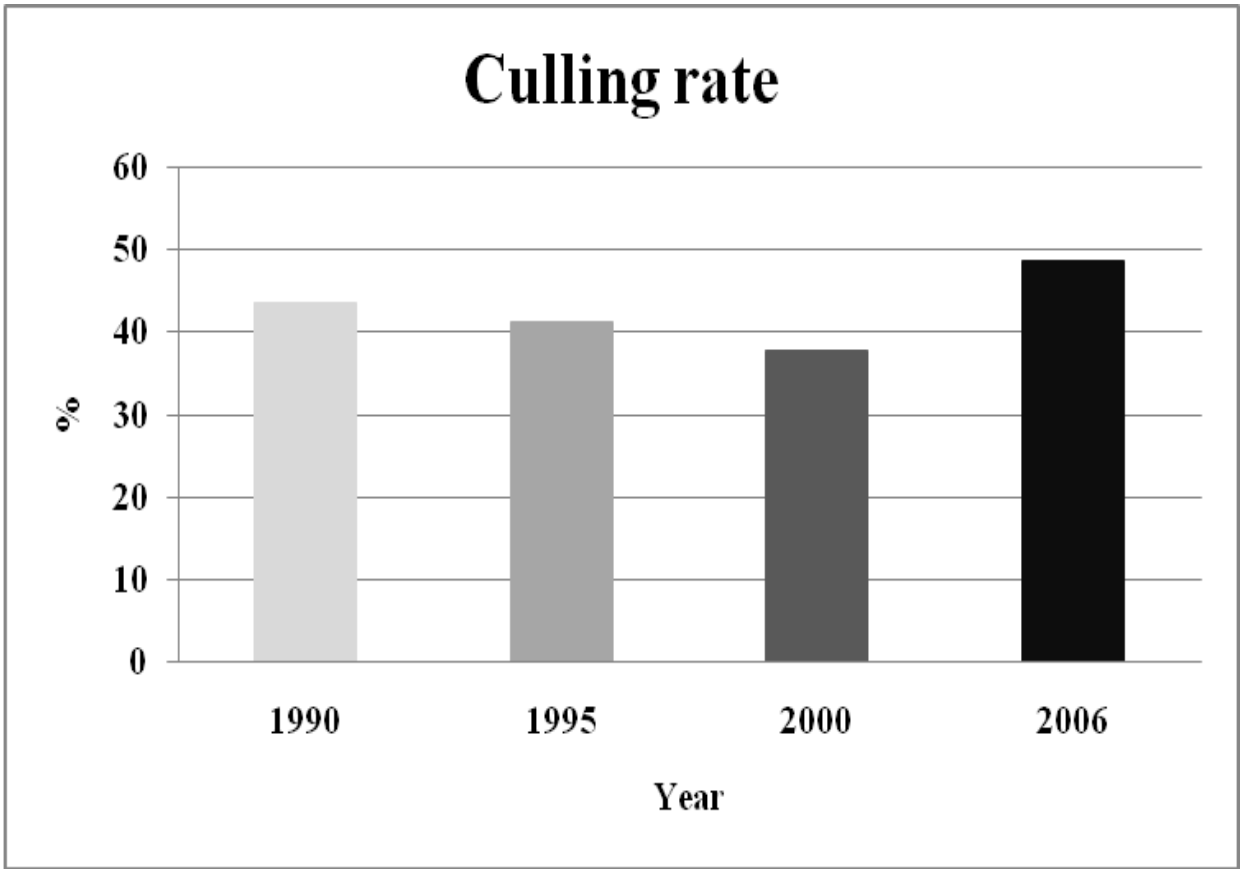

Figure 2.1. Culling rate of sows (Adapted from NAHMS, 2008)

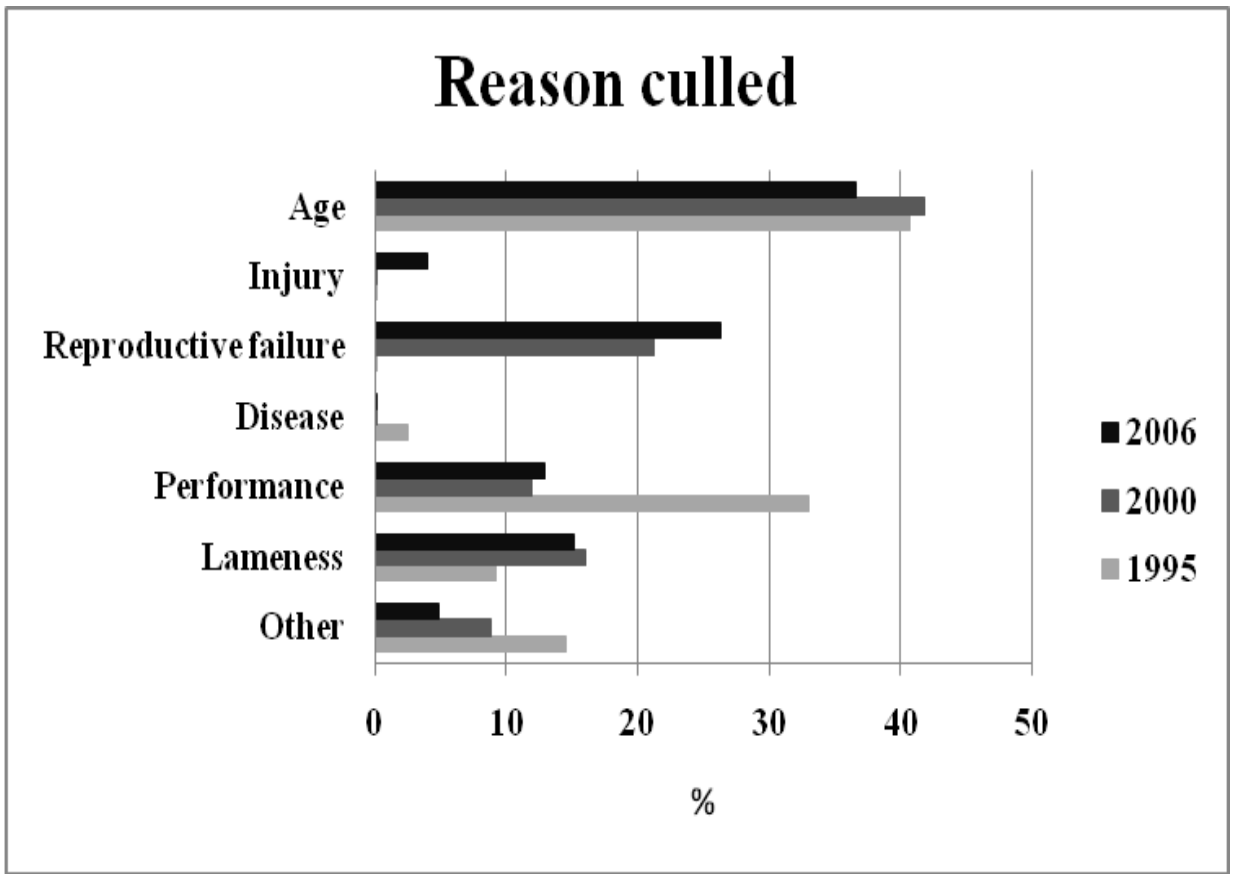

Figure 2.2. Reasons culled sows by producer (Adapted from NAHMS, 2008)

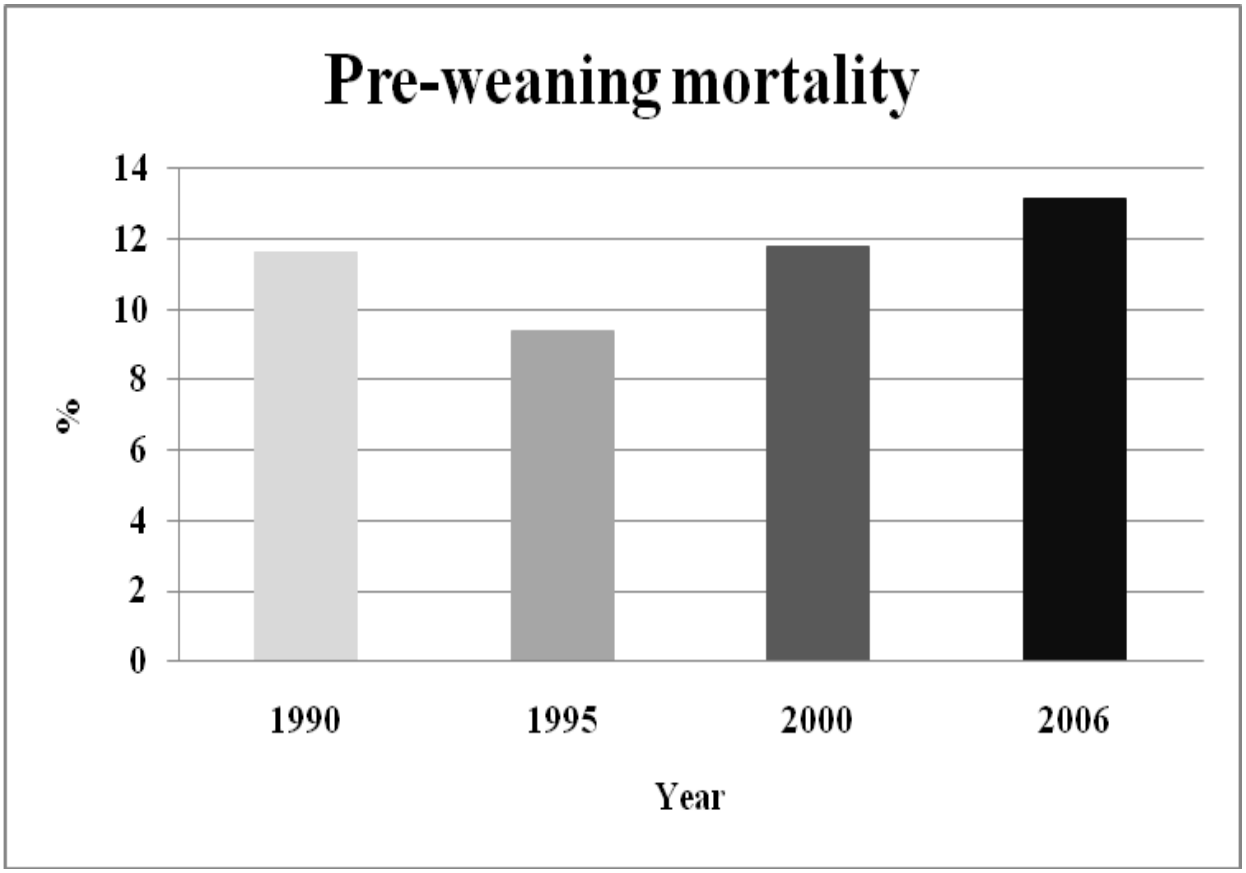

Figure 2.3. Pre-weaning pigs mortality (Adapted from NAHMS, 2008)

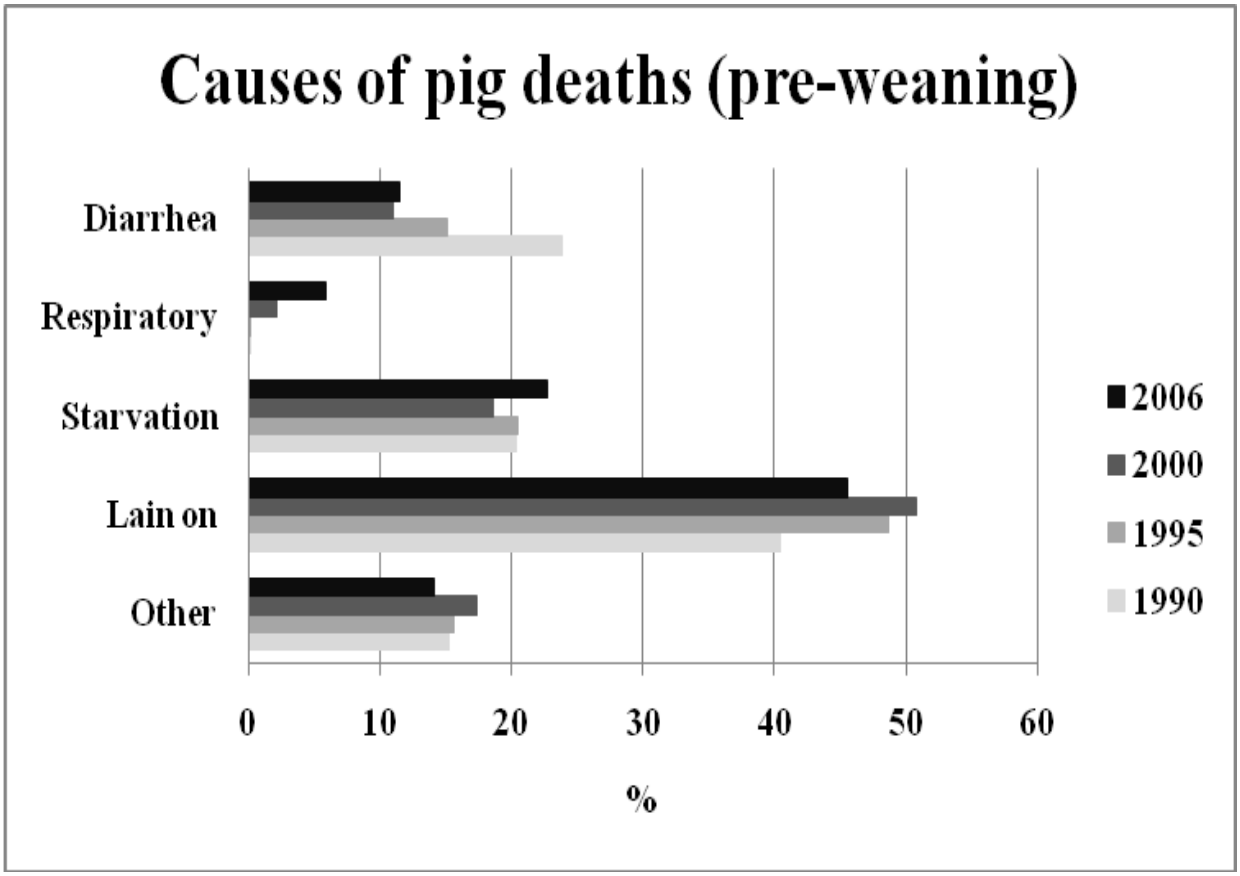

Figure 2.4. Causes of pig deaths by producer (Adapted from NAHMS, 2008)

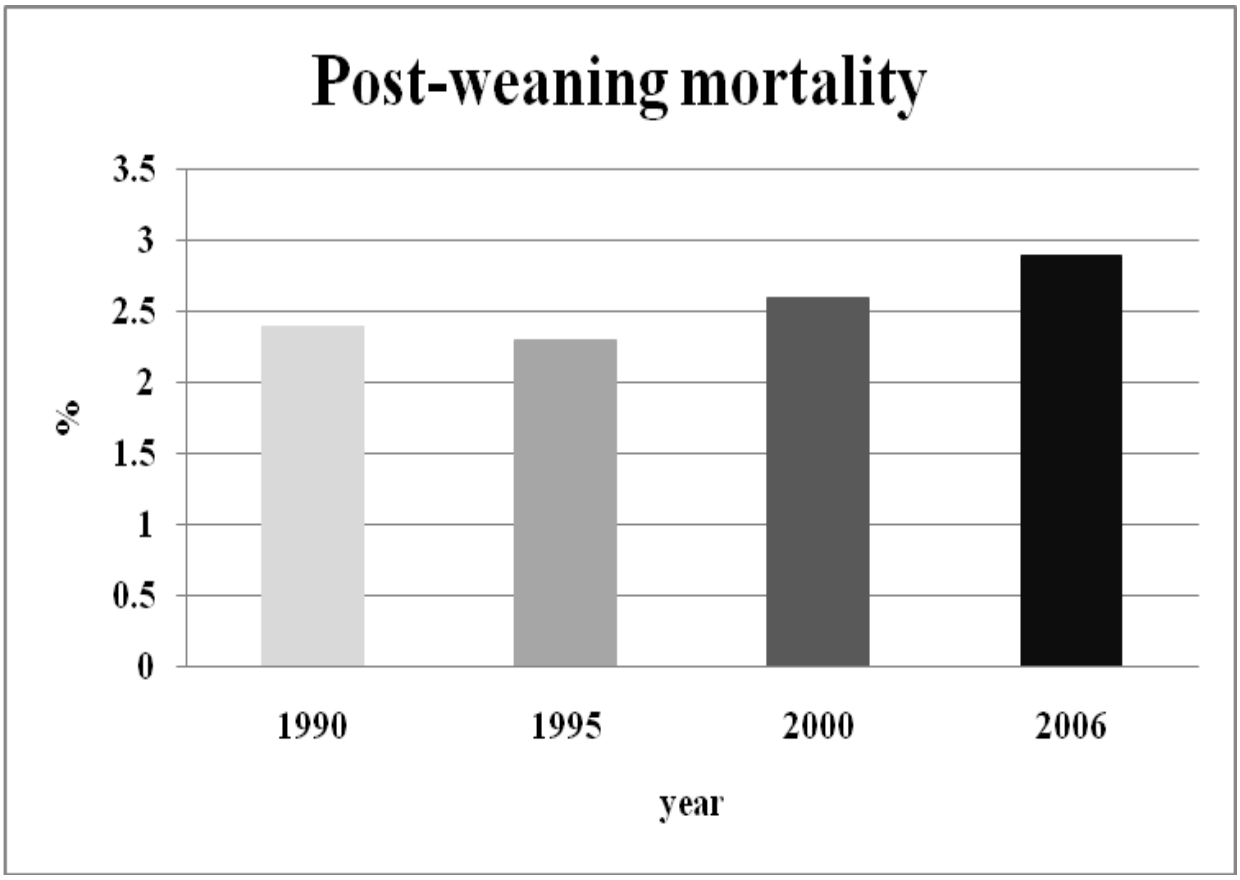

Figure 2.5. Post-weaning pigs mortality (Adapted from NAHMS, 2008)

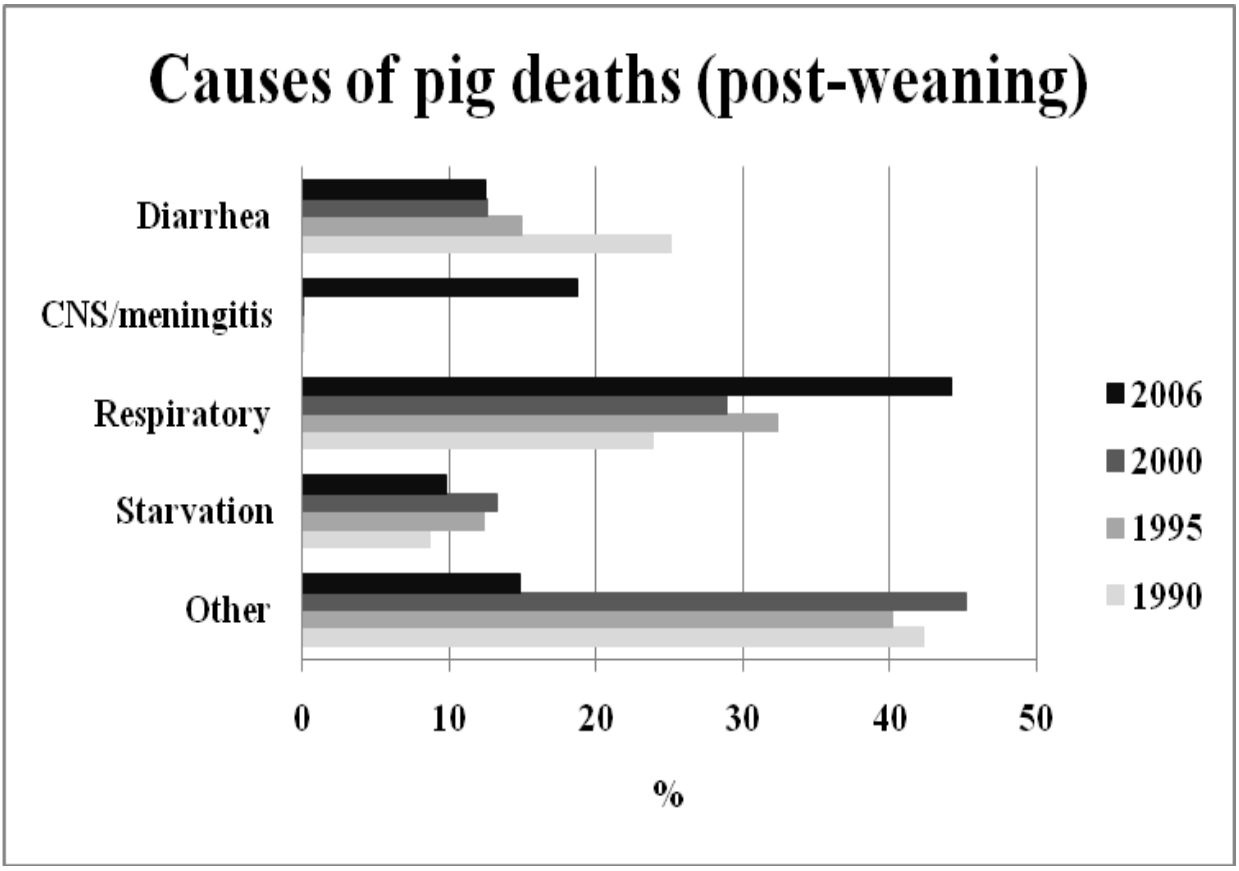

Figure 2.6. Causes of pig deaths by producer (Adapted from NAHMS, 2008)

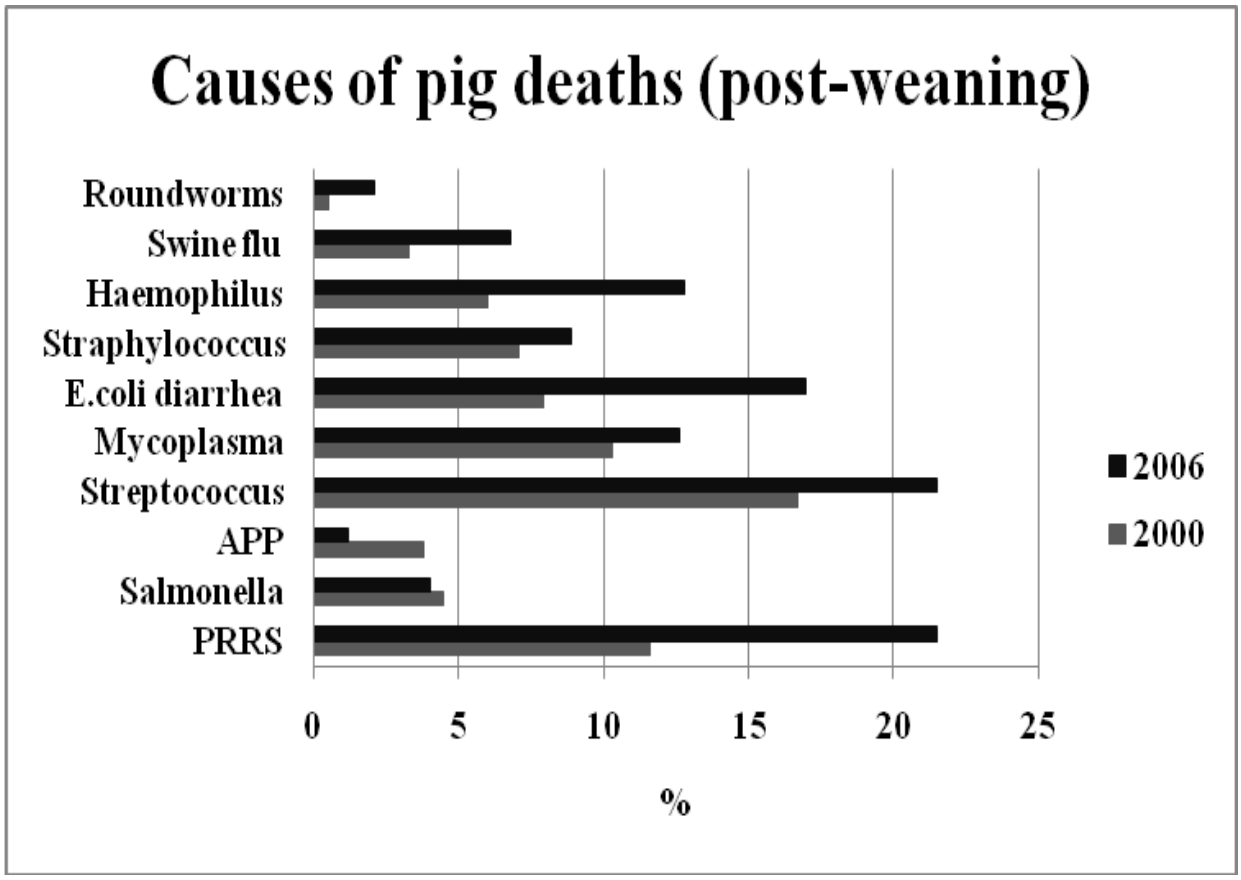

Figure 2.7. Causes of pig deaths by veterinarian or laboratory (Adapted from NAHMS, 2008)

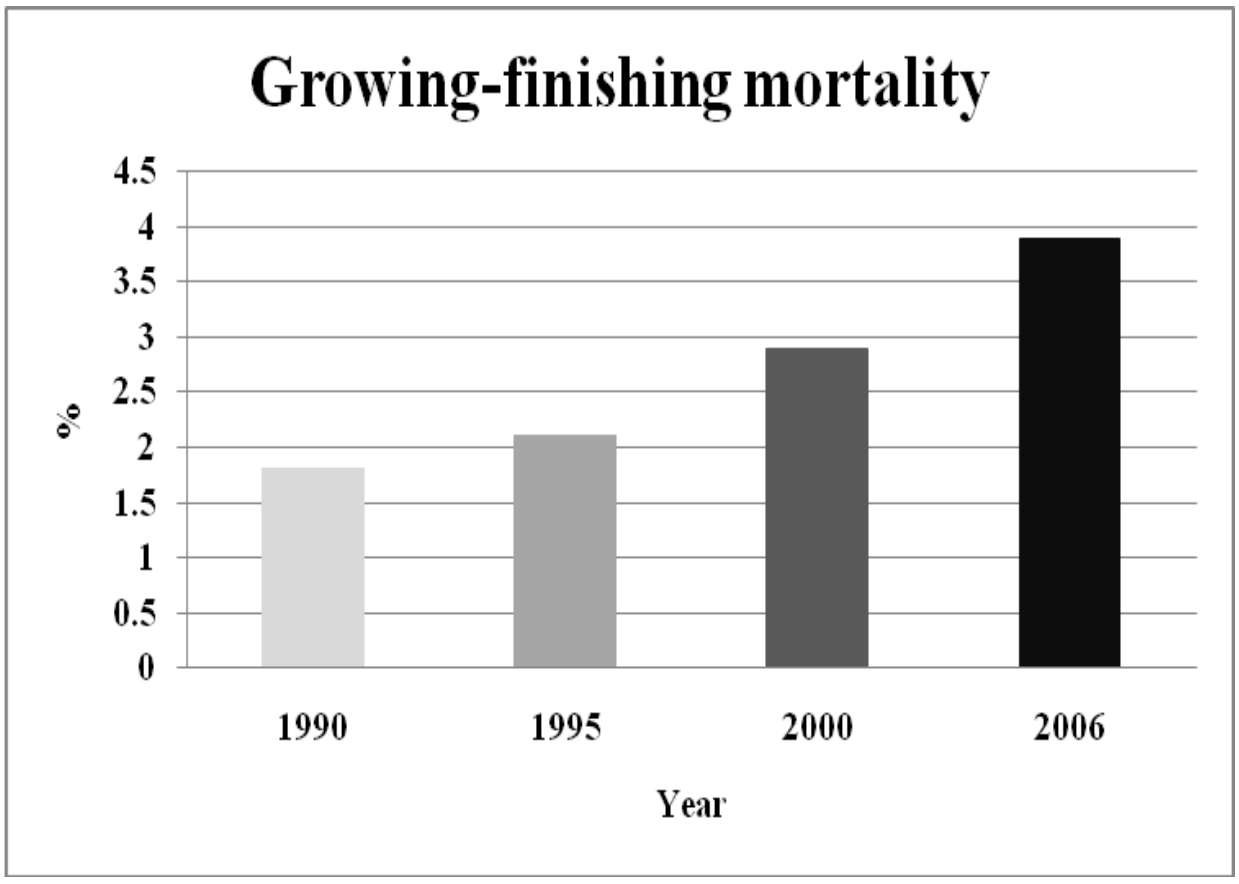

Figure 2.8. Growing-finishing pigs mortality (Adapted from NAHMS, 2008)

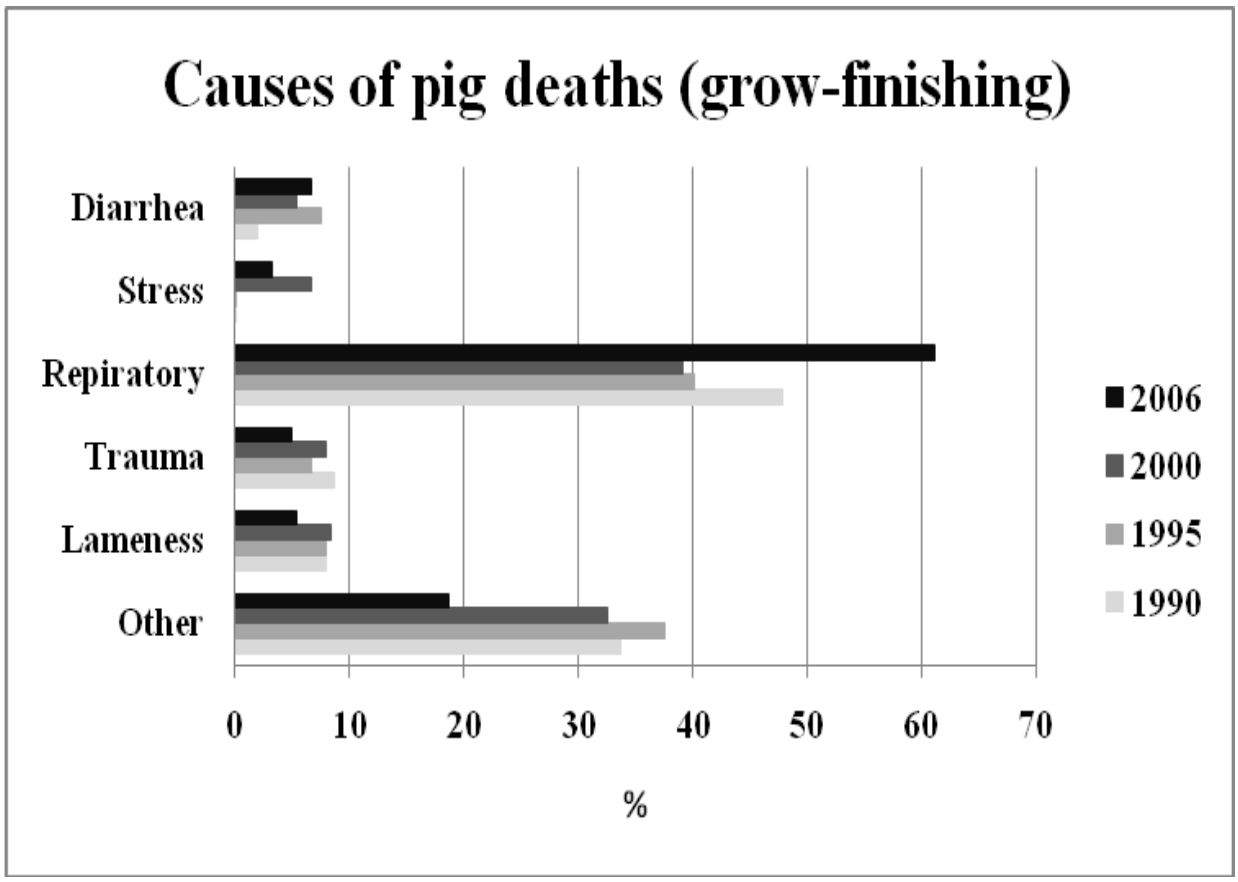

Figure 2.9. Causes of pig deaths by producer (Adapted from NAHMS, 2008)

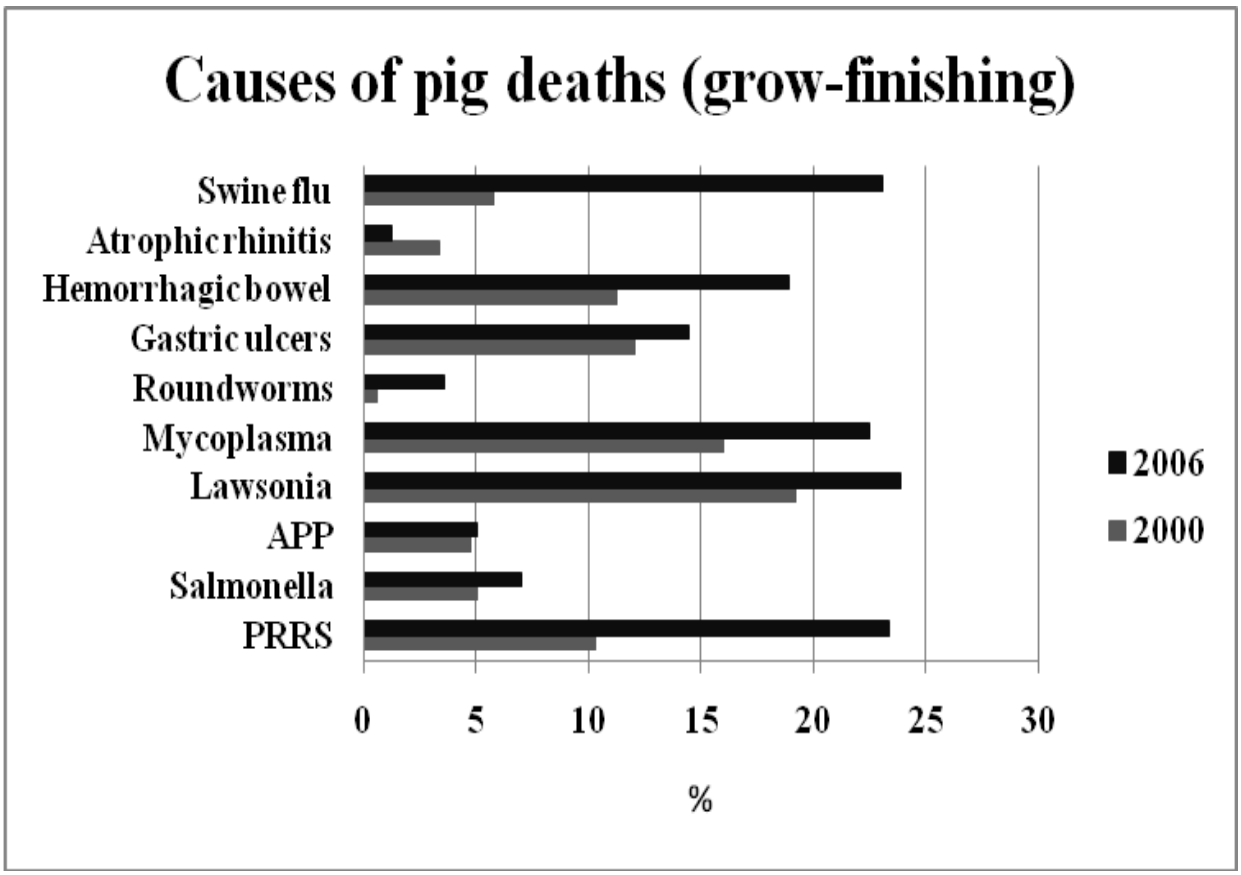

Figure 2.10. Causes of pig deaths by veterinarian or laboratory (Adapted from NAHMS, 2008)

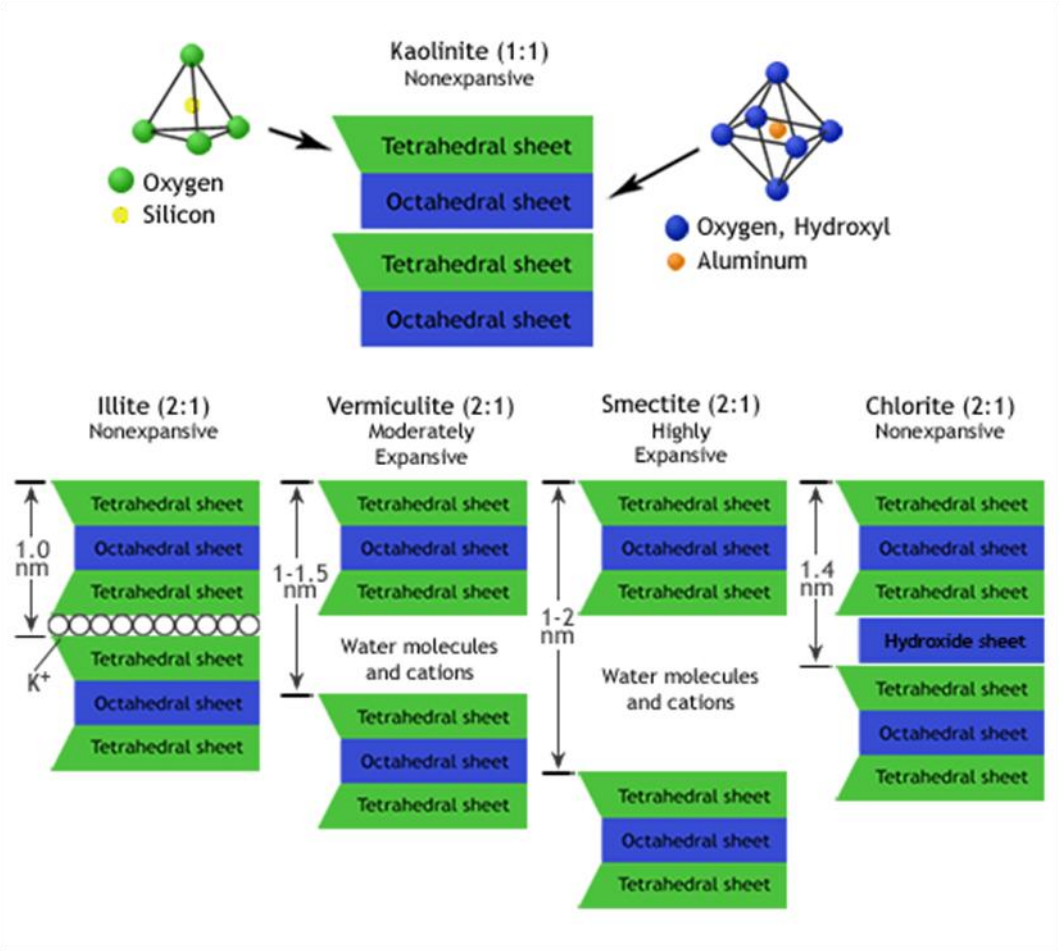

Figure 2.11. Structure of clays (Josh Lory, http://soils.missouri.edu/tutorial/page8.asp)

CHAPTER 3

EFFECTS OF DIETARY ANTIBIOTICS ON ILEAL AND FECAL MICROBIAL ECOLOGY OF PIGS

ABSTRACT

Two experiments were conducted to evaluate effects of virginiamycin (V) and carbadox (C) on ileal and fecal microbial ecology of pigs. Pigs were surgically equipped with a T-cannula in the distal ileum and assigned randomly to one of 3 dietary treatments. During a 6-wk experiment, all pigs were fed a corn-soybean meal diet (CON) during wk 1, 5, and 6 and their respective treatment diets during wk 2, 3, and 4. Pigs were allowed *ad libitum* access to feed and water. Ileal digesta and fecal samples were collected on d 6 and 7 of each period. The number of bacterial cells was counted after Gram's staining, and denaturing gradient gel electrophoresis (DGGE) was used to estimate the species diversity of the bacterial population (the number of bands) and quantitative measures of the similarity of population structures (banding pattern expressed by Sorenson′s pairwise similarity coefficients (Cs)) among pigs within treatments (INTRA) and between treatments (INTER). In the V experiment, 15 pigs (35 ± 2.7 kg BW) were used and treatments were CON, CON + 11 mg/kg of V (V11), and CON + 22 mg/kg of V (V22). The number of bacterial cells (log /g digesta) was reduced ($P < 0.05$) in ileal digesta during wk 2 (11.2 & 10.9 vs. 11.3 for V11 & V22 vs. CON, respectively) and wk 3 (11.2 & 11.2 vs. 11.5), and in feces during wk 4 (11.4 & 11.2 vs. 12.00) and overall period (11.7 & 11.7 vs. 11.9) when the V treatments were imposed. Pigs fed the V treatments had fewer bands ($P < 0.05$) in ileal

digesta during the overall period (23.6 & 22.1 vs. 26.8 for V11 & V22 vs. CON, respectively) than pigs fed the CON, suggesting that the V treatments may reduce some species of bacteria. In the C experiment, 15 pigs (9.6 ± 0.8 kg BW) were used and treatments were CON, CON + 27.5 mg/kg of C (C27.5), and CON + 55 mg/kg of C (C55). The INTRA Cs values of the C treatments were lower ($P < 0.05$) in ileal digesta during wk 2 (78 & 75 vs. 93 for C27.5 & C55 vs. CON, respectively) than those of the CON, but higher ($P < 0.05$) during wk 4 (89 & 95 vs. 80), suggesting that the C treatments eventually make pigs more uniform in ileal microbiota after an initial disruption. In a few cases, during the antibiotics feeding, specific bands were present in most pigs fed the CON, but absent from most pigs fed the V or C treatments. In conclusion, virginiamycin and carbadox modulated microbial populations in the digestive tract of pigs.

Key words: carbadox, denaturing gradient gel electrophoresis (DGGE), microbial ecology, pig, virginiamycin

INTRODUCTION

Antibiotics improve growth rate and efficiency as well as reproductive performance and thus they are called “growth promoters” (Cromwell, 2002; Dritz et al., 2002; Hardy, 2002). Antibiotics also improve animal health as they reduce mortality and morbidity by preventing or treating diseases (Gaskins et al., 2002).

There are several potential mechanisms of antibiotics (Visek, 1978). Antibiotics suppress or inhibit the growth of certain microorganisms or subclinical infection by damaging their cell wall formation, disrupting their nucleic acid synthesis, etc., resulting in reduced competition between host and microbes for nutrients and reduced microbial metabolites that depress host

growth (Gaskins et al., 2002; Hardy, 2002). Antibiotics also reduce intestinal density (thinner intestinal villi and total intestinal wall) by the loss of mucosal cell proliferation in the absence of luminal short chain fatty acids from microbial fermentation (Dibner and Richards, 2005; Niewold, 2007). For example, virginiamycin used for growing pigs can treat swine dysentery by inhibition of gram-positive bacterial growth by protein synthesis inhibition, and carbadox used for weaning pigs can treat swine dysentery and enteritis by inhibition of both gram positive and negative bacterial growth by DNA synthesis inhibition (Yen et al., 1985; Gaskins et al., 2002; Hardy, 2002; Stewart et al., 2010b).

Collier et al. (2003) found that antibiotics alter the intestinal microbiota of pigs and suggested these changes may relate to growth promotion. Therefore, two experiments evaluated effects of virginiamycin and carbadox on apparent ileal digestibility of AA (Stewart et al., 2010a,b) and the present measurements were made on the same pigs to evaluate effects of those antibiotics on ileal and fecal microbial ecology of pigs.

MATERIALS AND METHODS

The protocols for these experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. The experiments were conducted in the Swine Research Center at the University of Illinois, Urbana.

Animals, Diets, Housing, Experimental Design, and Sample Collection

Thirty crossbred pigs originating from the matings of Line 337 boars to C 22 sows (PIC, Hendersonville, TN) were used in two experiments. Fifteen pigs (BW = 35 ± 2.7 kg) for the virginiamycin experiment and 15 pigs (BW = 9.6 ± 0.8 kg) for the carbadox experiment were surgically equipped with T-cannulas in the distal ileum using procedures adapted from Stein et

al. (1998). Following the surgery, pigs were housed individually in 1.2-m x 1.8-m metabolism crates in an environmentally controlled room and had *ad libitum* access to feeder and water. Pigs were allowed a 7-d recovery period after surgery and were fed a standard diet during this period. Following the recovery period, pigs were allotted to 3 dietary treatments with five pigs per treatment in a completely randomized design.

For each experiment, a control diet (**CON**) based on corn and soybean meal without antibiotic growth promoters was formulated to meet or exceed all nutrient requirements for pigs of the relevant weights (Table 3.1; NRC, 1998). The other 2 dietary treatments in each experiment were additions of 2 levels of the respective antibiotics (11 and 22 mg/kg of virginiamycin; 27.5 and 55 mg/kg of carbadox; Phibro Animal Health, Ridgefield Park, NJ) to the corn-soybean meal control diet. Vitamins and minerals were included to meet or exceed the estimated requirements for growing pigs (NRC, 1998).

Pigs were fed a daily quantity of the assigned diet that supplied 3.5 times the estimated maintenance requirement for energy (i.e., 106 kcal ME/$kg^{0.75}$; NRC, 1998). The daily feed allotments were divided into 2 equal meals and fed at 0800 and 1700. Pigs were fed the dietary treatments during 6 periods of 7 d each. All pigs were fed the control diet during the initial period. Pigs in the control treatment were fed the control diet continuously and pigs in the virginiamycin and carbadox treatments were then fed their respective diets during 3 weekly periods. All pigs were fed the control diet during the final 2 weeks.

Ileal digesta were collected in plastic bags (Stein et al., 1999) for 8h on d6 and 7 of each period. Bags were removed when they were filled with digesta, or at least once every 30 min, and immediately stored at -20ºC. Feces were collected twice daily on d 6 and 7 of each period

and immediately stored at -20ºC. Ileal and fecal samples were thawed and mixed within animal and collection period, and sub-samples were taken and stored at -20ºC for microbial analyses.

Microbial Measurements

Two types of microbial measurements were made in ileal digesta and feces. Firstly, the total number of bacteria cells was measured by direct counts of microbes after Gram's staining. Secondly, the species composition of the population was assessed by a molecular method, denaturing gradient gel electrophoresis (**DGGE**).

Total Number of Microbes. For the direct counts of microbes, 10-fold serial dilutions of ileal digesta and fecal samples were prepared and a measured quantity applied to a Reichl slide. The gram stain was applied and the number of cells counted manually by use of a microscope (Carter, 1990).

Genomic DNA extraction and PCR-DGGE Analysis. Genomic DNA was isolated from approximately 250 mg of all of ileal digesta and fecal samples using a commercially available kit (MO BIO UltraPowerSoil^{TM} DNA isolation Kit; MO BIO Laboratories, Inc., Solana Beach, CA). The isolated DNA samples were standardized to 20 μg DNA/ml and PCR amplification was performed by using a PTC-100^{TM} Peltier Thermal Cycler (MJ Research, Inc., Boston, MA). The DNA was amplified using primers specific for the conserved sequences flanking the variable V3 region of 16S rDNA (341F: 5´CACGGGGGGGCCTACGGGAGGCAGCAG 3´ + 5´ 40 nucleotide GC clamp and 534R: 5´ ATTACCGCGGTGCTGG 3´) (Muyzer et al., 1998; Collier et al, 2003). Touchdown PCR was performed to reduce spurious PCR products (Muyzer and Smalla, 1998). After PCR amplification, the PCR products were verified using 1% agarose gel electrophoresis, followed by ethidium bromide staining and capturing the image under an ultraviolet (UV) light (Alpha Imager^{TM} IS-2200, Alpha Innotech Corp., San Leandro, CA).

After visual confirmation of PCR products, DGGE was performed using a DGGE-4801 Multiple Gel Caster (C.B.S. Scientific Company, Inc., Del Mar, CA). The PCR fragments were separated using a linear 35 to 60% denaturing gradient (100% denaturant is equivalent to 7 mol urea/L and 40% deionized formamide) formed in 8% polyacrylamide gels using the GM-500 Gradient Maker (C.B.S. Scientific Company, Inc., Del Mar, CA). Sample baterial V3 16S PCR products (10 μl) were loaded in each lane and bacterial standard ladders representing known bacterial strains were loaded to allow standardization of band migration and gel curvature among different gels (Simpson et al., 1999). The reference ladders consisted of the following species, listed in order from the top of the gel to the bottom: *Bacteroides vulgatus*, *Escherichia coli*, *Bacteroides fragilis*, *Porphyromonas sp.*, *Clostridium perfringens*, *Lactobacillus casei*, and *Enterococcus sp.*. After electrophoresis was performed at 60ºC at 150V for 7 h in 0.5X Tris-acetate-EDTA running buffer, gels were stored in 40% fixative (40% reagent grade methanol, 10% reagent grade acetic acid, 10% BioRad Fixative Enhancer Concentrate, and 40% deionized water) overnight. After fixation, gels were silver-stained and scanned using the BioRad GS-710 calibrated imaging densitometer (BioRad). Gel images were captured to estimate microbial richness and diversity.

Captured gel images were analyzed using the GelCompar II (version 4.5) software (Applied Maths, Inc., Austin, TX). This software was used to determine the number of bands produced by samples from each pig. A detectable band is created by a species that makes up approximately 1% or more of the total bacterial population (Muyzer et al., 1993). The software also calculates Sorenson's pairwise similarity coefficients (**Cs**) by comparing banding patterns among pigs within treatments (**INTRA**) and between treatments (**INTER**) as quantitative measures of the similarity of population structures (Simpson et al., 1999), and produces a

dendrogram based on the Cs values. A Cs value of 100 indicates the two samples being compared have exactly the same bands and a Cs value of 0 indicates the two samples share no bands.

The number of bands indicates microbial diversity as the number of dominant microbial species, except that multiple species may coincidentally occupy the same band. A low INTRA Cs value indicates the microbiota among pigs within a same treatment is not similar. High values of both measurements may be considered to indicate stability of the microbiota.

A low INTER Cs value indicates the microbiota among pigs between any two different treatments is not similar. Thus, average INTRA Cs values for the two treatments was used as the standard and compared with average INTER Cs value between the two treatments. If the INTER Cs value is lower than the INTRA Cs value, it indicates that the microbial populations are different between the two treatments and there is a treatment effect. If the INTER Cs value is equal to or higher than the INTRA Cs value, it indicates that the microbial populations are similar between the two treatments and there is no treatment effect.

Patterns of the DGGE bands were compared between CON and antibiotics treatments and then instances in which a band, representing one or more bacterial species, appeared or disappeared when one of the antibiotics was introduced into the diet, were identified.

Statistical Analyses

Data were analyzed using the PROC GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The experimental unit was the pig. For the number of bacterial cells and DGGE bands, and the INTRA Cs values, the statistical model included the effects of dietary treatments, period, and interaction between treatment and period. Specific contrasts were used to compare between CON

and antibiotics treatments and between different levels of antibiotics. Each INTER Cs value was compared by specific contrast to the 2 pertinent INTRA Cs values.

RESULTS

In the virginiamycin experiment, the virginiamycin treatments reduced ($P < 0.05$) the total number of bacterial cells in ileal digesta during 2 of the 3 wk (wk 2 and 3) of the virginiamycin feeding and in feces during the third week (wk 4) of virginiamycin feeding and in the mean of the overall 6-wk period compared with the CON (Figure 3.1). The counts of total bacteria cells in ileum were higher than those often reported, perhaps because ileal digesta was sampled from near the end of the ileum, but the counts in colon were in the expected range. The virginiamycin treatments reduced ($P < 0.05$) the number of DGGE bands in ileal digesta during the last wk only (wk 6) and overall period compared with the CON (Figure 3.2), but this pattern was not shown in feces (data not shown). The virginiamycin treatments produced modest clustering in the dendrogram and did not affect the INTRA or the INTER Cs values in either ileal digesta or feces (data not shown).

In the carbadox experiment, there were no treatment effects on the total number of bacterial cells or on the number of DGGE bands (data not shown). However, the carbadox treatments made a different cluster in the dendrogram for ileal digesta (Figure 3.3). In addition, the INTRA Cs values of the carbadox treatments were lower ($P < 0.05$) in ileal digesta during wk 2 than those of the CON, but were higher ($P < 0.05$) in ileal digesta during wk 4 than those of the CON (Figure 3.4), but this pattern was not shown in feces. This may indicate that the carbadox treatments gradually made pigs more similar to each other in the microbial populations after an

initial disruption. The carbadox treatments did not affect the INTER Cs values (Figure 3.5) in either ileal digesta or feces compared with the CON.

Based on the pattern of DGGE bands during period of feeding virginiamycin (wk 2 to 4), 3 specific bands in the ileal digesta (data not shown) and 4 specific bands in the feces were present in most pigs fed the CON, but absent from most pigs fed the virginiamycin treatments (Table 3.2). Similarly, in the carbadox experiment, these patterns were found in 4 specific bands in the ileal digesta (Table 3.3) and 1 specific band in the feces (data not shown). For example, band # 38 (Table 3.3) was found in 4 of 5 pigs fed the CON and all of the pigs fed the carbadox treatments during wk 1, when all pigs were fed the CON, and was still found in 4 of the 5 pigs fed the CON during wk 3, but not in any of the 10 pigs fed the carbadox treatments during wk 3. No bands appeared in a majority of the animals fed antibiotics, but not in the CON in either experiment.

DISCUSSION

The present experiments indicate that both virginiamycin and carbadox exert modest effects on microbial populations in the digestive tract of pigs, which is in agreement with reports by Collier et al. (2003), Dumonceaux et al. (2006), and Zhou et al. (2007). The virginiamycin treatments reduced total ileal and fecal microbial populations, which is not supported by previous data showing that virginiamycin does not affect total bacterial populations (Agudelo et al., 2007). Neither the present results nor a previous report (Zhou et al., 2007) showed effects of virginiamycin on microbiota by quantitative measures of population similarity. The carbadox treatments did not affect total ileal and fecal microbial populations, which is in agreement with data reported by White et al. (2002) and Davis et al. (2007). Carbadox homogenized ileal

microbiota, as it made pigs' ileal microbiota more similar to each other. Such effects of antibiotics have been previously reported by Collier et al. (2003) and Miguel et al. (2006). Both antibiotics largely eliminated some species of microbes during the feeding of the antibiotics, but further microbial analysis would be needed to verify these species. However, the antibiotic effects were not large enough to be detected by the INTRA or INTER Cs values in these experiments, perhaps because of natural individual variations of animals (Gong et al., 2005; Richard et al., 2005).

Several studies also support these observations that antibiotics change ileal microbiota (Castillo et al., 2006; Gong et al., 2008; Rettedal et al., 2009). Especially, the results from the present experiments are similar to those reported by Collier et al. (2003), who reported that antibiotics reduced and homogenized the ileal microbial population of pigs, but it was different from the microbiota of control pigs, with improved growth of beneficial commensal microbes, such as lactobacillus, to inhibit colonization of pathogenic microbes in the digestive tract. The modulation of ileal microbiota (homogeneity and/or reduction of the growth of microbial populations) may be an important mechanism of antibiotics for the improvement of animal growth, because it may reflect reduction of toxic metabolites from microbes and/or reduction of competition for energy and nutrients between host and microbes (Collier et al., 2003).

In addition, several studies showed that both virginiamycin and carbadox may affect intestinal immunity by the modulation of microbiota as the virginiamycin increased antibody responses (Brisbin et al., 2008) or as the carbadox changed lymphocyte subpopulation (Hahn et al., 2006; Davis et al., 2007). Several other studies also showed the modulation of microbiota may change intestinal immune responses as indicated by reduction of intraepithelial lymphocytes and lymphocytes in the lamina propria in the small intestine (Manzanilla et al., 2006) and

reduction of serum TNF-α concentration and ileal mRNA TNF-α expression (Weber and Kerr, 2008). Maybe the reduced microbial populations by antibiotics cause suppression of the intestinal immune responses and/or inhibition of intestinal infections, resulting in improvement of animal growth by reverted energy from the immune responses to the growth (Collier et al., 2003).

Therefore, the modulation of microbial populations by virginiamycin or carbadox in the present experiments may support improvement of apparent ileal digestibility of AA, the results from the companion experiments (Stewart et al., 2010a,b).

In conclusion, virginiamycin and carbadox modulated microbial populations in the digestive tract of pigs, especially in ileum, as indicated by homogeneity of ileal microbiota and/or reduction of ileal microbial populations. In addition, the modulation of microbiota of the digestive tract may contribute to improvement of apparent ileal digestibility of AA.

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Table 3.1. Ingredient composition of the experimental diets in virginiamycin and carbadox experiments (as-fed basis)

	Treatment					
	Virginiamycin experiment[1]			Carbadox experiment[2]		
Item	**CON**	**V11**	**V22**	**CON**	**C27.5**	**C55**
Ingredient, %						
Corn	67.55	67.55	67.55	60.25	60.25	60.25
Soybean meal, 48%	27.50	27.50	27.50	32.00	32.00	32.00
Soybean oil	1.00	1.00	1.00	3.00	3.00	3.00
Cornstarch	1.00	0.95	0.90	1.00	0.50	0
Virginimycin premix[3]	0	0.05	0.10	-	-	-
Carbadox premix[4]	-	-	-	0	0.50	1.00
Limestone	1.00	1.00	1.00	1.15	1.15	1.15
Monocalcium phosphate	0.85	0.85	0.85	1.40	1.40	1.40
Chromic oxide	0.40	0.40	0.40	0.50	0.50	0.50
Salt	0.40	0.40	0.40	0.40	0.40	0.40
Vitamin-micro mineral premix[5]	0.30	0.30	0.30	0.30	0.30	0.30
Calculated energy and nutrient levels						
ME, mcal ME/kg	3.31	3.31	3.31	3.43	3.43	3.43
Calcium, %	0.63	0.63	0.63	0.80	0.80	0.80
Phosphorus, %	0.55	0.55	0.55	0.69	0.69	0.69
Available phosphorus, %	0.23	0.23	0.23	0.32	0.32	0.32

Table 3.1. (cont.)

	Treatment					
	Virginiamycin experiment[1]			Carbadox experiment[2]		
Item	**CON**	**V11**	**V22**	**CON**	**C27.5**	**C55**
Analyzed nutrient levels						
Crude protein, %	18.43	17.58	17.96	19.85	21.97	20.59
Lysine, %	1.03	1.01	0.97	1.12	1.22	1.23

[1]CON = control diet; V11 = virginiamycin 11 mg/kg diet; V22 = virginiamycin 22 mg/kg diet.

[2]CON = control diet; C27.5 = carbadox 27.5 mg/kg diet; C55 = carbadox 55 mg/kg diet.

[3]Stafac, Phibro Animal Health Co., Fairfield, NJ.

[4]Mecadox, Phibro Animal Health Co., Fairfield, NJ.

[5]Provided per kilogram of diet: vitamin A, 11,121 IU as vitamin A acetate; vitamin D_3, 2,204 IU as D-activated animal sterol; vitamin E, 66 IU as alpha tocopherol acetate; vitamin K_3, 1.41 mg as menadione dimethylpyrimidinol bisulphate; thiamin, 0.24 mg as thiamine mononitrate; riboflavin, 6.58 mg; pyridoxine, 0.24 mg as pyridoxine hydrochloride; vitamin B_{12}, 0.031 mg; D-pantothenic acid, 23.5 mg as calcium pantothenate; niacin, 44mg; folic acid, 1.58 mg; biotin, 0.44 mg; choline, 0.924 mg as choline chloride; Cu, 10 mg as copper sulfate; Fe, 125 mg as iron sulfate; I, 1.26 mg as potassium iodate; Mn, 60 mg as manganese sulfate; Se, 0.30 mg as sodium selenite; Zn, 126 mg as zinc oxide.

Table 3.2. Effect of virginiamycin on the pattern of DGGE bands in feces

	Band No.[1]	**23**	**30**	**50**	**51**
Treatment[2]	**Period, wk**	***Number of pigs (of 5) showing the band*** [3]			
CON	1	4	0	4	4
V11	1	3	3	4	4
V22	1	2	5	4	5
CON	3 or 4	5	5	5	5
V11	3 or 4	1	1	2	1
V22	3 or 4	0	0	2	2

[1]Band number is for the specific band number in DGGE gel image.

[2]CON = control diet; V11 = virginiamycin 11 mg/kg diet; V22 = virginiamycin 22 mg/kg diet.

[3]About 50 bands were identified in DGGE gel image during virginiamycin feeding (wk 2 to 4). The number for each treatment indicates the number of pigs that had the specific band of total 5 pigs in each treatment.

Table 3.3. Effect of carbadox on the pattern of DGGE bands in ileal digesta

	Band No.[1]	**32**	**37**	**38**	**41**
Treatment[2]	**Period, wk**	***Number of pigs (of 5) showing the band*** [3]			
CON	1	4	3	4	4
C27.5	1	3	5	5	5
C55	1	2	4	5	5
CON	3	5	4	4	4
C27.5	3	1	1	0	1
C55	3	1	1	0	1

[1]Band number is for the specific band number in DGGE gel image.

[2]CON = control diet; C27.5 = carbadox 27.5 mg/kg diet; C55 = carbadox 55 mg/kg diet.

[3]About 40 bands were identified in DGGE gel image during carbadox feeding (wk 2 to 4). The number for each treatment indicates the number of pigs that had the specific band of total 5 pigs in each treatment.

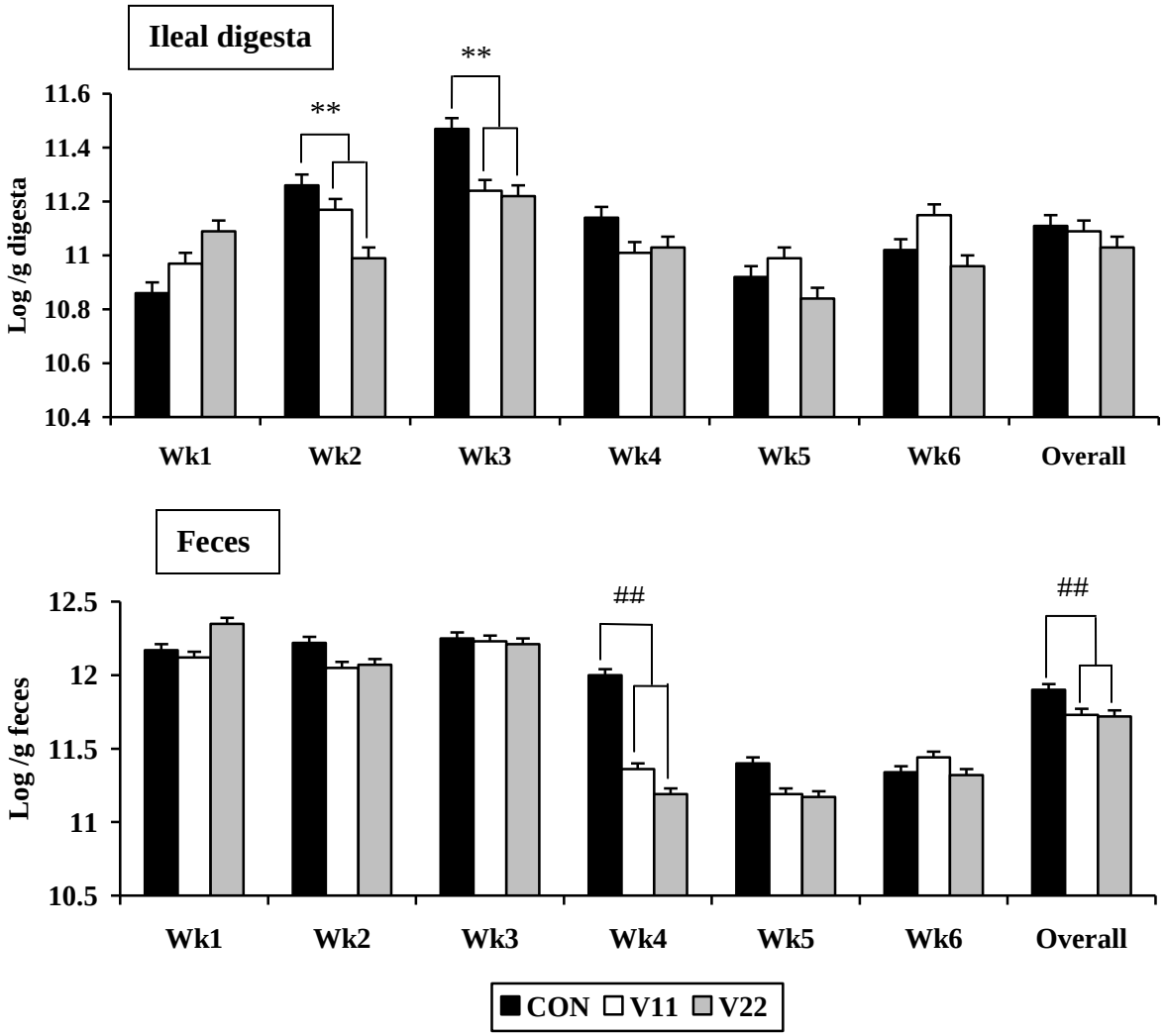

Figure 3.1. Effect of virginiamycin on the number of bacteria cells in ileal digesta and feces by direct counts of microbes after Gram's staining. **Contrast between CON and virginiamycin treatments in ileal digesta during wks 2 and 3 ($P < 0.05$). $^{\#\#}$ Contrast between CON and virginiamycin treatments in feces during wk 4 and overall period ($P < 0.05$). CON is for control diet, V11 is for virginiamycin 11 mg/kg diet, and V22 is for virginiamycin 22 mg/kg diet.

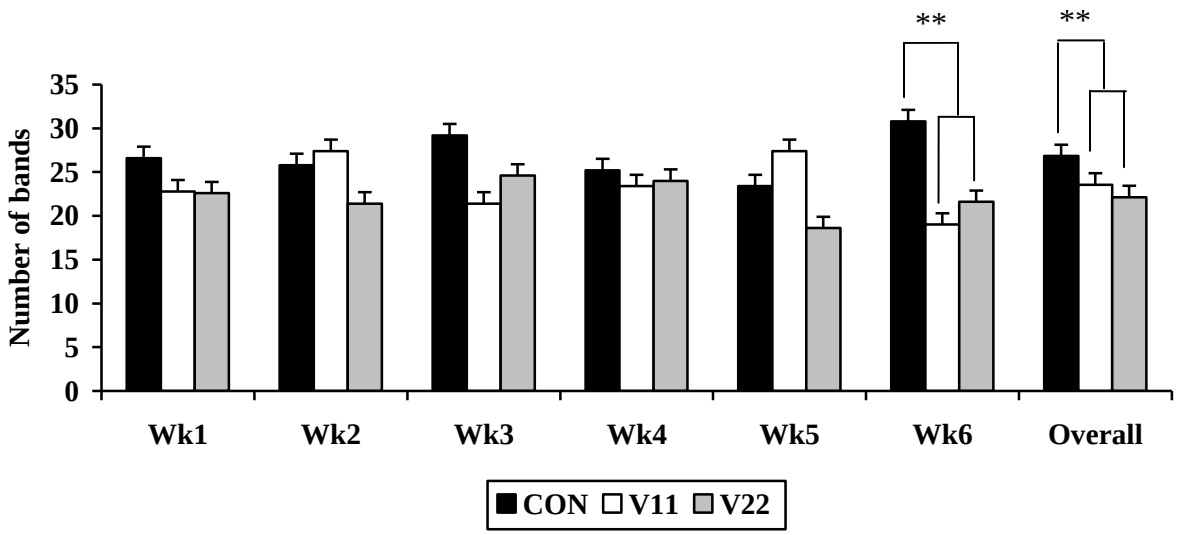

Figure 3.2. Effect of virginiamycin on the number of bands in ileal digesta by DGGE analysis. **Contrast between CON and virginiamycin treatments during wk 6 and overall period ($P < 0.05$). CON is for control diet, V11 is for virginiamycin 11 mg/kg diet, and V22 is for virginiamycin 22 mg/kg diet. There was no virginiamycin effect on the number of bands in feces ($P > 0.05$).

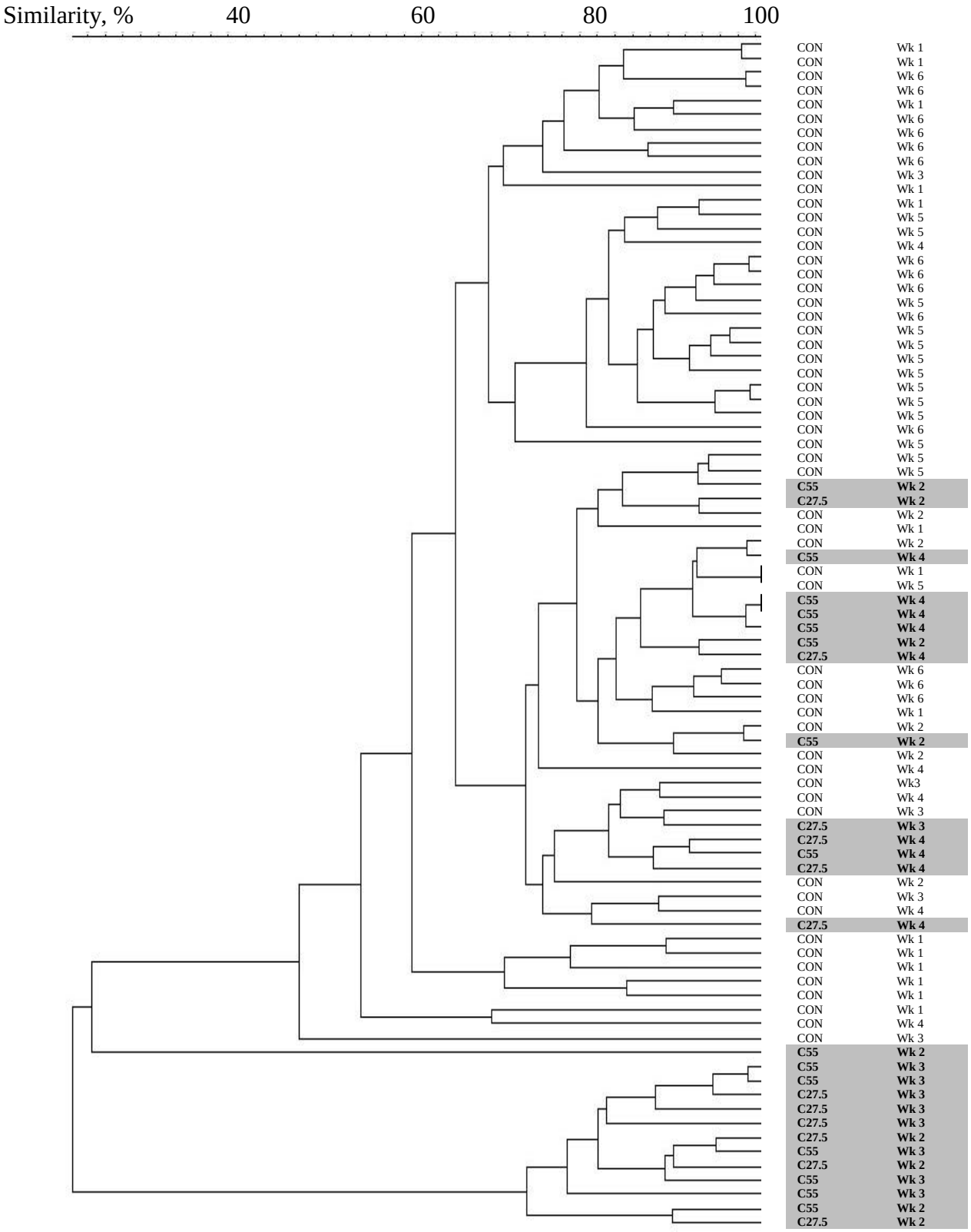

Figure 3.3. Effect of carbadox on ileal microbial ecology (dendrogram). CON is for control diet,

Figure 3.3. (cont.)

C27.5 is for carbadox 27.5 mg/kg diet, and C55 is for carbadox 55 mg/kg diet.

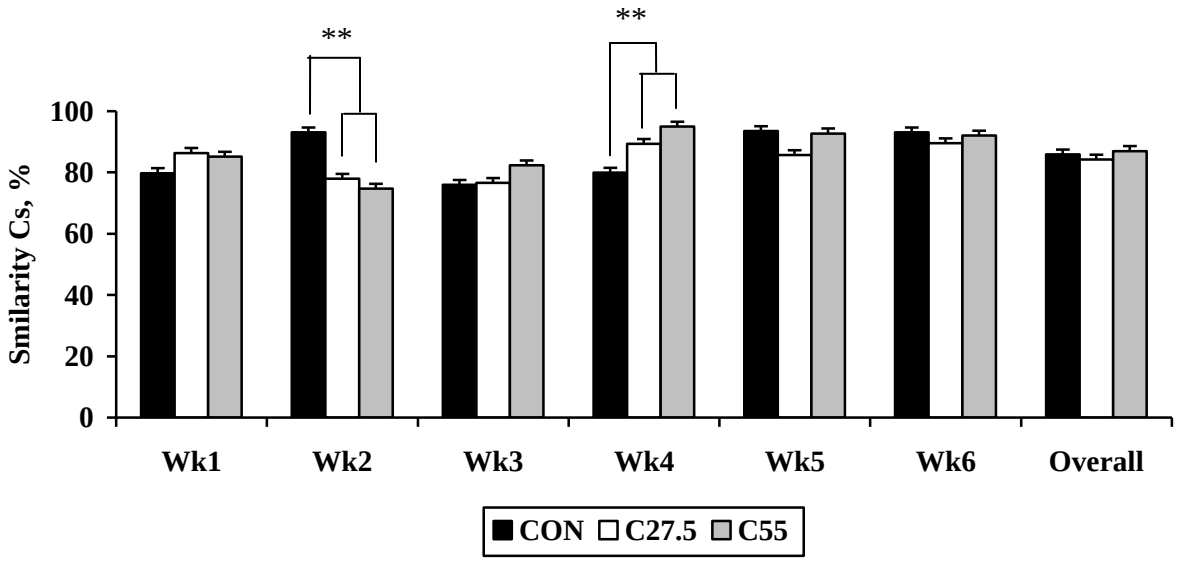

Figure 3.4. Effect of carbadox on the intratreatment (INTRA) Cs values in ileal digesta by DGGE analysis. **Contrast between CON and carbadox treatments during wks 2 and 4 ($P < 0.05$). CON is for control diet, C27.5 is for carbadox 27.5 mg/kg diet, and C55 is for carbadox 55 mg/kg diet. There was no carbadox effect on the INTRA Cs values in feces ($P > 0.05$).

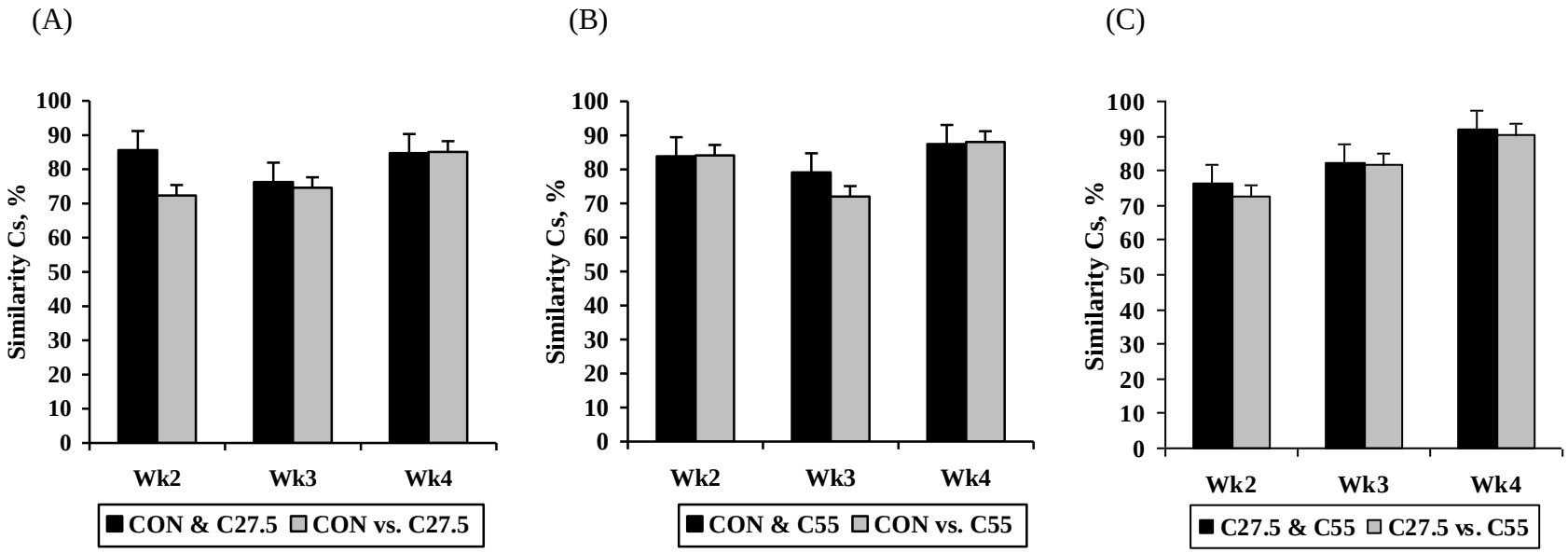

Figure 3.5. Effect of carbadox on the intertreatment (INTER) Cs values in ileal digesta by DGGE analysis. (A) Comparisons of similarity Cs values between average INTRA Cs values for CON and C27.5 (CON & C27.5) and average INTER Cs value between CON and C27.5 (CON vs. C27.5). (B) Comparisons of similarity Cs values between average INTRA Cs values for CON and C55 (CON & C55) and average INTER Cs value between CON and C55 (CON vs. C55). (C) Comparisons of similarity Cs values between average INTRA Cs values for C27.5 and C55 (C27.5 & C55) and average INTER Cs value between C27.5 and C55 (C27.5 vs. C55). CON is for control diet, C27.5 is for carbadox 27.5 mg/kg diet, and C55 is for carbadox 55 mg/kg diet. No differences were detected ($P > 0.05$).

CHAPTER 4

EFFECT OF DIETARY SPRAY-DRIED PLASMA ON PREGNANCY RATE OF MATED FEMALE MICE AFTER TRANSPORT AS A MODEL FOR STRESSED SOWS

ABSTRACT

A study was conducted to evaluate the effects of spray-dried plasma (SDP) on pregnancy rate of mated female mice after transport as a model for stressed sows. A total of 250 mated female mice (C57BL/6 strain, 16 ± 1.2 g BW; 4 replicate groups (block), 62 or 63 mice/group) were purchased and shipped from the vendor, Jackson Lab., Bar Harbor, ME to the university facility, Urbana, IL on the day the vaginal plug was found (gestation day (GD) 1), arriving at the laboratory on GD 3. They were weighed and housed in individual cages, randomly assigned to dietary treatments with or without 8% SDP (SDP or CON), and fed for 2 wk. The diets were formulated to similar ME, CP, and AA levels without antibiotics. On GD 16, pregnancy was determined on the basis of BW and shape of abdomen and later confirmed by inspection post-mortem. The SDP markedly improved ($P < 0.05$) pregnancy rate (49 vs. 11%) regardless of initial BW of mice (BW < 16 g: 36 vs. 4%; BW ≥ 16 g: 57 vs. 16%; no interactions between SDP and initial BW of mice) compared with the CON. In conclusion, SDP improved pregnancy rate of the mated female mice after transportation stress.

Key words: mice, pregnancy rate, spray-dried plasma, transportation stress

INTRODUCTION

In the livestock industry, reduction of conception or farrowing rate by various factors is an important economic factor. Especially, heat stress causes decreased implantation and pregnancy rate, and impairment of embryo development, resulting in low reproductive performance and economic losses (Biggers et al., 1987; Einarsson et al., 2008). In addition, transportation or relocation stress causes delayed puberty, anestrus, and decreased pregnancy rate during early pregnancy (Dalin et al., 1988; Rojanasthien and Einarsson, 1988; Perry, 2007).

There is now accumulating evidence that spray-dried plasma (**SDP**) may decrease wean-to-estrus interval and increase farrowing rate of sows, especially during summer when the sows may be heat-stressed (Crenshaw et al., 2007 and 2008; Fruge et al., 2009). Perhaps this benefit derives from improvement of the intestinal barrier function (Perez-Bosque et al., 2006; Lambert, 2009; Moreto and Perez-Bosque, 2009) which may be disrupted by heat stress, causing local or systemic inflammation. The SDP may provide benefits by moderating inflammatory responses (Jiang et al., 2000; Bosi et al., 2004; Nofrarias et al., 2006). However, there is no information about the SDP effect on pregnancy rate of breeding sows under transportation stress.

Therefore, the objective of this experiment was to evaluate the effects of SDP on pregnancy rate of mated female mice after transport as a model for stressed sows.

MATERIALS AND METHODS

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. The experiments were conducted in the mouse facility located in the Institute for Genomic Biology at the University of Illinois at Urbana-Champaign.

A total of 250 mated female mice (C57BL/6 strain; 16 ± 1.2 g BW; 4 replicate groups (block), 62 or 63 mice/group) were shipped from a vendor (The Jackson Laboratory, Bar Harbor, ME) to the university facility (Urbana, IL) on the day the vaginal plug was found (gestation day (**GD**) 1), arriving at the facility on GD 3 after 2 d transport by air and ground. They were weighed and housed in individual cages, randomly assigned to dietary treatments with or without 8% SDP (SDP or **CON**), and fed for 2 wk. The diets were formulated to meet or exceed NRC (1995) estimates of requirements of laboratory animals and to have similar ME, CP, and AA levels and no antibiotics (Table 4.1) and pelleted without heating (cold-pelleted) using a pellet press. On GD 16, pregnancy of the mice was determined on the basis of their BW and shape of abdomen (normal abdomen of non-pregnant mice vs. full, bulgy, rough, or bumpy abdomen of pregnant mice), and was confirmed later by inspection post-mortem. Measurements were pregnancy rate on GD 16.

Statistical Analysis

The experimental design was a completely randomized design and the mated female mouse was the experimental unit. Pregnancy rate on GD 16 was analyzed by chi-square test.

RESULTS

The pregnancy rate on GD 16 was dramatically higher ($P < 0.05$) among mice fed SDP compared to CON (Figure 4.1).

In addition, the average initial BW of mice on GD 3 of mice later determined to be pregnant was greater ($P < 0.05$) than that of non-pregnant mice (Figure 4.2), suggesting that heavier mice were more fertile under the conditions of these experiments. To ensure that the initial weight effect was not confounded with the SDP effect, the effect of SDP was examined in

mice lighter than 16 g and in those 16 g or heavier. The heavier mice were more likely to be pregnant ($P < 0.05$), and the benefit of SDP was strong ($P < 0.05$) in both weight groups (Figure 4.3). There was not an interaction between initial body weight and diet, indicating that confounding was not an issue (Figure 4.3).

DISCUSSION

The SDP markedly improved the pregnancy rate on GD 16 compared with the CON and this pattern was consistently shown in the all 4 groups (62 or 63 mice/group), plus in a group used in a preliminary experiment (data not shown). The transportation stress that presumably causes the failure of many of the mated female mice to be pregnant is somewhat chronic (De et al., 1993; Tuli et al., 1995; van Ruiven et al., 1998), occurring over a period of days, but perhaps SDP alleviates the chronic inflammation and contribute to improvement of the pregnancy rate because SDP can regulate inflammation (Bosi et al., 2004; Perez-Bosque et al., 2004, 2008) and/or provide other physiological benefits (Perez-Bosque et al., 2006; Moreto et al., 2008; Moreto and Perez-Bosque, 2009). However, the mechanism of SDP for this benefit against transportation stress causes chronic inflammation has not been investigated and thus further research is needed.

In conclusion, spray-dried plasma markedly improved pregnancy rate under the conditions of this experiment. The results may support a potential role of SDP in improving farrowing rate, especially when stress causes inflammation.

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Table 4.1. Ingredient composition of experimental diets (as-fed basis)

	Dietary treatment[1]	
Item	**CON**	**SDP**
Ingredient, %		
Dried skim milk	53.10	33.68
Corn starch	19.90	31.25
Sucrose	10.00	10.00
Spray-dried plasma[2]	0	8.00
Soybean oil	7.00	7.00
Cellulose	5.00	5.00
Mineral premix[3]	3.50	3.50
Vitamin premix[4]	1.00	1.00
DL-methionine	0.25	0.32
Choline bitartrate	0.25	0.25
Calculated energy and nutrient levels		
Energy, kcal ME/kg	3483	3558
Crude protein, %	18.28	18.00
Ash, %	4.44	3.57
Calcium, %	1.18	0.94
Phosphorus, %	0.70	0.64

[1]CON = control diet; SDP = spray-dried plasma diet.

[2]AP 920, American Protein Corporation, Inc., Ankeny, IA.

Table 4.1. (cont.)

[3]Dyets, Inc., Bethlehem, PA. Provided as milligrams per kilogram of diet: calcium, 5,000; phosphorus, 1,561; potassium, 3,600; sodium, 1,019; chloride, 1,571; sulfur, 300; magnesium, 507; iron, 35; copper, 6; manganese, 10; zinc, 30; chromium, 1; iodine, 0.2; selenium, 0.15; fluorine, 1; cobalt, 0.5; molybdenum, 0.15; silicon, 5; nickel, 0.5; lithium, 0.1; vanadium, 0.1.

[4]Dyets, Inc., Bethlehem, PA. Provided per kilogram of diet: thiamin HCl, 6 mg; riboflavin, 6 mg; pyridoxine HCl, 7 mg; niacin, 30 mg; calcium pantothenate, 16 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamin (vitamin B_{12}), 25 μg; vitamin A palmitate, 4,000 IU; vitamin E acetate, 75 IU; vitamin D_3, 1,000 IU; vitamin K_1, 0.75 mg.

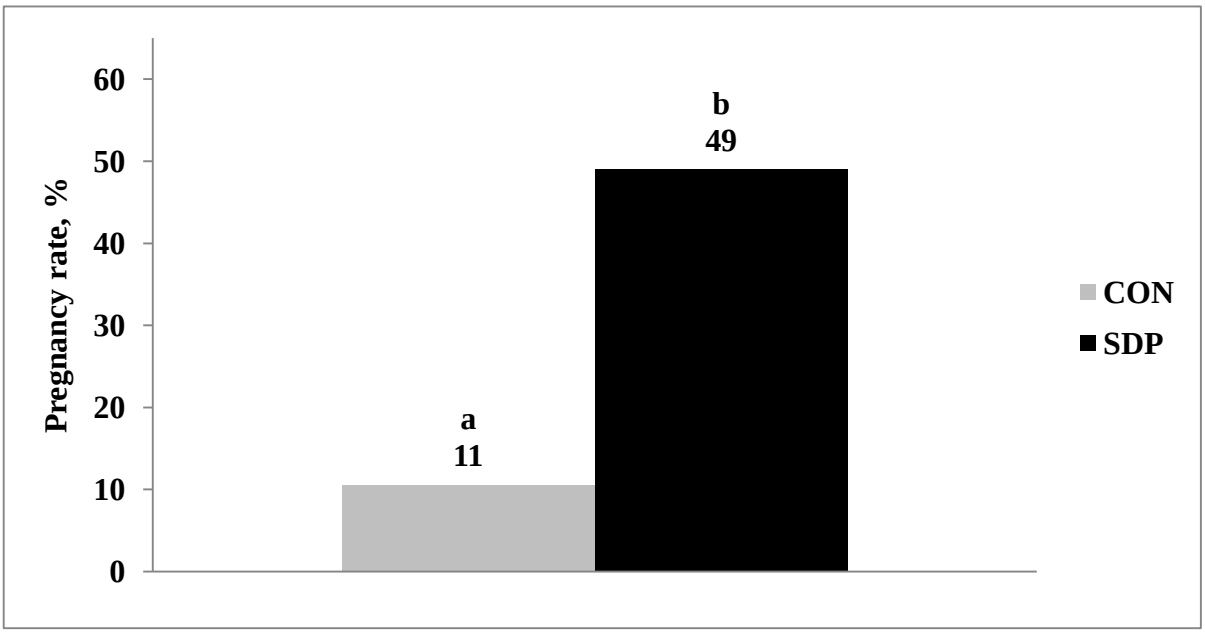

Figure 4.1. Effects of spray-dried plasma on pregnancy rate on gestation day 16 (GD 16). [ab]Means with different letters differ between dietary treatments ($P < 0.05$). CON and SDP are for control diet and spray-dried plasma diet, respectively. The day of detecting the vaginal plug was considered gestation day 1 (GD1) and the arrival day of mice to university facility was on GD 3. Data were analyzed by chi-square test.

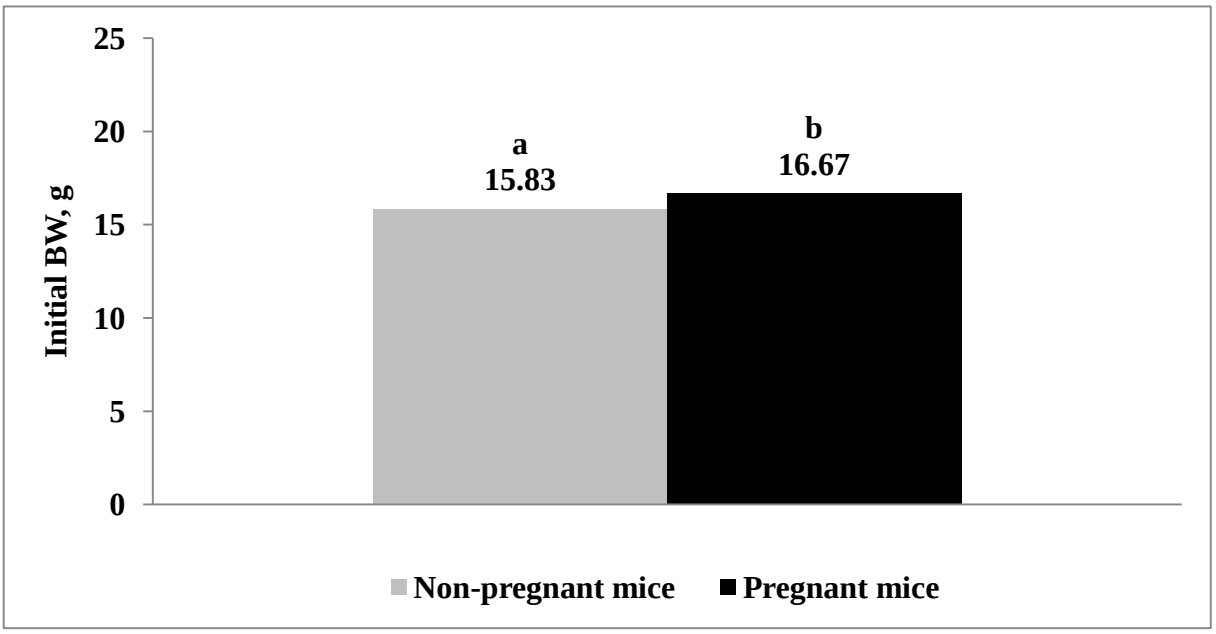

Figure 4.2. Initial BW on GD 3 of mice later determined to be non-pregnant or pregnant. [ab]Means with different letters differ between initial BW of non-pregnant mice and pregnant mice ($P < 0.05$). The day of detecting the vaginal plug was considered gestation day 1 (GD 1) and the initial BW was determined on the arrival day of mice to the university facility on GD 3. Data were analyzed by the PROC GLM procedure of SAS (SAS Inst. Inc., Cary, NC) as the initial BW was included in the statistical model.

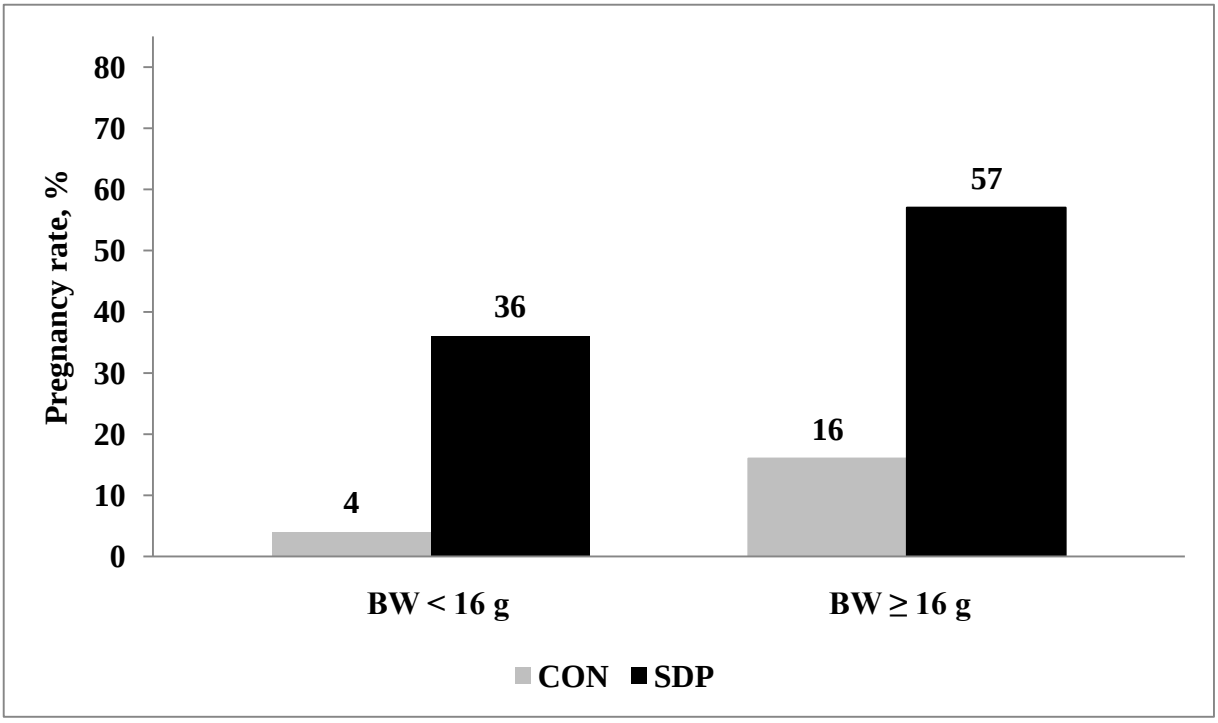

Figure 4.3. Effects of spray-dried plasma and initial BW of mice on gestation day 3 (GD 3) on their pregnancy rate on GD 16. There were effects of initial BW effect (BW < 16 g vs. BW ≥ 16 g; 15 vs. 32%, $P < 0.05$) and diet (10.6 vs. 49%, $P < 0.05$), but their interaction was not significant ($P = 0.45$). CON and SDP are for control diet and spray-dried plasma diet, respectively. The day of detecting the vaginal plug was considered gestation day 1 (GD1) and the arrival day of mice to university facility was on GD 3. Data were analyzed by chi-square test

CHAPTER 5

EFFECTS OF DIETARY SPRAY-DRIED PLASMA ON GROWTH, REPRODUCTIVE, AND IMMUNE RESPONSES OF PREGNANT MICE TO LIPOPOLYSACCHARIDE AS A MODEL FOR INFLAMMATION IN SOWS

ABSTRACT

A study was conducted to evaluate the effects of spray-dried plasma (SDP) on growth, reproductive, and immune responses of pregnant mice to lipopolysaccharide (LPS) as a model for inflammation in sows. A total of 250 mated female mice (C57BL/6 strain; 4 replicate groups, 62 or 63 mice/group) were shipped from a vendor to the university facility on the day the vaginal plug was found (gestation day (GD) 1), arriving at the facility on GD 3. They were housed in individual cages, randomly assigned to dietary treatments with or without 8% SDP, and fed for 15 d. On GD 17, the 61 pregnant mice (26.5 ± 1.65 g BW) were randomly assigned to intraperitoneal injections with or without 2 μg LPS in 200 μl PBS and euthanized 6 h (6H) or 24 h (24H) later. Measurements were growth performance, pregnancy loss, fetal death, and other reproductive responses, and maternal organ weight (Wt). In addition, uterus (U) and placenta (P) were collected from the 6H group mice only to measure pro-inflammatory (PRO; tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)) and anti-inflammatory cytokines (ANTI; interleukin-10 (IL-10) and transforming growth factor-β1 (TGF-β1)) by ELISA, and total protein (TP) using Bradford's reagent and BSA to normalize those cytokines. The SDP increased ($P < 0.05$) ADG (0.712 vs. 0.638 g/d) before the LPS challenge (GD 3 to 17) compared with the CON. The LPS

challenge on GD 17 increased ($P < 0.10$) pregnancy loss, fetal death, spleen Wt, and PRO in both U and P, and reduced growth performance and ANTI in the U only compared with the PBS challenge. The SDP increased BW gain (6H: 0.13 vs. -0.14 g, $P = 0.06$; 24H: 0.81 vs. 0.30 g, $P < 0.05$) and avg live fetal Wt (6H: 0.65 vs. 0.56 g, $P < 0.05$; 24H: 0.76 vs. 0.71 g; $P = 0.09$), and reduced spleen Wt (6H: 0.29 vs. 0.35% of BW, $P = 0.08$; interaction, $P = 0.09$) compared with the CON. In addition, the SDP reduced ($P < 0.05$) PRO (pg/mg TP) in both U (TNF-α: 3.83 vs. 6.93; IFN-γ: 0.97 vs. 2.37) and P (TNF-α: 4.15 vs. 5.71; IFN-γ: 0.19 vs. 0.46) and ANTI (ng/mg TP) in the U only (IL-10: 0.039 vs. 0.050; TGF-β1: 0.28 vs. 0.50) compared with the CON, and attenuated the LPS effect on PRO (interactions: TNF-α in the P ($P = 0.09$), IFN-γ in both U ($P = 0.08$) and P ($P < 0.05$)). In conclusion, SDP improved growth performance of pregnant mice before and after acute inflammation caused by the LPS, and their fetal Wt after the acute inflammation, and attenuated the acute inflammation, but did not affect pregnancy loss and fetal death after the acute inflammation.

Key words: mice, growth performance, immune responses, late-term pregnancy rate, reproductive responses, spray-dried plasma

INTRODUCTION

The swine industry uses spray-dried plasma (**SDP**) for newly weaned pigs because it dramatically increases growth rate and appears to protect the pigs from infectious disease (Pettigrew et al., 2006; Moreto and Perez-Bosque, 2009). There is now accumulating evidence that SDP may improve the reproductive performance of sows (Crenshaw et al., 2007 and 2008; Fruge et al., 2009). Although the mechanisms through which SDP provides benefits are not fully

understood, there are two at least potential protective mechanisms through which SDP may improve the sow reproductive performance. The first is that SDP improves the intestinal barrier function (Perez-Bosque et al., 2006; Moreto et al., 2008; Moreto and Perez-Bosque, 2009) that may be made dysfunctional by heat stress that causes local or systemic inflammation. The second is that SDP alters inflammatory responses (Jiang et al., 2000; Bosi et al., 2004; Nofrarias et al., 2006).

The implicit connection between inflammation and reproduction has not been thoroughly defined, especially in pigs. Evidence in mice and humans indicates that implantation of the embryo can be prevented by inflammation at the implantation site (Erlebacher et al., 2004; Salmon, 2004). Several studies (Rivera et al., 1998; Robertson et al., 2006, 2007) have shown that systemic inflammation can cause fetal death, fetal growth retardation, and even pregnancy loss during late pregnancy. Further, suppressing pro-inflammatory cytokines by administration of an anti-inflammatory cytokine (interleukin-10 (**IL-10**)) attenuated those reproductive losses (Robertson et al., 2006, 2007).

Therefore, this experiment evaluated the potential role of SDP in improving reproductive performance of sows by clarifying its impact on inflammatory damage to reproductive performance by using pregnant mice as a model for sows. The objective of this experiment was to determine whether SDP can moderate inflammation and ameliorate impairment of reproduction caused by lipopolysaccharide (**LPS**).

MATERIALS AND METHODS

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. The experiments

were conducted in the mouse facility located in the Institute for Genomic Biology at the University of Illinois at Urbana-Champaign.

Animals, Housing, Diet, and Experimental Design

A total of 250 mated female mice (C57BL/6 strain; 16 ± 1.2 g BW; 4 replicate groups, 62 or 63 mice/group) were shipped from a vendor (The Jackson Laboratory, Bar Harbor, ME) to the university facility (Urbana, IL) on the day the vaginal plug was found (gestation day (**GD**) 1), arriving at the facility on GD 3 after 2 d transport by air and ground. They were housed in individual cages, randomly assigned to dietary treatments with or without 8% SDP (SDP or **CON**), and fed for 15 d. The diets were formulated to meet or exceed NRC (1995) estimates of requirements of laboratory animals and to have similar ME, CP, and AA levels, and no antibiotics (Table 5.1) and pelleted without heating (cold-pelleted) using a pellet press.

On GD 17, the 61 pregnant mice (26.5 ± 1.65 g BW) were randomly assigned to intraperitoneal injections with or without 2 µg LPS in 200 µl PBS (LPS or **PBS**) to cause inflammation. The dose of LPS was determined by several preliminary experiments (no data presented) based on the report by Robertson et al. (2006). The mice were euthanized 6 h (**6H**; n = 17; 26.65 ± 1.67 g BW) or 24 h (**24H**; n = 44; 25.99 ± 1.60 g BW) after the LPS challenge by cervical dislocation under CO_2 anesthesia.

Measurements and Sample Collection

Measurements were growth performance before and after the injection challenge, late-term pregnancy loss, fetal death, total number of live and dead fetuses, average live fetal and placental weight (**Wt**), and organ Wt (intestine, liver, spleen, and lung) after the injection challenge. The total numbers of live and/or dead fetuses were recorded by checking movement of each fetus immediately after opening the body and then the live and/or dead fetuses,

placentae, and organs were collected and weighed. In addition, gestational tissues, uterus and placenta, were collected from the 6H group only, frozen in liquid nitrogen, and then stored at -80°C stored until cytokine measurements.

Cytokine and Protein Analyses

The frozen uterus and placental samples were weighed and cold dissolved protease inhibitor (5 ml/g sample; Complete Mini, EDTA-free; Roche Diagnostics, Mannheim, Germany) in PBS (1 tablet (3.7 mg protease inhibitor)/7 ml PBS) was added (Robertson et al., 2006). The samples were chopped by scissors, homogenized for 45 or 30 s for uterus or placental samples, respectively, using a high-speed homogenizer (Power Gen 125, Fisher Scientific, PA, USA), and thawed on ice. The samples were centrifuged at 10,000 x g for 20 min at 4°C and supernatants were collected and stored at -80°C for cytokine measurements. Each cytokine was measured in the tissue homogenates using mouse enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's procedure (tumor necrosis factor-α (**TNF-α**, KMC3011, Invitrogen Corporation, CA, USA); interferon-γ (**IFN-γ**, MIF00, R&D systems, MN, USA); interleukin-10 (**IL-10**, KMC0101, Invitrogen Corporation, CA, USA); transforming growth factor-β1 (**TGF-β1**, KAC1688, Invitrogen Corporation, CA, USA)). Total protein (**TP**) of the tissue homogenates was measured using Bradford's reagent and BSA (Bio-Rad Protein Assay, Bio-Rad Laboratories, CA, USA) following the manufacturer's procedure, and the data used to normalize the cytokine concentrations. A standard curve was included in each assay plate for cytokine and protein. Results were measured using microplate reader (Dynex Revelation Microtiter Plate Reader, Lab Recyclers, Inc., Gaithersburg, MD). All data for cytokine measurements were expressed as pg or ng cytokine/g sample as well as pg or ng cytokine/mg TP.

Statistical Analyses

Data were analyzed as a completely randomized design with a 2 x 2 factorial arrangement of treatments by the PROC GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The experimental unit was the pregnant mouse and litter. The statistical model for growth performance, numbers of total, live, and dead fetuses, fetal death, fetal and placental Wt and their ratio, organ Wt, and all cytokine as well as protein contents included the effects of diet, injection challenge, and their interaction. Late-term pregnancy loss after the injection challenge was analyzed by chi-square test.

RESULTS

The average initial BW of mice on GD 3 was similar between SDP and CON, but the SDP increased ($P < 0.05$) ADG and G:F of pregnant mice from GD 3 to 17 compared with the CON (Table 5.2).

The LPS challenge caused ($P < 0.05$) reduced growth performance (Table 5.2), lost pregnancy and fetuses (Table 5.3), reduced average placental Wt (Table 5.4), increased spleen Wt (Table 5.5), and increased pro-inflammatory cytokines in both uterus and placenta as well as reduced anti-inflammatory cytokines in the uterus only (Table 5.6).

The SDP improved ($P < 0.05$) ADG and G:F of the mice before and after the injection challenge and ADFI of the mice only from GD 17 to 6 h after the injection challenge compared with the CON (Table 5.2).

The SDP did not affect late-term pregnancy loss of the mice or their fetal death (24H group) after the injection challenge compared with the CON (Table 5.3). However, the SDP increased average fetal Wt at 6 h ($P < 0.05$) and 24 h ($P = 0.09$) after the injection challenge as

well as the ratio between fetal and placental Wt ($P = 0.07$) at 6 h only after the injection challenge (Table 5.4).

The SDP attenuated the LPS effect on spleen Wt of the mice at 6 h after the injection challenge (interaction, $P = 0.09$), but did not affect it at 24 h after the injection challenge and other organ Wt (Table 5.5).

The SDP reduced ($P < 0.05$) pro-inflammatory cytokines in both uterus and placenta and anti-inflammatory cytokines in the uterus only compared with the CON (Table 5.6). In addition, the SDP attenuated the LPS effect on pro-inflammatory cytokines (TNF-α in placenta (interaction, $P = 0.09$) and IFN-γ in uterus (interaction, $P = 0.08$) and placenta (interaction, $P < 0.05$); Table 5.6).

DISCUSSION

The SDP appeared to attenuate inflammation as indicated by reduced pro-inflammatory cytokines compared with the CON and this result is in agreement with data reported by Perez-Bosque et al. (2004, 2008) and Moreto et al. (2008). In addition, the SDP reduced anti-inflammatory cytokines compared with the CON, perhaps because of reduced pro-inflammatory cytokines (Touchette et al., 2002; Frank et al., 2003; Moreto and Perez-Bosque, 2009). However, this attenuation of inflammation was not enough to increase the proportion of mice that maintained late-term pregnancy after the LPS challenge. Only a few mice on the control treatment were pregnant, presumably the ones whose reproductive and immune systems were able to prevail in the face of what appears to have been a strong challenge from the transportation stress after mating. Perhaps they were then, for the same reasons, more able than

average mice to withstand the LPS challenge in late pregnancy, obscuring what may have otherwise been a benefit of SDP.

The SDP improved growth rate before and after the LPS challenge and this result is in agreement with several reviews (Coffey and Cromwell, 2001; van Dijk et al., 2001; Pettigrew, 2006). It suggests SDP provides physiological benefits beyond provision of bioavailable nutrients (NRC, 1998; Gottlob et al., 2006). These physiological benefits may include strengthening gut barrier function (Perez-Bosque et al. 2006; Lambert, 2009), antibacterial effects (Nollet et al., 1999; Owusu-Asiedu et al., 2003a,b; Niewold et al., 2007), regulating immunity (Bosi et al., 2004; Perez-Bosque et al., 2004, 2008), etc., in normal as well as challenging conditions. Challenging conditions may divert energy from growth to immunity, and SDP may alleviate that diversion (Touchette et al., 2002; Nofrarias et al., 2006).

The SDP tended to improve average fetal Wt, maybe because of the beneficial effects of SDP as mentioned in growth performance. However, a pig study did not show improvement of birth Wt of piglets (Fruge et al., 2009) and there is no corresponding information for mouse or rat. The question of whether SDP can increase birth Wt is not resolved. In addition, SDP may affect the ratio between fetal and placental Wt. The ratio has been used as an indicator of fetal growth in challenging conditions (Robertson et al., 2006, 2007), but the response of this ratio to LPS challenge has been inconsistent.

Organ Wt has been also used as an indicator of severity of inflammation. Greater organ Wt may indicate more severe inflammation as more immune cells are recruited into the organ (Rofe et al., 1996; Lin et al., 2007). The greater spleen Wt with the LPS challenge may indicate more immune cells are recruited in the spleen, and the SDP attenuated the spleen Wt at 6 h after

the LPS challenge and then did not affect the spleen Wt at 24 h after the LPS challenge, perhaps because of recovery.

In conclusion, spray-dried plasma attenuated inflammatory immune responses to LPS administration and markedly improved growth rate before and after acute inflammation, but it appeared not to affect late-term pregnancy loss and fetal death after acute inflammation.

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Table 5.1. Ingredient composition of experimental diets (as-fed basis)

	Dietary treatment[1]	
Item	**CON**	**SDP**
Ingredient, %		
Dried skim milk	53.10	33.68
Corn starch	19.90	31.25
Sucrose	10.00	10.00
Spray-dried plasma[2]	0	8.00
Soybean oil	7.00	7.00
Cellulose	5.00	5.00
Minineral premix[3]	3.50	3.50
Vitamin premix[4]	1.00	1.00
DL-methionine	0.25	0.32
Choline bitartrate	0.25	0.25
Calculated energy and nutrient levels		
Energy, kcal ME/kg	3483	3558
Crude protein, %	18.28	18.00
Ash, %	4.44	3.57
Calcium, %	1.18	0.94
Phosphorus, %	0.70	0.64

[1]CON = control diet; SDP = spray-dried plasma diet.

[2]AP 920, American Protein Corporation, Inc., Ankeny, IA.

Table 5.1. (cont.)

[3]Dyets, Inc., Bethlehem, PA. Provided as milligrams per kilogram of diet: calcium, 5,000; phosphorus, 1,561; potassium, 3,600; sodium, 1,019; chloride, 1,571; sulfur, 300; magnesium, 507; iron, 35; copper, 6; manganese, 10; zinc, 30; chromium, 1; iodine, 0.2; selenium, 0.15; fluorine, 1; cobalt, 0.5; molybdenum, 0.15; silicon, 5; nickel, 0.5; lithium, 0.1; vanadium, 0.1.

[4]Dyets, Inc., Bethlehem, PA. Provided per kilogram of diet: thiamin HCl, 6 mg; riboflavin, 6 mg; pyridoxine HCl, 7 mg; niacin, 30 mg; calcium pantothenate, 16 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamin (vitamin B_{12}), 25 μg; vitamin A palmitate, 4,000 IU; vitamin E acetate, 75 IU; vitamin D_3, 1,000 IU; vitamin K_1, 0.75 mg.

Table 5.2. Effects of spray-dried plasma on growth performance of pregnant mice from gestation day 3 (GD 3) to GD 17 and effects of spray-dried plasma and lipopolysaccharide on growth performance of pregnant mice from GD 17 to GD 18[1]

	Treatment[2]							
	CON		SDP			*P*-value[3]		
Item	PBS	LPS	PBS	LPS	SEM	Diet	Challenge	Interaction
GD 3 to GD17								
No. of mice[4]	6	11	18	26				
BW (GD 3), g	17.18	16.66	16.54	16.41	0.20	0.14	-	-
BW (GD 17), g	25.90	25.80	26.45	26.44	0.32	0.22	-	-
ADG, g/d	0.623	0.653	0.716	0.708	0.018	<0.05	-	-
ADFI, g/d	3.12	3.22	3.20	3.22	0.05	0.55	-	-
G:F	0.200	0.203	0.222	0.223	0.006	<0.05	-	-
During 6h after LPS challenge on GD 17 (6H)								
No. of mice[5]	3	4	5	5				
Final BW, g	26.70	25.20	27.28	27.28	0.76	0.12	0.36	0.36
ADG, g/d	0.000	-0.275	0.250	0.000	0.124	0.06	0.06	0.92
ADFI, g/d	0.100	0.025	0.300	0.100	0.049	<0.05	<0.05	0.25
G:F[6]	-	-	-	-		-	-	-

Table 5.2. (cont.)

Item	Treatment[2]				SEM	*P*-value[3]		
	CON		SDP					
	PBS	LPS	PBS	LPS		Diet	Challenge	Interaction
During 24 h after LPS challenge (24H)								
No. of mice[7]	3	4	13	10				
Final BW, g	25.47	26.13	27.28	26.49	0.95	0.15	0.93	0.33
ADG, g/d	0.567	0.025	1.015	0.613	0.078	<0.05	<0.05	0.58
ADFI, g/d	2.77	2.05	3.32	2.20	0.18	0.24	<0.05	0.50
G:F	0.206	0.012	0.314	0.277	0.029	<0.05	<0.05	0.11

[1]GD 1 = the day of detecting the vaginal plug; GD 3 = the arrival day of mice to university facility.

[2]CON = control diet; SDP = spray-dried plasma diet; PBS = intraperitoneal injection of phosphate-buffered saline on GD 17; LPS = intraperitoneal injection of lipopolysaccharide on GD 17.

[3]Diet = diet effect; Challenge = injection challenge effect; Interaction = interaction between diet and injection challenge.

[4]No. of mice = number of mice that maintained pregnancy until GD 17.

[5]No pregnant mouse in the 6H group had pre-delivery during 6h after injection on GD 17.

[6]Several mice lost BW and thus G:F could not be calculated.

[7]No. of mice = number of mice that maintained pregnancy during 24h after injection on GD 17.

Table 5.3. Effects of spray-dried plasma on pregnancy loss of pregnant mice (24H group) and their fetal death 24 h after lipopolysaccharide injection on GD 17[1]

	Treatment[2]							
	CON		SDP			*P*-value[3]		
Item	PBS	LPS	PBS	LPS	SEM	Diet	Challenge	Interaction
No. of mice (GD 17)[4]	3	7	13	21				
Pre-delivery[5]	0	3	0	11	-	0.92[6]	<0.05[6]	0.11[6]
Pregnancy loss, %	0	42.9[7]	0	52.4[7]				
No. of mice (GD 18)[8]	3	4	13	10				
No. of total fetuses/ litter	5.67	7.00	6.54	6.75	0.91	0.66	0.28	0.43
No. of live fetuses/ litter	5.67	6.50	6.46	6.50	0.93	0.58	0.55	0.58
No. of dead fetuses/ litter	0	0.50	0.08	0.25	0.10	0.61	0.06	0.34
Fetal death, %	0	6.70	1.28	3.87	1.60	0.77	0.08	0.43

[1]No pregnant mouse pre-delivery in the 6H group.

[2]CON = control diet; SDP = spray-dried plasma diet; PBS = intraperitoneal injection of phosphate-buffered saline on GD 17; LPS = intraperitoneal injection of lipopolysaccharide on GD 17.

[3]Diet = diet effect; Challenge = injection challenge effect; Interaction = interaction between diet and injection challenge.

[4]No. of mice = number of mice that maintained pregnancy until GD 17

[5]Pre-delivery = number of pregnant mice that did not maintain pregnancy during 24h after injection on GD 17.

Table 5.3. (cont.)

[6]Pregnancy loss during 24 h after injection was analyzed by chi-square test.

[7]No difference between CON and SDP within LPS treatment was found on pregnancy loss (42.9 vs. 52.4 %; $P = 0.80$) by chi-square test.

[8]No. of mice = number of mice that maintained pregnancy until 24h after injection.

Table 5.4. Effects of spray-dried plasma on fetal and placental weights from pregnant mice injected with lipopolysaccharide[1]

	Treatment[2]							
	CON		SDP			*P*-value[3]		
Item	PBS	LPS	PBS	LPS	SEM	Diet	Challenge	Inter.
At 6h after LPS injection on GD 17 (6H)								
No. of mice	3	4	5	5				
No. of live fetuses/litter	8.00	7.25	6.50	7.75	0.65	0.47	0.72	0.17
Total fetal wt, g	4.61	3.91	4.30	4.89	0.46	0.49	0.92	0.20
Avg fetal wt, g	0.577	0.537	0.660	0.634	0.034	<0.05	0.38	0.85
Total placental wt, g	0.90	0.86	0.71	0.81	0.07	0.16	0.75	0.37
Avg placental wt, g	0.113	0.119	0.112	0.104	0.0080	0.35	0.92	0.44
Ratio[4]	5.13	4.60	6.06	6.20	0.61	0.07	0.77	0.61
At 24 h after LPS injection on GD 17 (24H)								
No. of mice	3	4	13	10				
No. of live fetuses/litter	5.67	6.50	6.46	6.50	0.93	0.58	0.55	0.58
Total fetal wt, g	4.09	4.51	4.83	4.91	0.38	0.25	0.73	0.60
Avg fetal wt, g	0.719	0.697	0.767	0.752	0.019	0.09	0.54	0.91
Total placental wt, g	0.68	0.67	0.72	0.67	0.10	0.76	0.70	0.81
Avg placental wt, g	0.120	0.103	0.113	0.103	0.0056	0.60	0.08	0.63
Ratio[4]	6.01	6.76	6.99	7.46	0.76	0.17	0.31	0.81

Table 5.4. (cont.)

[1]GD 1 = the day of detecting the vaginal plug; GD 3 = the arrival day of mice to university facility.

[2]CON = control diet; SDP = spray-dried plasma diet; PBS = intraperitoneal injection of phosphate-buffered saline on GD 17; LPS = intraperitoneal injection of lipopolysaccharide on GD 17.

[3]Diet = diet effect; Challenge = injection challenge effect; Inter. = interaction between diet and injection challenge.

[4]Ratio = ratio between fetal and placental weight.

Table 5.5. Effects of spray-dried plasma on organ weights of pregnant mice injected with lipopolysaccharide[1]

	Treatment[2]							
	CON		SDP			*P*-value[3]		
Item	PBS	LPS	PBS	LPS	SEM	Diet	Challenge	Inter.
At 6h after LPS challenge on GD 17 (6H)								
No. of mice	3	4	5	5				
BW, g	26.70	25.20	27.28	27.28	0.76	0.12	0.36	0.36
Intestine, g[4]	2.335	2.053	2.170	1.801	0.157	0.23	0.07	0.79
Intestine wt, % of BW	8.80	8.17	7.98	6.62	0.67	0.12	0.18	0.61
Liver, g	1.318	1.211	1.275	1.293	0.060	0.76	0.49	0.33
Liver wt, % of BW	4.93	4.81	4.68	4.74	0.20	0.46	0.89	0.66
Spleen, mg	70.33	110.00	70.25	86.25	8.41	0.20	<0.05	0.20
Spleen wt, % of BW	0.26	0.44	0.26	0.32	0.031	0.08	<0.05	0.09
Lung, g	0.150	0.162	0.148	0.152	0.008	0.45	0.36	0.64
Lung wt, % of BW	0.56	0.64	0.54	0.56	0.031	0.11	0.16	0.32

Table 5.5. (cont.)

Item	Treatment[2]				SEM	*P*-value[3]		
	CON		SDP					
	PBS	LPS	PBS	LPS		Diet	Challenge	Inter.
At 24 h after LPS challenge on GD 17 (24H)								
No. of mice (GD 18)	3	4	13	10				
BW (GD 18), g	25.47	26.13	27.28	26.49	0.95	0.15	0.93	0.33
Intestine, g[4]	1.997	2.028	1.757	1.641	0.068	<0.05	0.62	0.40
Intestine wt, % of BW	7.93	7.79	6.46	6.21	0.25	<0.05	0.63	0.88
Liver, g	1.313	1.350	1.481	1.460	0.037	<0.05	0.87	0.54
Liver wt, % of BW	5.17	5.18	5.45	5.53	0.30	0.20	0.85	0.87
Spleen, mg	55.00	72.50	74.62	78.75	3.53	<0.05	0.10	0.12
Spleen wt, % of BW	0.22	0.28	0.27	0.30	0.013	0.11	0.08	0.19
Lung, g	0.137	0.155	0.155	0.149	0.011	0.51	0.54	0.20
Lung wt, % of BW	0.54	0.59	0.57	0.56	0.044	0.99	0.50	0.36

[1]GD 1 = the day of detecting the vaginal plug; GD 3 = the arrival day of mice to university facility.

[2]CON = control diet; SDP = spray-dried plasma diet; PBS = intraperitoneal injection of phosphate-buffered saline on GD 17; LPS = intraperitoneal injection of lipopolysaccharide on GD 17.

[3]Diet = diet effect; Challenge = injection challenge effect; Inter. = interaction between diet and injection challenge.

[4]Whole intestine with digesta was collected and weighed.

Table 5.6. Effects of spray-dried plasma on inflammatory responses in gestational tissues of pregnant mice at 6h after intraperitoneal injection of lipopolysaccharide on GD 17[1]

	Treatment[2]							
	CON		SDP			*P*-value[3]		
Item	**PBS**	**LPS**	**PBS**	**LPS**	**SEM**	**Diet**	**Challenge**	**Inter.**
No. of mice	3	4	5	5				
Total protein (TP), mg/g tissue								
Uterus	46.53	47.75	42.18	42.38	1.93	<0.05	0.75	0.82
Placenta	55.77	59.75	44.00	46.46	4.07	<0.05	0.49	0.87
Pro-inflammatory cytokines								
TNF-α								
Uterus, pg/g tissue	173.13	472.19	68.83	269.27	46.92	<0.05	<0.05	0.30
Uterus/TP, pg/mg	4.05	9.81	1.65	6.00	1.00	<0.05	<0.05	0.54
Placenta, pg/g tissue	91.35	527.62	55.23	347.21	29.96	<0.05	<0.05	<0.05
Placenta/TP, pg/mg	1.70	9.72	1.24	7.06	0.55	<0.05	<0.05	0.09
IFN-γ								
Uterus, pg/g tissue	14.23	200.06	4.56	78.25	25.52	<0.05	<0.05	0.07
Uterus/TP, pg/mg	0.326	4.149	0.108	1.837	0.515	<0.05	<0.05	0.08
Placenta, pg/g tissue	6.94	46.55	4.04	14.15	4.49	<0.05	<0.05	<0.05
Placenta/TP, pg/mg	0.129	0.789	0.082	0.307	0.073	<0.05	<0.05	<0.05

Table 5.6. (cont.)

Item	Treatment[2]				SEM	*P*-value[3]		
	CON		SDP					
	PBS	LPS	PBS	LPS		Diet	Challenge	Inter.
Anti-inflammatory cytokines								
IL-10								
Uterus, ng/g tissue	2.61	2.11	1.72	1.63	0.15	<0.05	0.10	0.26
Uterus/TP, ng/mg	0.057	0.044	0.040	0.038	0.0038	<0.05	0.09	0.67
Placenta, ng/g tissue	1.27	1.26	1.24	1.24	0.18	0.92	0.97	0.98
Placenta/TP, ng/mg	0.024	0.026	0.029	0.028	0.0050	0.27	0.80	0.92
TGF-β1								
Uterus, ng/g tissue	26.75	20.18	13.39	10.53	2.37	<0.05	0.10	0.49
Uterus/TP, ng/mg	0.571	0.421	0.317	0.248	0.045	<0.05	<0.05	0.71
Placenta, ng/g tissue	27.56	27.52	13.48	14.08	2.73	<0.05	0.93	0.92
Placenta/TP, ng/mg	0.492	0.454	0.299	0.300	0.037	<0.05	0.66	0.66

[1]GD 1 = the day of detecting the vaginal plug; GD 3 = the arrival day of mice to university facility.

[2]CON = control diet; SDP = spray-dried plasma diet; PBS = intraperitoneal injection of phosphate-buffered saline on GD 17; LPS = intraperitoneal injection of lipopolysaccharide on GD 17.

[3]Diet = diet effect; Challenge = injection challenge effect; Inter. = interaction between diet and injection challenge.

CHAPTER 6

EFFECTS OF DIETARY CLAYS ON DIARRHEA OF NEWLY WEANED PIGS EXPERIMENTALLY INFECTED WITH A PATHOGENIC *ESCHERICHIA COLI*

ABSTRACT

Two experiments were conducted to determine whether 3 different clays in the nursery diet reduce diarrhea of weaned pigs experimentally infected with a pathogenic *E. coli*. Weaned pigs (21 d old) were housed in individual pens of disease containment chambers for 16 d (4 d before and 12 d after the first challenge (d 0)). The treatments were in a factorial arrangement: 1) with or without an *E. coli* challenge (F-18 *E. coli* strain; toxins LT, STb and SLT-2; 10^{10} cfu/3 ml oral dose daily for 3 d from d 0) and 2) dietary treatments. The ADG, ADFI, and G:F were measured for each interval (d 0 to 6, 6 to 12, and 0 to 12). Diarrhea score (DS; 1 = normal; 5 = watery diarrhea) was recorded for each pig daily. Feces were collected on d 0, 3, 6, 9, and 12 and plated on blood agar to differentiate β-hemolytic coliforms (HC) from total coliforms (TC) and on MacConkey agar to verify *E. coli*. Their populations on blood agar were assessed visually using a score (0 = no growth; 8 = very heavy bacterial growth) and expressed as a ratio of HC to TC scores (RHT). Blood was collected on d 0, 6, and 12 to measure total and differential white blood cell (WBC) counts, packed cell volume (PCV), and total protein (TP). Exp. 1 was conducted using 48 pigs (6.9 ± 1.0 kg BW) and 4 diets (a nursery basal diet (CON), CON + 0.3% smectite (S), CON + 0.6% S, and CON until d 0 and then CON + 0.3% S). The S treatments did not affect growth rate of the pigs for the overall period. In the *E. coli* challenged group, the S

treatments reduced DS for the overall period (1.77 vs. 2.01; $P < 0.05$) and RHT on d 6 (0.60 vs. 0.87; $P < 0.05$) and d 9 (0.14 vs. 0.28; $P = 0.08$), and altered differential WBC on d 6 (neutrophils, 48 vs. 39%, $P = 0.09$; lymphocytes, 49 vs. 58%, $P = 0.08$) compared with the control treatment. Exp. 2 was conducted using 128 pigs (6.7 ± 0.8 kg BW) and 8 diets (CON and 7 clay treatments (0.3% clay smectite, kaolinite, and zeolite individually and all possible combinations to total 0.3% of the diet)). The clay treatments did not affect growth rate of the pigs. In the *E. coli* challenged group, the clay treatments reduced DS for the overall period (1.63 vs. 3.00; $P < 0.05$), RHT on d 9 (0.32 vs. 0.76; $P < 0.05$) and d 12 (0.13 vs. 0.39; $P = 0.09$), and total WBC on d 6 (15.2 vs. 17.7 $x10^3/\mu L$; $P = 0.07$) compared with the control treatment. In conclusion, clays alleviated diarrhea of weaned pigs experimentally infected by a pathogenic *E. coli*.

Key words: clay, diarrhea, *Escherichia coli*, weaned pigs

INTRODUCTION

Clays (hydrated aluminosilicates) are naturally occurring materials composed primarily of fine-grained minerals, and have specific structures of porous aluminosilicate layers (Guggenheim and Martin, 1995; Papaioannou et al., 2005; Williams et al., 2009). Clays bind mycotoxins (Lemke et al., 1998, 2001) that are detrimental to animal production and health and thus they have been widely used in animal diets for that purpose (Lindemann et al. 1993; Schell et al., 1993a,b).

In addition, several literature reviews suggest that clays may have antibacterial or antidiarrheic effects (Carretero, 2002; Tateo and Summa, 2007; Williams et al., 2009). For

example, Ramu et al. (1997) reported clays adsorb and inactivate the heat-labile enterotoxins of *E. coli* and the cholera enterotoxins of *Vibrio cholerae*. Moreover, two other studies reported a natural French clay inhibits growth of pathogenic *E. coli* and other antibiotic-susceptible or -resistant bacteria (Haydel et al., 2008; Williams et al., 2008).

These beneficial effects of clays may provide some protective effects against enteric diseases in humans (Gomes and Silva, 2007) and pigs (Papaioannou et al., 2005). Specifically, some studies showed clays attenuate overall disorder of diarrhea-predominant irritable bowel syndrome (Chang et al., 2007) and severity of acute diarrhea of children (Madkour et al., 1993; Dupont et al., 2009). Single studies with pigs showed clays reduced post-weaning diarrhea of nursery pigs (Papaioannou et al., 2004) and the colonization of pathogenic *E. coli* (Trckova et al., 2009).

However, different clays with different chemical structures may have different activities (Bergaya and Lagaly, 2006; Williams et al., 2009). Moreover, the experimental evidence for an antidiarrheic effect of clays in pig diets is limited. Therefore, the objective of these studies was to determine whether 3 different clays reduce diarrhea of weaned pigs experimentally infected with a pathogenic *E. coli*.

MATERIALS AND METHODS

The protocol for these experiments was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. The experiments were conducted in disease containment chambers of the Edward R. Madigan Laboratory building at the University of Illinois at Urbana-Champaign.

Animals, Housing, Diet, and Experimental Design

The same number of barrows and gilts (PIC C-22 female x PIC line 337 male) with similar weight were selected at weaning and assigned to treatments in a randomized complete block design with sex (barrow and gilt) by weight (heavy, middle, and light weights) as the blocks and pig as the experimental unit. Pigs were housed in individual pens of disease containment chambers for 16 d (4 d before (acclimation period) and 12 d after the first challenge (**d 0**)). There were 4 individual pens in each disease containment chamber and 8 chambers in each suite to provide 1 suite each for unchallenged and *E. coli* challenged groups.

The treatments were in a factorial arrangement (with or without *E. coli* challenge treatment and dietary treatments). The *E. coli* used for the challenge, (isolate # UI-VDL 05-27242) was an F-18 fimbria+ *E. coli* strain that produced heat-labile toxin, heat-stable toxin, and Shiga-like toxin (Perez-Mendoza et al., 2010) isolated from a field disease outbreak and provided at 10^{10} cfu per 3 ml dose in phosphate-buffered saline (**PBS**) to cause mild diarrhea (Perez-Mendoza et al., 2010). The unchallenged treatment (**sham**) was a 3 ml dose of PBS. Both the *E. coli* and sham inoculations were given orally to pigs daily for 3 consecutive days beginning 4 d after weaning (d 0). Three clays used in these experiments belonged to classes of smectite (2:1 layer structure), kaolinite (1:1 layer structure), and zeolite (framework structure). The researchers were blind to the identity of the clays until the data were analyzed.

The complex nursery basal diet was formulated to meet or exceed NRC (1998) estimates of requirements of weanling pigs (Table 6.1). It did not include spray-dried plasma, antibiotics, or zinc oxide to avoid their antibacterial or physiological effects. The experimental diets were introduced at weaning.

In Exp. 1, a total of 48 pigs (6.9 ± 1.0 kg initial BW, 21 d old; 7.4 ± 1.1 kg d 0 BW, 25 d old) were used and 4 dietary treatments were the complex nursery basal diet throughout the

experiment (**CON**), CON + 0.3% smectite throughout the experiment (**0.3S**), CON + 0.6% smectite throughout the experiment (**0.6S**), and CON until d 0 and then CON + 0.3% smectite (**B/0.3S**).

In Exp. 2, a total of 128 pigs (6.7 ± 0.8 kg initial BW, 21 d old; 7.1 ± 0.8 kg d 0 BW, 25 d old) were used in 2 groups of 64 separated by time and 8 dietary treatments were the basal diet (**CON**), CON + 0.3% smectite (**S**), CON + 0.3% kaolinite (**K**), CON + 0.3% zeolite (**Z**), CON + 0.15% of each smectite and kaolinite (**SK**), CON + 0.15% of each smectite and zeolite (**SZ**), CON + 0.15% of each kaolinite and zeolite (**KZ**), and CON + 0.1% of each smectite, kaolinite, and zeolite (**SKZ**).

Sample Collection, Analyses, and Measurements

Pigs and feeders were weighed on the day of weaning (d -4), the day of the first inoculation (d 0), d 6, and d 12. Growth performance (ADG, ADFI, and G:F) was measured for each interval from d 0 to 6, 6 to 12, and 0 to 12.

Clinical observations (diarrhea and alertness scores) were recorded daily beginning on the first day of challenge (d 0). Diarrhea score of each pig was assessed visually each day by 2 independent evaluators with a score from 1 to 5 (1 = normal feces, 2 = moist feces, 3 = mild diarrhea, 4 = severe diarrhea, and 5 = watery diarrhea). Frequency of diarrhea was calculated by counting pig days with diarrhea score of 3 or higher. Alertness score of each pig was assessed visually each day with a score from 1 to 3 (1 = normal, 2 = slightly depressed or listless, and 3 = severely depressed or recumbent). Every pig was alert throughout the experiment and was given an alertness score of 1 (normal) each day, thus those data are not reported. The *E. coli* infection model was achieved successfully in both Exp. 1 and 2, but the *E. coli* challenge was more severe in Exp. 2 than in Exp. 1 based on the diarrhea score and frequency of diarrhea. The pigs in Exp.

1 were born to sows immunized against F-4 *E. coli*. Immunity to the F-4 fimbrial antigen should not be protective against the F-18 challenge strain used in these experiments, but the vaccine also contained the heat-labile toxin that is expressed by the challenge organism used in these experiments, so it may have offered some protection. Heat-labile toxin is a potent immunogen. The pigs in Exp. 1 also received antibiotic injections after birth and antibiotics in their creep feed, and these factors may have provided some protection. The sows and pigs used in Exp. 2 did not receive either the vaccines, the antibiotic injections, or the antibiotics in creep feed.

Prior to weaning, fecal samples of sows that produced the piglets destined for these experiments were collected to verify absence of β-hemolytic coliforms by plating on blood and MacConkey agars (more detailed procedures are presented below). No β-hemolytic coliforms were detected in the sows' feces. Fecal samples were collected from the rectum of each pig on d 0, 3, 6, 9, and 12 and kept on ice during transport to the laboratory. When it was not possible to get a bulk sample because of the absence of feces or watery diarrhea, a cotton swab was used to collect the sample. Samples were processed within 2 h after collection. Each sample was plated on blood agar to differentiate β-hemolytic coliforms (generally gray and shiny colonies; complete lysis of red blood cells surrounding colonies) from non-β-hemolytic coliforms. Growth on MacConkey agar was compared to blood agar to support that hemolytic colonies on the blood agar were correctly identified as *E. coli* (generally flat pink colonies). Plates were incubated at 37 °C and were read 24 h after plating. Populations of both total coliforms and β-hemolytic coliforms on blood agar were assessed visually, assigning a score from 0 through 8, where 0 corresponds to no growth and 8 to very heavy bacterial growth. The results were then expressed as a ratio of the β-hemolytic coliforms score to the total coliforms score, as an approximation of the proportion of *E. coli* that were β-hemolytic coliforms. When atypical colonies were detected

on blood agar, they were isolated, grown, and plated sequentially on MacConkey and blood agars to determine whether the colonies were β-hemolytic coliforms, and the results were further verified using triple sugar iron and lysine iron agars. When the isolated colonies were verified as β-hemolytic coliforms through the above methods, they were finally tested by polymerase chain reaction to determine whether the colonies were F-18 *E. coli*.

Two blood samples (whole blood and serum) were collected from the jugular vein of each pig on d 0, 6, and 12 to measure total and differential white blood cell counts, packed cell volume, and total protein by the Veterinary Clinical Pathology Laboratory at the University of Illinois at Urbana-Champaign. Total and differential white blood cell (**WBC**) counts and packed cell volume (**PCV**) by hematocrit concentration were analyzed on a multiparameter, automated hematology analyzer calibrated for porcine blood (CELL-DYN 3700, Abbott Laboratories, Abbott Park, IL). Serum total protein (**TP**) was analyzed on an automated biochemistry analyzer (HITACHI 917, Roche Diagnostics GmbH, Mannheim, Germany). Both PCV and TP were used as indicators for dehydration.

Statistical Analyses

Data were analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). Pig was the experimental unit. The statistical model included effects of *E. coli* challenge, diet, and their interaction as fixed effects and block as a random effect. Specific contrasts were used to test comparisons between the control and the clay treatments collectively within each challenge treatment. In addition, differences among the clay treatments within each challenge treatment were tested by pair-wise comparisons when the overall main effects were significant. The Chi-square test was used for the frequency of diarrhea.

RESULTS

Experiment 1

The *E. coli* challenge did not affect growth performance (Table 6.2), but tended (P = 0.06) to reduce ADFI from d 0 to 6. The S treatments did not affect growth rate over the entire experimental period, but the unchallenged pigs fed the S treatments grew more slowly than the control treatment (P = 0.08) during the early part of the experiment, and more rapidly later (P < 0.05). Among the unchallenged pigs, the S treatments also improved (P < 0.05) feed efficiency during the last 6 days and over the entire experimental period. There were no differences among the S treatments.

The *E. coli* challenge did not affect diarrhea scores or frequency of diarrhea (Table 6.3), but addition of the S to the diet reduced (P < 0.05) the diarrhea score and frequency of diarrhea. These effects were identified (P < 0.05) during the period from d 3 to 6 and 0 to 12 after the sham challenge, and during the periods from d 7 to 9, 10 to 12, and 0 to 12 after the *E. coli* challenge. There were no differences among the S treatments.

The *E. coli* challenged pigs had populations of β-hemolytic coliforms in their feces at all time periods after the *E. coli* challenge, but those organisms were never found in feces from the unchallenged pigs (Table 6.4). Among the *E. coli* challenged pigs, feeding the S treatments reduced (P < 0.05) the proportion of β-hemolytic coliforms on d 6 and tended (P = 0.08) to reduce it on d 9. There were no differences among the S treatments.

The *E. coli* challenge tended (P = 0.08) to increase the number of total WBC on d 6 (Table 6.5), but the S treatments did not affect WBC. There were no differences among the S treatments. The *E. coli* challenge increased (P < 0.05) PCV on d 6 and tended (P = 0.07) to

increase it on d 12, but did not affect TP (Table 6.6). The S treatments did not affect PCV and TP. There were no differences among the S treatments.

Experiment 2

One *E. coli* challenged pig in SZ treatment did not grow over the entire experimental period and its growth rate was detected as an outlier by statistical analysis. Thus, data from the pig were not used on any measurements. Several *E. coli* challenged pigs lost BW from d 0 to 6, rendering the G:F values meaningless, so those data are not presented.

The *E. coli* challenge reduced ($P < 0.05$) ADFI and ADG during all stages and G:F during the overall period (Table 6.7). The clay treatments did not affect growth rate compared with the control treatment and there were no differences among the clay treatments.

The *E. coli* challenge increased ($P < 0.05$) diarrhea scores for the entire experimental period except during the period from d 10 to 12 and increased ($P < 0.05$) the frequency of diarrhea (Table 6.8). The clay treatments in the *E. coli* challenged pigs reduced the diarrhea score ($P < 0.05$) in most time periods and the frequency of diarrhea ($P < 0.05$) for the entire experimental period. In addition, the clay treatments tended ($P = 0.06$) to reduce the diarrhea score in the sham group from d 7 to 9. Significant differences ($P < 0.05$) among the clay treatments were inconsistent over the time, but in general the treatments providing either Z or a combination of S and Z showed the least frequent diarrhea.

The populations of β-hemolytic coliforms increased ($P < 0.05$) markedly after the *E. coli* challenge, plateaued, and then declined ($P < 0.05$), but the number of these organisms in the sham group remaining low (Table 6.9). Among the *E. coli* challenged pigs, the clay treatments reduced ($P < 0.05$) the proportion of β-hemolytic coliforms on d 9 and tended ($P = 0.09$) to reduce it on d 12. There were no differences among the clay treatments.

The *E.coli* challenge increased ($P < 0.05$) the number of total WBC on d 6 and the clay treatments tended ($P = 0.07$) to reduce them (Table 6.10). There were no differences among the clay treatments. The *E. coli* challenge increased ($P < 0.05$) PCV and TP on d 6 (Table 6.11), but the clay treatments did not affect these measures of dehydration. There were no differences among the clay treatments.

In summary, the *E. coli* infection reduced growth performance and increased diarrhea score and frequency of diarrhea in 1 of 2 experiments. In both experiments, it increased populations of β-hemolytic coliforms and measures of dehydration and altered WBC populations. The clay treatments reduced the diarrhea score, the frequency of diarrhea, and the populations of β-hemolytic coliforms in both experiments.

DISCUSSION

The results from both experiments show clearly that clays reduce diarrhea in the face of an experimental challenge with enterotoxigenic *E. coli* as indicated by reductions in both diarrhea scores and frequency of diarrhea. These results are supported by observations reported by Papaioannou et al. (2004) showing clinoptilolite (natural zeolite) in the diet reduced diarrhea of weaned pigs in normal conditions. Other research also showed benefits of clays for humans suffering enteric diseases (Madkour et al., 1993; Chang et al., 2007; Dupont et al., 2009).

The effects of clays were not artifacts of the visual diarrhea scores, as shown by changes in more objective measures. For example, the proportions of β-hemolytic coliforms in feces were consistently lower in pigs fed clays. This finding is supported by observations reported by Trckova et al. (2009), who found that kaolinite in the diet reduced the colonization and shedding

of pathogenic *E. coli* after weaned pigs were experimentally infected with an enterotoxigenic F-18 *E. coli*.

The chemical structures (Bergaya and Lagaly, 2006; Williams et al., 2009) of clays provide ion exchange and/or adsorption properties (Carretero, 2002; Papaioannou et al., 2005; Tateo and Summa, 2007) consistent with several potential mechanisms by which clays may reduce diarrhea. First, clays may attract bacterial cells with enough physical force to tear cell membrane, resulting in lysis of the bacterial cells (Papaioannou et al., 2005; Williams et al., 2009). Second, clays may adsorb or detoxify bacterial toxins and protect intestinal permeability and damage by the toxins (Droy-Lefaix and Tateo, 2006; Williams and Haydel, 2010). Third, clays may adhere to gastrointestinal mucous membranes and reinforce the physical mucous barrier, resulting in some protection against enteric diseases caused by bacteria and/or toxins (Droy-Lefaix and Tateo, 2006; Tateo and Summa, 2007). Fourth, clays can absorb water and thus influence the presentation of diarrhea directly (Carretero, 2002; Carretero et al., 2006). Fifth, clays' ion exchange capacity may modify the characteristics of the intestinal environment, such as pH or oxidation state, influencing the growth of specific bacteria (Williams et al., 2008; Williams and Haydel, 2010).

Clays did not improve growth performance in either of our experiments. Previous reports of effects of clays on growth performance have been inconsistent, showing either improvements (Pond et al., 1988; Papaioannou et al., 2004; Alexopoulos et al., 2007) or no effects (Shurson et al., 1984; Ward et al., 1991; Parisini et al., 1999). Although clays can protect against enteric disease, they may also bind nutrients or exert other effects in the digestive tract that may be either beneficial or detrimental (Shurson et al., 1984; Pond et al., 1988). Clearly, more research is needed in this area.

The *E. coli* infection model was achieved successfully in both experiments as indicated by diarrhea scores, frequency of diarrhea, populations of β-hemolytic coliforms, and/or other measures. The *E. coli* infection was less severe in Exp. 1, maybe because of protective interventions.

A total of 19 of the 128 pigs in Exp. 2 died (6 pigs in the sham challenged group and 13 pigs in the *E. coli* challenged group), but they were spread across dietary treatments. Clinical signs included labored breathing and neurological disorders, and diagnostic examination confirmed pulmonary edema. These symptoms may have resulted from the Shiga-like toxin (SLT-2) produced by the challenge organism. In addition, it was routinely confirmed sows were negative for fecal β-hemolytic coliforms before their pigs were used in experiments. However, typically β-hemolytic coliforms are found in feces of the sham challenged pigs (or pigs before challenge) in experiments of this type, perhaps because the sows harbor low, undetectable, levels of the organisms and the stresses of weaning and transport allow those strains to proliferate in the pigs. Exp. 1 is unusual in our experience in showing no β-hemolytic coliforms in feces of the sham challenged pigs.

In conclusion, clays alleviated diarrhea of weaned pigs experimentally infected by a pathogenic *E. coli*, as shown by reduction of diarrhea scores, frequency of diarrhea, and fecal β-hemolytic coliforms. We did not find clear differences in protection against diarrhea among the 3 different clays we tested, suggesting that all 3 of them may be beneficial. The responses to the clay treatments in both experiments provide strong confidence that the clay products can be useful in maintaining health of pigs, especially prevention of diarrhea. Therefore, it will be important to understand the mechanisms through which clays exert these effects.

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Table 6.1. Ingredient composition of basal diet in Exp. 1 and 2 (as-fed basis)

Item	Basal diet
Ingredient, %	
Corn	40.93
Dried whey	20.00
Soybean meal, 47%	10.00
Fishmeal	10.00
Lactose	7.22
Soy protein concentrate	5.00
Poultry byproduct meal	3.22
Soybean oil	2.92
Mineral premix[1]	0.35
Vitamin premix[2]	0.20
L-Lysine·HCl	0.06
DL-Methionine	0.05
L-Threonine	0.03
L-Tryptophan	0.02

Table 6.1. (cont.)

Item	Basal diet
Calculated energy and nutrient levels	
Energy, kcal ME/kg	3480
Crude protein, %	22.53
Fat, %	6.48
Calcium, %	0.80
Phosphorus, %	0.73
Available phosphorus, %	0.51
Lysine, %	1.50
Lactose, %	21.00

[1]Provided as milligrams per kilogram of diet: sodium chloride, 3,000; zinc, 100 from zinc oxide; iron, 90 from iron sulfate; manganese, 20 from manganese oxide; copper, 8 from copper sulfate; iodine, 0.35 from calcium iodide; selenium, 0.30 from sodium selenite.

[2]Provided per kilogram of diet: retinyl acetate, 2,273 μg; cholecalciferol, 17 μg; DL-α-tocopheryl acetate, 88 mg; menadione sodium bisulfite complex, 4 mg; niacin, 33 mg; D-Ca-pantothenate, 24 mg; riboflavin, 9 mg; vitamin B_{12}, 35 μg; choline chloride, 324 mg.

Table 6.2. Effect of smectite on growth performance of pigs challenged with a pathogenic *E. coli*[1] (Exp. 1)

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Effect[3]			CON vs. S[4]	
Item	CON	0.3S	0.6S	B/0.3S	CON	0.3S	0.6S	B/0.3S	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 0 to 6														
ADG, g/d	398	323	307	370	410	322	313	370	35	0.85	<0.05	0.99	0.08	<0.05
ADFI, g/d	752	627	533	577	565	520	492	563	65	0.06	0.17	0.54	<0.05	0.59
G:F	0.57	0.56	0.59	0.70	0.75	0.62	0.62	0.70	0.08	0.19	0.44	0.65	0.59	0.24
d 6 to 12														
ADG, g/d	489	578	558	606	525	531	567	594	46	0.76	0.32	0.30	<0.05	0.82
ADFI, g/d	1264	1053	1014	936	1067	1053	939	1128	103	0.78	0.33	0.30	<0.05	0.82
G:F	0.40	0.61	0.56	0.65	0.52	0.56	0.61	0.54	0.06	0.92	0.12	0.26	<0.05	0.53
d 0 to 12														
ADG, g/d	448	450	435	488	467	427	440	482	31	0.95	0.36	0.90	0.75	0.62
ADFI, g/d	1008	843	775	748	815	787	715	847	74	0.31	0.16	0.27	<0.05	0.70
G:F	0.44	0.58	0.56	0.67	0.60	0.58	0.61	0.58	0.05	0.45	0.29	0.14	<0.05	0.83

[1]n = 48 (6 pigs/ treatment).

Table 6.2. (cont.)

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = basal diet throughout the experiment; 0.3S = basal diet plus 3 kg smectite/ ton throughout the experiment; 0.6S = basal diet plus 6 kg smectite/ ton throughout the experiment; B/0.3S = basal diet until challenge, then basal diet plus 3 kg smectitie/ ton.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and the 3 smectite treatments within challenge treatments.

Table 6.3. Effect of smectite on diarrhea score of pigs challenged with a pathogenic *E. coli*[1] (Exp. 1)

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Effect[3]			CON vs. S[4]	
Item	**CON**	**0.3S**	**0.6S**	**B/0.3S**	**CON**	**0.3S**	**0.6S**	**B/0.3S**	**SEM**	***E.coli***	**Diet**	**E*D**	**Sham**	***E. coli***
d 0 to 2[5]	1.00	1.17	1.00	1.00	1.00	1.17	1.00	1.00	0.08	1.00	0.13	1.00	0.57	0.57
d 3 to 6	2.33	1.46	1.38	1.83	2.21	1.71	2.17	1.83	0.21	0.13	<0.05	0.15	<0.05	0.21
d 7 to 9	2.33	2.00	2.11	2.11	2.50	2.00	2.11	2.06	0.14	0.79	<0.05	0.88	0.13	<0.05
d 10 to 12	2.17	2.06	2.06	2.11	2.28	2.00	2.06	2.00	0.07	0.78	<0.05	0.42	0.25	<0.05
d 0 to 12	1.99	1.66	1.62	1.77	2.01	1.72	1.86	1.73	0.08	0.18	<0.05	0.30	<0.05	<0.05
Pig days[6]	72	72	72	72	72	72	72	72						
Diarrhea days[7]	17	5	4	10	13	3	9	4						
Frequency, %[8]	24	7	6	14	18	4	13	6	-	0.36	<0.05	<0.05	<0.05	<0.05

[1]n = 48 (6 pigs/ treatment).

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = basal diet throughout the experiment; 0.3S = basal diet plus 3 kg smectite/ ton throughout the experiment; 0.6S = basal diet plus 6 kg smectite/ ton throughout the experiment; B/0.3S = basal diet until challenge, then basal diet plus 3 kg smectitie/ ton.

Table. 6.3. (cont.)

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and the 3 smectite treatments within challenge treatments.

[5]Diarrhea score = 1, normal feces, 2, moist feces, 3, mild diarrhea, 4, severe diarrhea, 5, watery diarrhea.

[6]Pig days = number of pigs x the number of days of diarrhea scoring.

[7]Diarrhea days = number of pig days with diarrhea score ≥ 3. Statistical analysis was conducted by chi-square test.

[8]Frequency (frequency of diarrhea during the entire experimental period) = diarrhea*100 / pig days.

Table 6.4. Effect of smectite on culture score of feces from pigs challenged with a pathogenic *E. coli*[1] (Exp. 1)

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Effect[3]			CON vs. S[4]	
Item	CON	0.3S	0.6S	B/0.3S	CON	0.3S	0.6S	B/0.3S	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 0														
Coliform[5,6]	6.7	6.0	6.8	6.7	5.8	5.8	6.2	6.7	0.28	<0.05	0.06	0.41	0.61	0.24
β-hemolytic[5,6]	0	0	0	0	0	0	0	0	-	-	-	-	-	-
β-hemo., ratio[5,6]	0	0	0	0	0	0	0	0	-	-	-	-	-	-
d 3														
Coliform	4.3	3.2	4.7	3.8	6.0	5.0	5.8	4.7	0.55	<0.05	0.08	0.79	0.49	0.20
β-hemolytic	0	0	0	0	5.5	4.7	5.0	4.2	0.50	<0.05	0.38	0.38	-	<0.05
β-hemo., ratio	0	0	0	0	0.91	0.93	0.86	0.89	0.05	<0.05	0.92	0.92	-	0.86
d 6														
Coliform	3.0	2.5	2.3	2.5	4.2	4.3	3.7	4.2	0.70	<0.05	0.83	0.95	0.46	0.88
β-hemolytic	0	0	0	0	3.5	2.8	2.8	2.8	0.65	<0.05	0.94	0.94	-	0.38
β-hemo., ratio	0	0	0	0	0.87	0.58	0.60	0.62	0.11	<0.05	0.47	0.47	-	<0.05

Table 6.4. (cont.)

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Effect[3]			CON vs. S[4]	
Item	**CON**	**0.3S**	**0.6S**	**B/0.3S**	**CON**	**0.3S**	**0.6S**	**B/0.3S**	**SEM**	***E.coli***	**Diet**	**E*D**	**Sham**	***E. coli***
d 9														
Coliform	3.5	2.3	4.3	2.0	3.2	4.8	3.5	5.2	0.73	<0.05	0.87	<0.05	0.45	0.11
β-hemolytic	0	0	0	0	0.8	0.5	0.7	0.8	0.26	<0.05	0.91	0.91	-	0.59
β-hemo., ratio	0	0	0	0	0.28	0.09	0.16	0.16	0.07	<0.05	0.60	0.60	-	0.08
d 12														
Coliform	4.3	3.7	2.8	3.0	4.0	5.5	2.0	4.5	0.66	0.22	<0.05	<0.05	0.11	1.00
β-hemolytic	0	0	0	0	0.8	0.2	0.7	0.7	0.32	<0.05	0.73	0.73	-	0.37
β-hemo., ratio	0	0	0	0	0.24	0.03	0.21	0.14	0.09	<0.05	0.66	0.66	-	0.29

[1]n = 48 (6 pigs/ treatment).

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = basal diet throughout the experiment; 0.3S = basal diet plus 3 kg smectite/ ton throughout the experiment; 0.6S = basal diet plus 6 kg smectite/ ton throughout the experiment; B/0.3S = basal diet until challenge, then basal diet plus 3 kg smectitie/ ton.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

Table 6.4. (cont.)

[4]Contrast between CON and the 3 smectite treatments within challenge treatments.

[5]Score of bacterial growth = 0, none, 1, rare, 2, a few, 3, light, 4, very light, 5, moderate, 6, very moderate, 7, heavy, 8, very heavy.

[6]Coliform = total coliforms; β-hemolytic = β-hemolytic coliforms; β-hemo., ratio = ratio of β-hemolytic coliforms score to total coliforms score.

Table 6.5. Effect of smectite on total and differential white blood cells of pigs challenged with a pathogenic *E. coli*[1] (Exp. 1)

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Effect[3]			CON vs. S[4]	
Item	CON	0.3S	0.6S	B/0.3S	CON	0.3S	0.6S	B/0.3S	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 0														
WBC, x10^3/uL[5]	11.1	10.4	12.5	10.1	9.3	12.3	9.7	10.5	1.32	0.51	0.73	0.24	0.94	0.29
Neu, %[5]	55.0	38.8	51.0	49.3	47.7	53.2	49.2	53.0	5.16	0.54	0.70	0.20	0.15	0.49
Lym, %[5]	41.8	55.7	44.7	48.3	47.8	44.3	47.5	45.0	4.91	0.67	0.74	0.30	0.17	0.69
Mono, %[5]	2.8	3.7	3.5	2.0	3.0	2.0	3.2	1.2	0.86	0.28	0.23	0.75	0.83	0.38
Eos, %[5]	0.3	0.8	0.2	0.3	1.0	0.3	0.2	0.5	0.29	0.69	0.34	0.26	0.74	<0.05
Baso, %[5]	0	0.2	0.2	0	0.5	0	0	0.3	0.16	0.25	0.65	0.07	0.53	<0.05
d 6														
WBC, x10^3/uL	14.6	15.6	15.4	13.9	17.5	16.8	15.7	17.1	1.53	0.08	0.95	0.72	0.83	0.59
Neu, %	44.5	34.0	37.2	45.2	39.2	47.3	51.3	44.7	4.46	0.08	0.73	0.07	0.26	0.09
Lym, %	51.0	61.3	57.7	52.0	57.5	49.2	44.7	52.5	4.52	0.13	0.76	0.07	0.22	0.08
Mono, %	2.7	4.0	3.7	1.7	2.67	3.00	3.17	1.50	0.83	0.48	0.10	0.93	0.65	0.91
Eos, %	1.2	0.3	0.8	0.7	0.7	0.5	0.5	1.3	0.38	1.00	0.36	0.36	0.18	0.78
Baso, %	0	0.2	0.2	0	0	0	0	0	0.08	0.14	0.51	0.51	0.22	1.00

Table 6.5. (cont.)

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Effect[3]			CON vs. S[4]	
Item	**CON**	**0.3S**	**0.6S**	**B/0.3S**	**CON**	**0.3S**	**0.6S**	**B/0.3S**	**SEM**	***E.coli***	**Diet**	**E*D**	**Sham**	***E. coli***
d 12														
WBC, x10^3/uL	21.2	18.2	19.5	17.8	17.9	17.8	17.9	14.7	1.74	0.09	0.26	0.83	0.18	0.56
Neu, %	55.0	43.8	34.5	44.0	49.5	46.7	37.0	42.2	5.12	0.89	<0.05	0.83	<0.05	0.21
Lym, %	39.7	50.8	59.5	51.7	45.0	48.3	57.2	52.7	5.16	0.92	<0.05	0.86	<0.05	0.20
Mono, %	3.2	3.8	2.8	2.7	2.5	3.5	4.2	3.5	0.92	0.65	0.79	0.65	0.96	0.25
Eos, %	1.2	1.0	2.7	1.3	1.7	1.5	1.5	1.5	0.55	1.00	0.36	0.30	0.39	0.77
Baso, %	0	0	0.5	0	0.3	0	0	0.3	0.13	0.66	0.30	<0.01	0.28	0.15

[1]n = 48 (6 pigs/ treatment).

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = basal diet throughout the experiment; 0.3S = basal diet plus 3 kg smectite/ ton throughout the experiment; 0.6S = basal diet plus 6 kg smectite/ ton throughout the experiment; B/0.3S = basal diet until challenge, then basal diet plus 3 kg smectitie/ ton.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and the 3 smectite treatments within challenge treatments.

[5]WBC = white blood cell; Neu = neutrophil; Lym = lymphocyte; Mono = monocyte; Eos = eosinophil; Baso = basophil.

Table 6.6. Effect of smectite on packed cell volume and total protein of pigs challenged with a pathogenic *E. coli*[1] (Exp. 1)

	Treatment[2]								*P*-value					
	Sham				*E. coli*					Effect[3]			CON vs. S[4]	
Item	CON	0.3S	0.6S	B/0.3S	CON	0.3S	0.6S	B/0.3S	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 0														
PCV, %[5]	34.9	33.7	34.3	33.2	35.8	36.1	35.1	34.6	1.35	0.13	0.72	0.92	0.43	0.72
TP, g/dL[5]	4.6	4.9	4.4	4.7	4.7	4.7	4.7	4.9	0.13	0.39	0.14	0.47	0.42	0.50
d 6														
PCV, %	37.0	37.2	37.2	35.8	39.5	38.6	39.4	38.8	0.83	<0.05	0.60	0.81	0.78	0.54
TP, g/dL	4.2	4.6	4.3	4.3	4.4	4.4	4.5	4.5	0.13	0.29	0.48	0.25	0.19	0.54
d 12														
PCV, %	39.6	37.8	38.8	38.4	40.1	40.0	39.1	40.2	0.90	0.07	0.68	0.68	0.23	0.70
TP, g/dL	4.4	4.5	4.2	4.4	4.4	4.3	4.4	4.5	0.12	0.88	0.26	0.48	0.81	0.97

[1]n = 48 (6 pigs/ treatment).

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = basal diet throughout the experiment; 0.3S = basal diet plus 3 kg smectite/ ton throughout the experiment; 0.6S = basal diet plus 6 kg smectite/ ton throughout the experiment; B/0.3S = basal diet until challenge, then basal diet plus 3 kg smectitie/ ton.

Table 6.6. (cont.)

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and the 3 smectite treatments within challenge treatments.

[5]PCV = packed cell volume; TP = total protein.

Table 6.7. Effect of different clays on growth performance of pigs challenged with a pathogenic *E. coli* (Exp. 2)

	Treatment[1]																	*P*-value				
	Sham								*E. coli*									Effect[2]			CON vs. Clays[3]	
Item	CON	S	K	Z	SK	SZ	KZ	SKZ	CON	S	K	Z	SK	SZ[4]	KZ	SKZ	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 0 to 6																						
No. of pigs[5]	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	8						
No gain[6]	0	0	0	0	0	0	0	0	0	0	2	1	0	0	1	1						
ADG, g/d	208	200	233	258	227	240	258	196	117	110	67	127	121	141	88	169	38	<0.05	0.93	0.60	0.58	0.99
ADFI, g/d	546	519	735	588	735	625	617	694	577	560	556	519	492	529	475	592	69	<0.05	0.51	0.34	0.15	0.51
G:F[7]	0.41	0.41	0.37	0.45	0.39	0.44	0.48	0.37	-	-	-	-	-	-	-	-	0.11	-	0.84	-	0.96	-
d 6 to 12																						
No. of pigs[5]	7	7	7	7	8	7	7	8	6	7	6	7	6	5	6	7						
No gain[6]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
ADG, g/d	424	417	424	507	442	395	457	425	350	336	331	395	328	283	431	314	73	<0.05	0.68	0.99	0.83	0.95
ADFI,g/d	867	811	1017	929	973	1052	1052	948	886	800	925	910	897	713	781	991	92	<0.05	0.46	0.19	0.22	0.77
G:F	0.49	0.54	0.43	0.56	0.46	0.40	0.48	0.47	0.42	0.41	0.37	0.44	0.36	0.41	0.55	0.34	0.09	0.08	0.67	0.88	0.86	0.91
d 0 to 12																						
No. of pigs[5]	7	7	7	7	8	7	7	8	6	7	6	7	6	5	6	7						
No gain[6]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0						
ADG, g/d	317	317	341	389	334	323	360	310	238	227	215	257	221	200	269	250	50	<0.05	0.84	0.99	0.62	0.95
ADFI, g/d	711	654	871	752	854	825	819	821	764	691	750	713	694	617	638	789	75	<0.05	0.40	0.34	0.19	0.38
G:F	0.46	0.49	0.42	0.51	0.42	0.42	0.49	0.42	0.34	0.34	0.28	0.36	0.32	0.33	0.42	0.34	0.08	<0.05	0.80	0.99	0.94	0.95

Table 6.7. (cont.)

[1]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = basal diet; S = 0.3% smectite; K = 0.3% kaolinite; Z = 0.3% zeolite; SK = 0.15% smectite + 0.15% kaolinite; SZ = 0.15% smectite + 0.15% zeolite; KZ = 0.15% kaolinite + 0.15% zeolite; SKZ = 0.1% smectite + 0.1% kaolinite + 0.1% zeolite.

[2]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[3]Contrast between control and all clay treatments within challenge treatments.

[4]Data from one pig in treatment SZ of *E. coli* group were not used because its growth rate was detected as an outlier by statistical analysis.

[5]No. of pigs = number of live pigs.

[6]No gain = number of pigs which did not gain BW.

[7]G:F from d 0 to 6 for *E. coli* group are not presented because several pigs lost BW, making the ratio meaningless.

Table 6.8. Effect of different clays on diarrhea score of pigs challenged with a pathogenic *E. coli* (Exp. 2)

	Treatment[1]																	*P*-value				
	Sham								*E. coli*									Effect[2]			CON vs. Clays[3]	
Item	**CON**	**S**	**K**	**Z**	**SK**	**SZ**	**KZ**	**SKZ**	**CON**	**S**	**K**	**Z**	**SK**	**SZ[4]**	**KZ**	**SKZ**	**SEM**	***E.coli***	**Diet**	**E*D**	**Sham**	***E. coli***
No. of pigs	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	8						
d 0 to 2[5]	1.00	1.00	1.06	1.06	1.06	1.00	1.06	1.00	3.46	1.63^{ab}	1.75^{ab}	1.56^{ab}	2.13^{a}	1.43^{b}	1.46^{b}	1.50^{b}	0.22	<0.05	<0.05	<0.05	0.87	<0.05
No. of pigs	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	8						
d 3 to 6	1.49	1.38	1.25	1.17	1.38	1.00	1.09	1.21	3.52	2.39^{abc}	2.50^{ab}	1.86^{c}	2.03^{bc}	1.89^{bc}	2.64^{a}	2.30^{abc}	0.24	<0.05	<0.05	<0.05	0.22	<0.05
No. of pigs[6]	8	7	7	7	8	8	8	8	7	7	7	7	7	5	6	7						
d 7 to 9	1.73	1.64	1.19	1.31	1.29	1.46	1.15	1.33	3.23	1.81^{ab}	2.07^{a}	1.27^{b}	1.43^{b}	1.50^{ab}	2.04^{a}	1.40^{b}	0.24	<0.05	<0.05	<0.05	0.06	<0.05
No. of pigs[6]	7	7	7	7	8	7	7	8	6	7	6	7	6	5	6	7						
d 10 to 12	1.69	1.43	1.69	1.50	1.52	1.43	1.45	1.58	1.61	1.31	1.45	1.14	1.06	1.10	1.47	1.14	0.21	<0.05	0.33	0.83	0.35	0.07
No. of pigs[6]	7	7	7	7	8	7	7	8	6	7	6	7	6	5	6	7						
d 0 to 12[7]	1.47	1.34	1.21	1.26	1.33	1.23	1.20	1.29	3.00	1.74^{ab}	1.78^{ab}	1.50^{ab}	1.46^{b}	1.45^{b}	1.88^{a}	1.60^{ab}	0.15	<0.05	<0.05	<0.05	0.15	<0.05
Pigs days[8]	95	92	92	92	96	94	94	96	87	90	90	91	89	75	87	92						
Diarrhea[9]	7	7	8	4	6	3	3	4	62	24	25	11	18	9	25	18						
Freq., %[10]	7	8	9	4	6	3	3	4	71	27^{a}	28^{a}	12^{b}	20^{ab}	12^{b}	29^{a}	20^{ab}	-	<0.05	<0.05	<0.05	0.45	<0.05

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

[1]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = basal diet; S = 0.3% smectite; K = 0.3% kaolinite; Z = 0.3% zeolite; SK = 0.15% smectite + 0.15% kaolinite; SZ = 0.15% smectite + 0.15% zeolite; KZ = 0.15% kaolinite + 0.15% zeolite; SKZ = 0.1% smectite + 0.1% kaolinite + 0.1% zeolite.

Table 6.8. (cont.)

[2]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[3]Contrast between control and all clay treatments within challenge treatments.

[4]Data from one pig in treatment SZ of *E. coli* group was not used because growth rate data was detected as an outlier by statistical analysis.

[5]Diarrhea score = 1, normal feces, 2, moist feces, 3, mild diarrhea, 4, severe diarrhea, 5, watery diarrhea.

[6]No. of pigs = number of live pigs.

[7]The average of diarrhea scores from d 0 to 12 was calculated using only pigs that survived until d 12.

[8]Pig days = number of pigs x the number of days of diarrhea scoring.

[9]Diarrhea = number of pig days with diarrhea score ≥ 3. Statistical analysis was conducted by chi-square test.

[10]Freq. (frequency of diarrhea during the entire experimental period) = diarrhea*100 / pig days.

Table 6.9. Effect of different clays on culture score of feces from pigs challenged with a pathogenic *E. coli* (Exp. 2)

	Treatment[1]																	*P*-value				
	Sham								*E. coli*									Effect[2]			CON vs. Clays[3]	
Item	CON	S	K	Z	SK	SZ	KZ	SKZ	CON	S	K	Z	SK	SZ[4]	KZ	SKZ	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 0																						
No. of pigs	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	8						
Coliform[5,6]	6.4	6.8	6.0	6.0	6.3	6.4	7.0	7.0	6.6	6.0	7.0	5.1	6.0	6.1	6.5	5.1	0.57	0.13	0.50	0.29	0.85	0.27
β-hemolytic[5,6]	0	0	0	0	0.9	0	0	0	0	0	0	0	0	0	0	0	0.18	0.18	0.08	0.08	0.47	0.99
β-hemo., ratio[5,6]	0	0	0	0	0.15	0	0	0	0	0	0	0	0	0	0	0	0.03	0.16	0.06	0.06	0.45	0.99
d 3																						
No. of pigs	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	8						
Coliform	4.8	5.9	5.6	5.6	6.6	5.6	6.5	5.6	7.6	7.9	7.6	7.4	7.6	7.6	7.4	7.6	0.44	<0.05	0.44	0.33	<0.05	0.92
β-hemolytic	0.8	0.6	1.0	0.1	2.0	0	0	0.5	7.6	7.9	7.6	7.4	7.6	7.6	7.4	7.6	0.58	<0.05	0.40	0.64	0.79	0.95
β-hemo., ratio	0.13	0.13	0.13	0.02	0.25	0	0	0.08	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.09	<0.05	0.65	0.63	0.60	0.99
d 6																						
No. of pigs	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	8						
Coliform	6.1	6.5	5.9	5.6	6.5	5.8	6.5	6.0	7.3	7.5	7.8	7.6	7.8	7.4	7.6	7.3	0.57	<0.05	0.94	0.97	0.98	0.59
β-hemolytic	1.8	1.9	1.0	1.4	2.0	1.0	0.9	1.0	7.3	7.3	7.4	7.3	7.6	6.8	6.5	6.9	1.08	<0.05	0.88	0.99	0.61	0.86
β-hemo., ratio	0.25	0.23	0.13	0.19	0.25	0.13	0.13	0.13	1.00	0.97	0.95	0.95	0.98	0.89	0.86	0.94	0.14	<0.05	0.86	0.99	0.45	0.56
d 9																						
No. of pigs[7]	8	7	7	7	8	8	8	8	7	7	7	7	7	5	6	7						
Coliform	6.8	6.4	5.8	6.3	6.3	6.8	6.9	6.0	6.3	7.1	7.3	6.0	6.9	6.8	7.0	6.1	0.75	0.33	0.86	0.81	0.51	0.50
β-hemolytic	2.0	2.1	1.1	1.3	1.9	3.9	1.0	1.9	5.0	3.3	3.0	0.9	3.2	2.8	2.1	1.3	0.83	0.15	0.31	0.62	0.91	<0.05
β-hemo., ratio	0.25	0.26	0.16	0.16	0.25	0.50	0.13	0.23	0.76	0.43	0.40	0.13	0.42	0.40	0.27	0.22	0.20	0.06	0.18	0.50	0.95	<0.05

Table 6.9. (cont.)

	Treatment[1]																	*P*-value				
	Sham								*E. coli*									Effect[2]			CON vs. Clays[3]	
Item	CON	S	K	Z	SK	SZ	KZ	SKZ	CON	S	K	Z	SK	SZ[4]	KZ	SKZ	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 12																						
No. of pigs[7]	7	7	7	7	8	7	7	8	6	7	6	7	6	5	6	7						
Coliform	5.7	5.7	5.7	4.6	4.3	5.9	5.6	5.9	5.2	6.7	5.5	5.7	5.5	6.6	7.2	4.8	0.93	0.26	0.44	0.56	0.68	0.38
β-hemolytic	1.5	1.9	1.7	1.3	1.3	2.0	1.7	3.4	2.3	1.1	1.2	0.5	0.6	0.6	1.2	0.5	1.20	0.09	0.95	0.80	0.67	0.19
β-hemo., ratio	0.19	0.27	0.22	0.18	0.22	0.29	0.25	0.43	0.39	0.15	0.15	0.09	0.09	0.09	0.18	0.14	0.16	0.17	0.93	0.81	0.60	0.09

[1]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = basal diet; S = 0.3% smectite; K = 0.3% kaolinite; Z = 0.3% zeolite; SK = 0.15% smectite + 0.15% kaolinite; SZ = 0.15% smectite + 0.15% zeolite; KZ = 0.15% kaolinite + 0.15% zeolite; SKZ = 0.1% smectite + 0.1% kaolinite + 0.1% zeolite.

[2]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[3]Contrast between control and all clay treatments within challenge treatments.

[4]Data from one pig in treatment SZ of *E. coli* group were not used because its growth rate was detected as an outlier.

[5]Score of bacterial growth = 0, none, 1, rare, 2, a few, 3, light, 4, very light, 5, moderate, 6, very moderate, 7, heavy, 8, very heavy.

[6]Coliform = total coliforms; β-hemolytic = β-hemolytic coliforms; β-hemo., ratio = ratio of β-hemolytic coliforms score to total coliforms score.

[7]No. of pigs = number of live pigs.

Table 6.10. Effect of different clays on total and differential white blood cell of pigs challenged with a pathogenic *E. coli* (Exp. 2)

	Treatment[1]																	*P*-value				
	Sham								*E. coli*									Effect[2]			CON vs. Clays[3]	
Item	CON	S	K	Z	SK	SZ	KZ	SKZ	CON	S	K	Z	SK	SZ[4]	KZ	SKZ	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 0																						
No. of pigs	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	8						
WBC, x10^3/uL[5]	10.0	7.4	8.8	10.0	7.5	7.1	8.4	9.6	6.4	10.8	7.4	9.1	7.9	6.7	6.1	8.1	1.13	0.12	0.12	0.05	0.14	0.16
Neu, %[5]	51.5	39.1	47.1	47.4	44.6	46.2	40.1	48.9	46.0	55.5	43.2	54.8	48.6	41.6	46.6	46.4	4.33	0.27	0.58	0.10	0.11	0.64
Lym, %[5]	43.6	56.8	49.0	47.0	51.0	47.4	55.0	46.6	50.9	39.8	51.5	42.9	48.6	55.8	49.9	49.9	4.32	0.67	0.66	0.07	0.12	0.58
Mono, %[5]	4.5	3.5	3.8	4.9	3.0	5.3	3.9	3.9	2.4	3.9	5.0	2.0	2.5	2.6	3.5	3.4	0.92	<0.05	0.81	0.22	0.61	0.40
Eos, %[5]	0.4	0.8	0.1	0.3	1.0	0.4	0.4	0.1	0.3	0.4	0.1	0.3	0.1	0	0	0.4	0.23	<0.05	0.31	0.30	0.78	0.68
Baso, %[5]	0	0	0	0.3	0.3	0.3	0	0.4	0	0.1	0.1	0.1	0	0	0	0	0.12	0.07	0.44	0.13	0.14	0.65
d 6																						
No. of pigs	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	8						
WBC, x10^3/uL	12.6	12.5	13.3	13.8	11.7	12.2	13.0	12.1	17.7	15.2	15.9	15.7	15.2	14.7	15.0	14.6	1.36	<0.05	0.78	0.94	0.98	0.07
Neu, %	46.0	42.0	42.6	44.8	36.5	42.1	41.0	43.9	41.1	42.1	45.3	47.3	45.1	46.4	43.8	45.8	3.58	0.19	0.84	0.72	0.25	0.27
Lym, %	47.6	53.4	52.0	49.3	57.6	50.0	52.6	47.5	53.1	51.9	51.0	47.3	49.0	48.6	51.6	49.3	3.74	0.56	0.74	0.72	0.27	0.38
Mono, %	4.9	4.2	4.1	4.6	5.1	7.4	5.1	6.9	5.0	4.9	3.4	4.9	5.0	4.8	4.4	3.9	1.13	0.11	0.48	0.46	0.65	0.59
Eos, %	0.9	0.4	1.3	1.3	0.8	0.8	1.3	0.8	0.5	1.0	0.4	0.5	0.8	0.1	0.1	1.1	0.37	<0.05	0.91	0.17	0.90	0.82
Baso, %	0.3	0	0	0	0	0	0	0.8	0	0.1	0	0.1	0	0	0.1	0	0.11	0.15	<0.05	<0.05	0.21	0.64

Table 6.10. (cont.)

	Treatment[1]																	*P*-value				
	Sham								*E. coli*									Effect[2]			CON vs. Clays[3]	
Item	CON	S	K	Z	SK	SZ	KZ	SKZ	CON	S	K	Z	SK	SZ[4]	KZ	SKZ	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 12																						
No. of pigs[6]	7	7	7	7	8	7	7	8	6	7	6	7	6	5	6	7						
WBC, x10^3/uL	16.6	17.0	17.7	15.2	14.6	17.1	12.1	13.5	18.4	18.4	17.5	18.4	17.1	15.0	15.4	15.4	2.24	0.14	0.36	0.90	0.53	0.48
Neu, %	43.5	40.2	48.7	49.0	44.6	46.8	45.0	40.6	54.1	51.0	53.0	54.1	46.9	42.0	41.6	47.2	5.58	0.10	0.49	0.67	0.77	0.28
Lym, %	46.4	51.3	43.8	43.8	47.8	43.6	47.6	49.0	42.2	44.0	40.8	40.8	48.0	53.4	53.0	48.0	5.80	0.87	0.58	0.72	0.95	0.41
Mono, %	8.1	7.3	6.3	5.7	5.8	7.5	6.4	8.6	3.6	4.3	5.2	4.5	4.6	3.8	5.3	3.9	2.18	<0.05	0.99	0.91	0.49	0.68
Eos, %	1.9	1.1	1.3	1.4	1.5	1.6	0.8	1.8	0.2	0.3	0.7	0.6	0.5	0.6	0.8	0.9	0.47	<0.05	0.91	0.79	0.24	0.40
Baso, %	0.1	0.1	0	0.1	0.3	0.1	0.2	0	0	0.3	0.3	0	0.2	0.4	0	0	0.16	0.76	0.48	0.38	0.90	0.29

[1]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = basal diet; S = 0.3% smectite; K = 0.3% kaolinite; Z = 0.3% zeolite; SK = 0.15% smectite + 0.15% kaolinite; SZ = 0.15% smectite + 0.15% zeolite; KZ = 0.15% kaolinite + 0.15% zeolite; SKZ = 0.1% smectite + 0.1% kaolinite + 0.1% zeolite.

[2]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[3]Contrast between control and all clay treatments within challenge treatments.

[4]Data from one pig in treatment SZ of *E. coli* group were not used because its growth rate was detected as an outlier by statistical analysis.

[5]WBC = white blood cell; Neu = neutrophil; Lym = lymphocyte; Mono = monocyte; Eos = eosinophil; Baso = basophil.

[6]No. of pigs = number of live pigs.

Table 6.11. Effect of different clays on packed cell volume and total protein of pigs challenged with a pathogenic *E. coli* (Exp. 2)

	Treatment[1]																*P*-value					
	Sham								*E. coli*									Effect[2]			CON vs. Clays[3]	
Item	**CON**	**S**	**K**	**Z**	**SK**	**SZ**	**KZ**	**SKZ**	**CON**	**S**	**K**	**Z**	**SK**	**SZ[4]**	**KZ**	**SKZ**	**SEM**	***E.coli***	**Diet**	**E*D**	**Sham**	***E. coli***
d 0																						
No. of pigs	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	8						
PCV, %[5]	33.1	32.0	32.3	35.0	32.2	35.1	35.6	35.0	32.4	33.1	33.1	34.3	34.9	33.9	34.4	33.8	1.51	0.94	0.46	0.83	0.61	0.33
TP, g/dL[5]	5.0	4.9	5.0	4.9	5.0	4.9	5.0	5.0	4.8	4.8	5.1	4.9	5.1	5.0	5.1	5.3	0.14	0.12	0.56	0.81	0.96	0.06
d 6																						
No. of pigs	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	8						
PCV, %	34.6	33.7	35.7	33.7	33.7	33.9	35.6	34.6	35.3	36.0	34.8	37.2	35.6	36.9	36.4	34.9	1.59	<0.05	0.97	0.70	0.88	0.60
TP, g/dL	4.7	4.6	4.8	4.6	4.6	4.6	4.5	4.6	4.7	4.9	5.2	4.8	4.8	4.9	5.1	4.7	0.16	<0.05	0.32	0.43	0.38	0.12
d 12																						
No. of pigs[6]	7	7	7	7	8	7	7	8	6	7	6	7	6	5	6	7						
PCV, %	36.5	35.3	37.3	38.2	35.6	36.5	35.1	36.3	35.0	32.6	33.1	33.8	32.1	34.2	34.1	33.9	2.10	<0.05	0.89	0.98	0.95	0.44
TP, g/dL	4.7	4.5	4.5	4.7	4.5	4.7	4.5	4.5	4.5	4.7	4.7	4.5	4.6	4.5	4.6	4.4	0.16	0.73	0.96	0.67	0.50	0.51

[1]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = basal diet; S = 0.3% smectite; K = 0.3% kaolinite; Z = 0.3% zeolite; SK = 0.15% smectite + 0.15% kaolinite; SZ = 0.15% smectite + 0.15% zeolite; KZ = 0.15% kaolinite + 0.15% zeolite; SKZ = 0.1% smectite + 0.1% kaolinite + 0.1% zeolite.

[2]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[3]Contrast between control and all clay treatments within challenge treatments.

Table 6.11. (cont.)

[4]Data from one pig in treatment SZ of *E. coli* group were not used because its growth rate was detected as an outlier by statistical analysis.

[5]PCV = packed cell volume; TP = total protein.

[6]No. of pigs = number of live pigs.

CHAPTER 7

EFFECTS OF DIETARY SPRAY-DRIED EGG ON GROWTH PERFORMANCE AND HEALTH OF WEANED PIGS

ABSTRACT

Four experiments were conducted to evaluate the nutrient contributions and physiological health benefits of spray-dried egg (SDE) containing only unfertilized eggs as a protein source in nursery pig diets. In all experiments, each pen within a block (BW x sex) housed the same number of barrows and gilts and dietary treatments were formulated to the same ME and standardized ileal digestible lysine levels. In Exp. 1 and 2 (168 and 140 pigs, respectively, 5 kg BW, 16 d old; 14 replicates/experiment) conducted in a university farm, treatments were with or without 5% SDE in a nursery control diet including antibiotics and zinc oxide. Pigs were fed for 10 d after weaning to measure ADG, ADFI, and G:F. The SDE increased ($P < 0.05$) ADG (Exp. 1: 243 vs. 204 g/d; Exp. 2: 204 vs. 181 g/d) and ADFI (Exp. 1: 236 vs. 204 g/d; Exp. 2: 263 vs. 253 g/d) compared with the control diet, but did not affect G:F. In Exp. 3 (1008 pigs, 5.2 kg BW, 20 d old; 12 replicates) conducted in a commercial farm, treatments were in a factorial arrangement (with or without SDE and high or low level of spray-dried plasma (SDP) in a nursery control diet including antibiotics and zinc oxide). Pigs were fed for 6 wk using a 4-phase feeding program (phases of 1, 1, 2, and 2 wk, respectively) with declining diet complexity to measure ADG, ADFI, G:F, removal rate (mortality plus morbidity), and frequency of medical treatments per pen and day (MED). The SDE increased ($P < 0.05$) ADFI during phase 1 only

(180 vs. 164 g/d) compared with the diets without the SDE, but did not affect growth performance during any other intervals. The SDE reduced MED during phase 1 (0.75 vs. 1.35%; $P < 0.05$) and overall period (0.84 vs. 1.01%; $P = 0.062$) compared with the diets without the SDE, but did not affect removal rate. In Exp. 4 (160 pigs, 6.7 kg BW, 21 d old; 10 replicates) conducted in a university farm to determine whether SDE can replace SDP, treatments were in a factorial arrangement (with or without SDP or SDE in a nursery control diet excluding antibiotics and zinc oxide). Pigs were fed for 6 wks using the same feeding program used in Exp. 3 to measure ADG, ADFI, and G:F. The SDE increased ($P < 0.05$) ADFI during phase 1 only (195 vs. 161 g/d) compared with the diets without SDE, but did not affect growth performance during any other intervals. In conclusion, SDE can be an efficacious protein and energy source in nursery pig diets and improves health and, in some cases, increases growth rate.

Key words: health, nursery pigs, performance, spray-dried egg

INTRODUCTION

The post-weaning period has been emphasized not only to maintain or improve the health of weaned pigs because of potential stresses by weaning and their immature immune system, but also to provide adequate nutrients to pigs because of their immature digestive tracts (Pluske et al., 2002; Lalles et al., 2007). Thus, high-quality protein ingredients, such as spray-dried plasma, milk products, or fishmeal, have been used in nursery pig diets to minimize disease problems and to maximize growth performance in spite of their relatively high cost (Pettigrew, 2006; Stein and Kil, 2006). For example, spray-dried plasma significantly increases growth rate and provides

protection against disease (Pettigrew, 2006). Recent dramatic increases in prices of protein products for nursery pig diets have exacerbated the challenge.

The spray-dried egg (**SDE**) product tested here is produced from only eggs without shell that are below the USDA Grade B standards (Norberg et al., 2004; AAFCO, 2008; Table 7.1). It is an excellent nutrient source (Norberg et al., 2004; Harmon and Richert, 2007): 1) highly digestible, 2) balanced AA, 3) high fat, and 4) high ME. Beyond the provision of bioavailable nutrients, SDE may also provide specific physiological benefits because it contains 1) immunoglobulin antibodies (IgY in egg yolk) (Rose et al., 1974; Akita and Nakai, 1992; Harmon et al., 2002) and 2) lysozyme, an antimicrobial protein (Cunningham et al., 1991; Ibrahim et al., 1996; Schmidt et al., 2007). Therefore, SDE may perform two important roles in nursery pig diets, both provision of bioavailable nutrients and specific physiological benefits to improve health.

However, there is little empirical evidence that SDE improves growth performance and health of nursery pigs. Therefore, the objective of present studies was to verify the nutrient contributions and physiological health benefits of SDE in nursery pig diets.

MATERIALS AND METHODS

The protocols for these experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Purdue University at West Lafayette and University of Illinois at Urbana-Champaign. Two experiments were conducted in university swine research farms and one experiment was conducted in a commercial pig nursery in IL.

In all experiments, each pen within a block housed the same number of barrows and gilts, and pigs had *ad-libitum* access to feed and water. Dietary treatments within each experiment and

phase were formulated to the same ME and standardized ileal digestible lysine levels and to meet or exceed NRC (1998) estimates of nutrient requirements and targets for ratios of other AA to lysine on a standardized ileal digestible basis (Tables 7.2, 7.3, and 7.4).

Experiment 1 and 2

These experiments were conducted at Purdue University. A total of 168 and 140 weaned pigs with average initial BW of 5 kg and 16 d old were used in Exp.1 and 2, respectively, to evaluate the effect of SDE on growth performance. Treatments were with or without 5% SDE in a nursery control diet including antibiotics and zinc oxide (Table 7.2). Pigs were assigned to each pen by BW groups (block) and each pen was assigned to the dietary treatments. There were 2 rooms with 7 replications per room and 6 and 5 pigs/pen in Exp. 1 and 2, respectively.

Experiment 3

This experiment was conducted in a commercial pig nursery (Blunier Pork Farm, Forrest, IL). A total of 1008 weaned pigs that averaged 21 d old (5.2 ± 0.7 kg BW; PIC x Monsanto) were used in Exp. 3 to verify the nutrient contributions that Exp. 1 and 2 showed and to evaluate potential health benefits of SDE. There were 4 rooms adapted by installation of individual pen feeders and nipple drinkers and each room had 12 nursery pens. About 275 pigs were placed in 4 pens at the center of each room and separated into 3 weight blocks (heavy, medium, or light) as follows. The largest pigs were selected individually by eye and placed rotationally into the 4 pens (20 or 21 pigs/pen) designated for the heaviest weight block. Then, the smallest pigs were selected and placed into the 4 pens designated for the lightest weight block. After the selection of the smallest pigs, the remaining pigs in the center of the room were designated for the medium weight block. All pigs not selected were moved from the room. Within block, pigs were moved among pens to equalize the number of barrows and gilts across those 4 pens. After all

assignments of pigs in each room, all pigs designated for the experiment were weighed by pen, and if necessary, some pigs were traded among pens within the weight block to ensure the pen weight difference between any 2 pens was less than 5% of the average pen weight. When pigs were traded among pens, pigs were re-weighed in those pens. Treatments were in a 2 x 2 factorial arrangement with or without 6% SDE and two different concentrations of spray-dried plasma (**SDP**; 6% (**HSDP**) and 3% (**LSDP**)) in a nursery control diet including antibiotics and zinc oxide (Table 7.3). Pigs were fed for 6 wk using a 4-phase feeding program. Each of the treatments consisted of a series of 3 diets appropriate for pigs of increasing age and was fed for the following period after weaning, phase 1 (wk 1), phase 2 (wk 2), and phase 3 (wk 3 and 4) and then a common corn-soybean meal diet was fed for an additional 2 wk (phase 4; wk 5 and 6) to all pigs. Measurements were ADG, ADFI, G:F, removal rate (mortality plus morbidity), and frequency of medical treatments per pen and day (**MED**) for each phase and the overall period. Decisions to remove or treat sick pigs were according to standard practice of the farm. All pig deaths, removals, and medical treatments were recorded.

Experiment 4

Exp. 4 was conducted at the University of Illinois. A total of 160 weaned pigs with average age of 21 d (6.7 ± 1.0 kg BW; Line 337 x C 22, PIC) were used to evaluate whether SDE can replace spray-dried plasma in nursery pig diets. There were 40 pens total, 10 pens/treatment. Treatments were in a 2 x 2 factorial arrangement with or without 6% SDP and with or without 6% SDE in a nursery control diet and did not include antibiotics or zinc oxide (Table 7.4). Pigs were assigned to each pen by BW (3 blocks: heavy, medium, and light) and fed for 6 wk using the same 4-phase feeding program used in Exp. 3 to measure ADG, ADFI, and G:F for each phase and the overall period.

Statistical Analyses

Data were analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The experimental unit was the pen. For Exp. 1 and 2, the statistical model included effects of diet as a fixed effect and block as a random effect. For Exp. 3, the statistical model included effects of SDE, SDP level, and interaction as fixed effects and block as a random effect, and the chi-square test was used for removal rate and MED. For Exp. 4, the statistical model included the effect of SDE, SDP, and interaction as fixed effects and block as a random effect.

RESULTS

In both Exp. 1 and 2, the SDE increased ADG and ADFI ($P < 0.05$) compared with the control diets, but did not affect G:F (Figure 7.1).

In Exp. 3, the SDE increased ($P < 0.05$) ADFI during phase 1 only compared with the diets without the SDE (Table 7.5), but did not affect growth performance during any other intervals. The SDE increased ADG ($P < 0.05$) and G:F ($P = 0.061$) during phase 4 (Table 7.5), while all pigs consumed a common corn-soybean meal diet, apparently a carry-over effect. There were negative SDE effects on G:F during phase 2 ($P = 0.064$) and 3 ($P = 0.096$) compared with diets without the SDE. The SDE reduced MED during phase 1 ($P < 0.05$) and overall period ($P = 0.062$) compared with the diets without the SDE (Figure 7.2). In addition, the SDE reduced MED in the presence of the low level of SDP and increased MED in the presence of the high level of SDP (phase 2: interaction, $P < 0.05$). However, the SDE did not affect removal rate (Figure 7.3). No interactions between SDE and SDP level were detected on growth performance during any interval (Table 7.5).

In Exp. 4, the SDP increased ($P < 0.05$) ADFI during phase 1 only compared with the diets without the SDE, but did not affect growth performance during any other interval (Table 7.6). There were negative SDE effects ($P < 0.05$) on G:F during phases 2 and 3, and overall period compared with diets without the SDE (Table 7.6). The reduction of G:F by SDE in phase 3 was stronger in the presence of SDP than in its absence (interaction, $P = 0.063$).

DISCUSSION

The present experiments showed that SDE either increased growth rate (in Exp. 1 and 2 and Phase 4 of Exp. 3) or did not change it (in Exp. 3 and 4 over the entire period). It reduced feed efficiency during some phases of Exp. 3 and 4. Previous studies found a similar range of effects on growth performance. When the SDE replaced soybean meal in the present Exp. 1 and 2 and in the previous work by DeRouchey et al. (2003), growth rate was increased. When it replaced poultry by-product meal (the present Exp. 3), growth rate was unchanged. When it replaced SDP growth rate was usually not changed (Norberg et al., 2001; Figueiredo et al., 2003; the present Exp. 4), but in one case (Schmidt et al., 2003), was reduced. It is possible that a benefit of SDE is more likely in the absence of other dietary factors that promote health, such as SDP, antibiotics and zinc oxide, but that is not shown clearly in either the present results or the previously published studies. The reduction of G:F when SDE was fed is consistent with energy or nutrient contribution of SDE less than assumed in formulation of the diets. The formulations were based on ME for SDE of 5,000 kcal/kg as-fed basis (Harmon and Richart, 2007). Otherwise, the growth data suggest SDE is an efficacious nutrient source, in agreement with previous information.

Generally, SDE contains a large proportion of egg white (albumen) (Rose et al., 1974; Schmidt et al., 2003), which has an excellent amino acid profile with a relatively high level of methionine, tryptophan, and valine (DeRouchey et al., 2003; Figueiredo et al., 2003; Harmon and Richert, 2007) compared with other protein sources for nursery pigs such as soybean meal, fishmeal, dried whey, and spray-dried plasma (NRC, 1998). The egg product is also highly digestible, with nutrient digestibility values similar to those of soybean meal and plasma protein in pig (Schmidt et al., 2003) and duck (Norberg et al., 2004) diets. In addition, SDE contains a higher fat content, about 30% (Norberg et al., 2001; Figueiredo et al., 2003; Norberg et al., 2004), and therefore a higher metabolizable energy content, estimated to be about 5000 kcal/kg (Harmon and Richert, 2007), than other protein ingredients for nursery pigs (NRC, 1998).

The present Exp. 3 showed SDE reduced MED during the first wk after weaning and the overall period, indicating that it improved pig health. This observation indicates that SDE provides some physiological benefits to young animals beyond the bioavailable nutrient contributions. It may contribute some protection against disease because of specific components of SDE. Firstly, SDE contains immunoglobulin antibodies (IgY) (Harmon et al., 2002). The level of IgY in egg yolk has been estimated to be about 30,000 mg/kg (Rose et al., 1974; Harmon et al., 2002) or 12,000 mg/kg (Akita and Nakai, 1992). The SDE used in the present studies contained 11,800 mg/kg IgY, as analyzed by binding affinity for both peptidoglycan and bacterial lipopolysaccharide antigens through competitive binding assays by M. E. Spurlock (Iowa State University, Ames, IA, personal communication). Chicken IgY antibodies are structurally similar to the IgG antibodies produced by mammals in response to conventional immunization methods. They do not interfere with mammalian IgG, or do not activate mammalian complement (Tini et al., 2002). The specificity of the egg antibodies is unknown.

Secondly, SDE also contains lysozyme, an antimicrobial protein (Schmidt et al., 2007). It can damage bacterial cell walls (Cunningham et al., 1991) and this catalytic activity can reduce survival rate of gram positive or negative bacteria (Ibrahim et al., 1996; Schmidt et al., 2007). However, to our knowledge the present experiments are the first to show a practical benefit of SDE on pig health.

In conclusion, SDE is an efficacious nutrient source in nursery pig diets. Perhaps more importantly, it may also provide physiological benefits that specifically improve the health of pigs.

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Table 7.1. Analyzed nutrient composition of spray-dried egg compared with soybean meal and spray-dried plasma (as-fed basis)

Nutrient, %	**Spray-dried egg**	**Soybean meal, 48%**[1]	**Spray-dried plasma**[1]
DM	93.00	90.00	92.00
CP	49.73	47.50	78.00
Fat	26.14	3.00	2.00
Lysine	3.72	3.02	6.84
Calcium	0.36	0.34	0.15
Phosphorus	0.76	0.69	1.71

[1]Data from Nutrient Requirement of Swine (NRC, 1998)

Table 7.2. Ingredient composition of experimental diets used in Exp. 1 and 2 (as-fed basis)

	Exp. 1		Exp. 2	
Item	**CON**[1]	**SDE**[1]	**CON**[1]	**SDE**[1]
Ingredient, %				
Corn	35.44	35.10	47.34	50.07
Soybean meal, 48%	29.85	23.30	23.08	16.51
Dried whey	15.00	15.00	15.00	15.00
Meat and bone meal	5.00	5.00	-	-
Poultry byproduct meal	-	-	5.00	5.00
Fishmeal	5.00	5.00	4.00	4.00
Soy hulls	0.76	0.56	-	-
Spray-dried egg	0	5.00	0	5.00
Animal fat	5.51	4.51	2.52	1.48
Di-calcium phosphate	1.34	4.33	1.41	1.29
Limestone	0.54	0.51	0.03	0.07
Mecadox	0.25	0.25	0.25	0.25
Zinc oxide	0.20	0.20	0.20	0.20
Salt	0.20	0.20	0.20	0.20
Vitamin premix[2]	0.30	0.30	0.30	0.30
Mineral premix[3]	0.15	0.15	0.15	0.15
Selenium premix[4]	0.05	0.05	0.05	0.05
L-Lysine·HCl	0.33	0.34	0.41	0.41
DL-Methionine	0.08	0.20	0.06	0.02

Table 7.2. (cont.)

Item	Exp. 1		Exp. 2	
	CON[1]	SDE[1]	CON[1]	SDE[1]
Calculated energy and nutrient levels				
ME, mcal/kg	3.30	3.30	3.35	3.35
Lysine, %	1.62	1.62	1.62	1.62
Met/Cys, %	0.82	0.82	0.82	0.82
Threonine, %	0.92	0.92	0.92	0.92
Tryptophan, %	0.27	0.27	0.26	0.26

[1]CON = control diet; SDE = spray-dried egg diet.

[2]Provided per kilogram of diet: vitamin A, 6,108 IU from retinyl acetate; vitamin D_3, 600 IU; vitamin E, 23 IU from DL-α-tocopheryl acetate; menadione sodium bisulfate, 1.2 mg; vitamin B_{12}, 31 μg; riboflavin, 6 mg; D-pantothenic acid, 22.5 mg; niacin, 35 mg.

[3]Provided as milligrams per kilogram of diet: copper, 10 from copper oxide; iron, 100 from iron sulfate; manganese, 27.5 from manganese oxide; iodine, 1.4 from calcium iodide; zinc, 60 from zinc oxide.

[4]Provided per kilogram of diet: selenium, 300 μg from sodium selenite.

Table 7.3. Ingredient composition of experimental diets in Exp. 3 (as-fed basis)

	Treatments[1]										
	P1				P2				P3		P4
Item	HSDP		LSDP		HSDP		LSDP		H/LSDP		CS
	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	
Ingredient, %											
Corn	41.38	42.00	41.00	42.17	46.65	47.11	46.07	46.52	56.61	56.71	67.37
Dried Whey	16.00	16.00	16.00	16.00	14.00	14.00	14.00	14.00	10.00	10.00	0
SBM[2]	10.00	10.00	10.00	10.00	18.00	18.00	18.00	18.00	24.00	24.00	28.66
Lactose	9.80	9.80	9.80	9.80	4.20	4.20	4.20	4.20	0	0	0
SDP[3]	6.00	6.00	3.00	3.00	3.00	3.00	1.50	1.50	0	0	0
SDE[4]	0	6.00	0	6.00	0	4.00	0	4.00	0	2.00	0
PBM[5]	4.77	1.39	8.29	4.37	3.48	1.18	5.65	3.60	2.54	0.80	0
Fishmeal	3.00	3.00	3.00	3.00	2.00	2.00	2.00	2.00	2.00	2.00	0
SPC[6]	3.00	1.00	3.00	1.00	2.00	0.70	2.00	0.70	0	0	0
Soybean oil	3.00	1.34	3.00	1.39	3.00	1.90	3.00	1.83	1.00	0.49	0
Limestone	0.84	0.99	0.50	0.72	0.79	0.87	0.49	0.70	0.67	0.73	1.38
Di-cal.[7]	0	0.29	0	0.26	0.49	0.71	0.62	0.60	0.91	1.08	1.18
Carbadox	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.5
Zinc Oxide	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.21	0.21	0
Min premix[8]	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Vit premix[9]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
DL-Methionine	0.16	0.15	0.27	0.13	0.18	0.13	0.24	0.14	0.16	0.09	0.05
L-Lysine·HCl	0.07	0.07	0.14	0.17	0.22	0.22	0.23	0.22	0.29	0.29	0.26
L-Tryptophan	0.01	0	0.03	0.02	0.02	0.01	0.03	0.02	0.02	0.01	0
L-Threonine	0	0	0	0	0	0	0	0	0.04	0.04	0.05

Table 7.3. (cont.)

Item	Treatments[1]										
	P1				P2				P3		P4
	HSDP		LSDP		HSDP		LSDP		H/LSDP		CS
	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	
Calculated energy and nutrient levels											
ME, mcal/kg	3.47	3.47	3.47	3.47	3.44	3.44	3.44	3.44	3.33	3.33	3.29
CP, %	21.98	21.68	22.08	21.39	21.67	21.43	21.93	21.80	20.69	20.56	19.53
Lysine, %	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.40	1.40	1.12
Calcium, %	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Phosphorus, %	0.60	0.63	0.61	0.63	0.64	0.66	0.67	0.65	0.67	0.69	0.63
Available P, %	0.41	0.40	0.42	0.40	0.40	0.40	0.44	0.40	0.40	0.40	0.32

[1]P1 = phase 1 (wk 1), P2 = phase 2 (wk 2), P3 = phase 3 (wk 3 and 4), and P = phase 4 (wk 5 and 6); HSDP = high concentration of spray-dried plasma; LSDP = low concentration of spray-dried plasma; CON = control diet; SDE = spray-dried egg diet; CS = corn-soybean meal based diet.

[2]SBM = soybean meal, dehulled, 48%.

[3]SDP = spray-dried plasma (Appetein; APC, Inc., Ankeny, IA).

[4]SDE = spray-dried egg (Rose Acre Farms, Seymour, IN).

[5]PBM = poultry byproduct meal, 65% (Griffin Industries, Inc., Cold Spring, KY).

[6]SPC = soy protein concentrate (Soycomil-K; ADM, Decatur, IL).

[7]Di-cal. = calcium phosphate, dibasic.

[8]Provided as milligrams per kilogram of diet: sodium chloride, 3,000; zinc, 100 from zinc oxide; iron, 90 from iron sulfate; manganese, 20 from manganese oxide; copper, 8 from copper sulfate; iodine, 0.35 from calcium iodide; selenium, 0.30 from sodium selenite.

Table 7.3. (cont.)

[9]Provided per kilogram of diet: retinyl acetate, 2,273 μg; cholecalciferol, 17 μg; DL-α-tocopheryl acetate, 88 mg; menadione sodium bisulfite complex, 4 mg; niacin, 33 mg; D-Ca-pantothenate, 24 mg; riboflavin, 9 mg; vitamin B_{12}, 35 μg; choline chloride, 324 mg.

Table 7.4. Ingredient composition of experimental diets in Exp. 4 (as-fed basis)

	Treatments[1]												
	P1				P2				P3				P4
	CON		SDP		CON		SDP		CON		SDP		CS
Item	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	
Ingredient, %													
Corn	39.81	41.28	41.09	41.78	45.85	46.96	46.98	47.38	56.08	56.64	56.85	57.13	70.31
Dried Whey	16.00	16.00	16.00	16.00	14.00	14.00	14.00	14.00	10.00	10.00	10.00	10.00	0
SBM[2]	10.00	10.00	10.00	10.00	18.00	18.00	18.00	18.00	20.45	20.45	20.45	20.45	26.18
Lactose	9.80	9.80	9.80	9.80	4.20	4.20	4.20	4.20	0	0	0	0	0
SDP[3]	0	0	6.00	6.00	0	0	4.00	4.00	0	0	2.00	2.00	0
SDE[4]	0	6.00	0	6.00	0	4.00	0	4.00	0	2.00	0	2.00	0
PBM[5]	5.19	7.59	2.05	0.71	2.17	4.04	0.91	0.05	1.60	2.53	1.76	1.63	0
Fishmeal	5.00	5.00	5.00	5.00	3.00	3.00	3.00	3.00	1.00	1.00	1.00	1.00	0
SPC[6]	7.50	0	3.68	0	5.89	0.60	2.48	0	4.04	1.39	1.49	0	0
Soybean oil	5.11	3.02	4.84	3.00	4.48	3.04	4.21	3.00	3.82	3.11	3.63	3.00	0
Limestone	0.43	0.31	0.85	0.94	0.62	0.57	0.89	0.93	0.72	0.70	0.84	0.85	1.09
Di-cal.[7]	0.14	0	0	0.06	0.72	0.54	0.50	0.59	1.19	1.09	1.00	1.01	1.36
Min Premix[8]	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Vit Premix[9]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
DL-Methionine	0.24	0.21	0.09	0.13	0.19	0.17	0.10	0.13	0.16	0.15	0.13	0.10	0.07
L-Lysine·HCl	0.20	0.18	0.05	0.03	0.28	0.27	0.18	0.17	0.33	0.32	0.28	0.27	0.34
L-Tryptophan	0.03	0.05	0	0	0.02	0.03	0	0	0.02	0.02	0.01	0.01	0.01
L-Threonine	0	0.01	0	0	0.03	0.03	0	0	0.04	0.05	0.01	0	0.09

Table 7.4. (cont.)

	Treatments[1]												
	P1				P2				P3				P4
	CON		SDP		CON		SDP		CON		SDP		CS
Item	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	SDE-	SDE+	
Calculated energy and nutrient levels													
ME, mcal/kg	3.60	3.60	3.6	3.60	3.55	3.55	3.55	3.55	3.50	3.50	3.50	3.50	3.31
CP, %	21.82	21.82	21.82	21.82	21.63	21.63	21.63	21.63	20.34	20.34	20.34	20.34	18.73
Lysine, %	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.40	1.40	1.40	1.40	1.12
Calcium, %	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.84	0.80
P, %	0.62	0.63	0.62	0.62	0.66	0.66	0.64	0.66	0.68	0.68	0.66	0.67	0.63
Avail P, %	0.40	0.42	0.41	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.32

[1]P1 = phase 1 (wk 1), P2 = phase 2 (wk 2), P3 = phase 3 (wk 3 and 4), and P = phase 4 (wk 5 and 6); SDP = spray-dried plasma; CON = control diet; SDE = spray-dried egg diet; CS = corn-soybean meal based diet.

[2]SBM = soybean meal, dehulled, 48%.

[3]SDP = spray-dried plasma (Appetein; APC, Inc., Ankeny, IA).

[4]SDE = spray-dried egg (Rose Acre Farms, Seymour, IN).

[5]PBM = poultry byproduct meal, 65% (Griffin Industries, Inc., Cold Spring, KY).

[6]SPC = soy protein concentrate (Soycomil-K; ADM, Decatur, IL).

[7]Di-cal. = calcium phosphate, dibasic.

[8]Provided as milligrams per kilogram of diet: sodium chloride, 3,000; zinc, 100 from zinc oxide; iron, 90 from iron sulfate; manganese, 20 from manganese oxide; copper, 8 from copper sulfate; iodine, 0.35 from calcium iodide; selenium, 0.30 from sodium selenite.

[9]Provided per kilogram of diet: retinyl acetate, 2,273 μg; cholecalciferol, 17 μg; DL-α-tocopheryl acetate, 88 mg; menadione sodium bisulfite complex, 4 mg; niacin, 33 mg; D-Ca-pantothenate, 24 mg; riboflavin, 9 mg; vitamin B_{12}, 35 μg; choline chloride, 324 mg.

Table 7.5. Effect of spray-dried egg on growth performance (Exp. 3)[1]

	Treatments[2]							
	HSDP		LSDP			*P*-value[3]		
Item	**SDE-**	**SDE+**	**SDE-**	**SDE+**	**SEM**	**SDE**	**SDP**	**SDE*SDP**
Day 1 to 7 (Phase 1)								
No. of pigs	251	252	252	252				
ADG, g/d	117	124	110	114	6.24	0.34	0.17	0.84
ADFI, g/d	170	185	157	174	4.10	< 0.05	< 0.05	0.80
G:F	0.684	0.672	0.697	0.659	0.026	0.33	0.99	0.61
Day 7 to 14 (Phase 2)								
No. of pigs	250	248	251	252				
ADG, g/d	286	283	293	276	6.06	0.11	0.99	0.23
ADFI, g/d	354	355	347	362	8.03	0.35	0.94	0.37
G:F	0.806	0.798	0.848	0.771	0.021	0.064	0.72	0.16
Day 14 to 28 (Phase 3)								
No. of pigs	242	239	242	248				
ADG, g/d	329	319	343	334	7.14	0.21	< 0.05	0.92
ADFI, g/d	511	502	521	531	7.34	0.86	< 0.05	0.28
G:F	0.644	0.636	0.657	0.630	0.011	0.096	0.69	0.44

Table 7.5. (cont.)

	Treatments[2]							
	HSDP		LSDP			*P*-value[3]		
Item	**SDE-**	**SDE+**	**SDE-**	**SDE+**	**SEM**	**SDE**	**SDP**	**SDE*SDP**
Day 28 to 42 (Phase 4)								
No. of pigs	237	236	241	247				
ADG, g/d	490	515	477	507	13.01	< 0.05	0.40	0.84
ADFI, g/d	863	892	863	880	21.63	0.31	0.78	0.78
G:F	0.568	0.577	0.555	0.577	0.0077	0.061	0.43	0.42
Day 1 to 42 (Overall period)								
No. of pigs	237	236	241	247				
ADG, g/d	337	342	338	344	6.36	0.38	0.84	0.92
ADFI, g/d	540	549	541	557	9.85	0.20	0.63	0.71
G:F	0.625	0.625	0.627	0.618	0.0051	0.34	0.66	0.51

[1]Each value is the mean of 12 replicates.

[2]P1 = phase 1 (wk 1), P2 = phase 2 (wk 2), P3 = phase 3 (wk 3 and 4), and P = phase 4 (wk 5 and 6); HSDP = high concentration of spray-dried plasma; LSDP = low concentration of spray-dried plasma; CON = control diet; SDE = spray-dried egg diet.

[3]SDE = SDE effect; SDP = SDP effect; SDE*SDP = interaction between SDE and SDP.

Table 7.6. Effect of spray-dried egg on growth performance (Exp. 4)[1]

	Treatments[2]							
	CON		SDP			*P*-value[3]		
Item	**SDE-**	**SDE+**	**SDE-**	**SDE+**	**SEM**	**SDE**	**SDP**	**SDE*SDP**
Day 1 to 7 (Phase 1)								
No. of pigs	40	40	40	40				
ADG, g/d	50	67	108	120	14	0.30	< 0.05	0.87
ADFI, g/d	140	176	181	214	14	< 0.05	< 0.05	0.94
G:F	0.356	0.361	0.567	0.554	0.052	0.94	< 0.05	0.86
Day 7 to 14 (Phase 2)								
No. of pigs	40	40	40	40				
ADG, g/d	163	142	149	144	15	0.38	0.69	0.59
ADFI, g/d	256	273	266	291	14	0.15	0.33	0.79
G:F	0.623	0.512	0.561	0.486	0.038	< 0.05	0.25	0.64
Day 14 to 28 (Phase 3)								
No. of pigs	40	40	39	40				
ADG, g/d	494	499	517	502	18	0.78	0.47	0.58
ADFI, g/d	696	712	675	724	25	0.17	0.78	0.57
G:F	0.710	0.697	0.768	0.695	0.015	< 0.05	0.081	0.063

Table 7.6. (cont.)

	Treatments[2]							
	CON		SDP			*P*-value[3]		
Item	**SDE-**	**SDE+**	**SDE-**	**SDE+**	**SEM**	**SDE**	**SDP**	**SDE*SDP**
Day 28 to 42 (Phase 4)								
No. of pigs	40	39	39	40				
ADG, g/d	669	698	674	648	18	0.94	0.20	0.13
ADFI, g/d	1074	1074	1078	1055	30	0.70	0.81	0.71
G:F	0.624	0.652	0.625	0.614	0.010	0.41	0.089	0.063
Day 1 to 42 (Overall period)								
No. of pigs	40	39	39	40				
ADG, g/d	423	433	440	427	12	0.91	0.66	0.36
ADFI, g/d	656	682	662	677	18	0.27	0.98	0.79
G:F	0.645	0.637	0.665	0.630	0.008	< 0.05	0.40	0.107

[1]Each value is the mean of 10 replicates.

[2]P1 = phase 1 (wk 1), P2 = phase 2 (wk 2), P3 = phase 3 (wk 3 and 4), and P = phase 4 (wk 5 and 6); CON = control diet; SDP = spray-dried plasma diet; SDE = spray-dried egg diet.

[3]SDE = SDE effect; SDP = SDP effect; SDE*SDP = interaction between SDE and SDP.

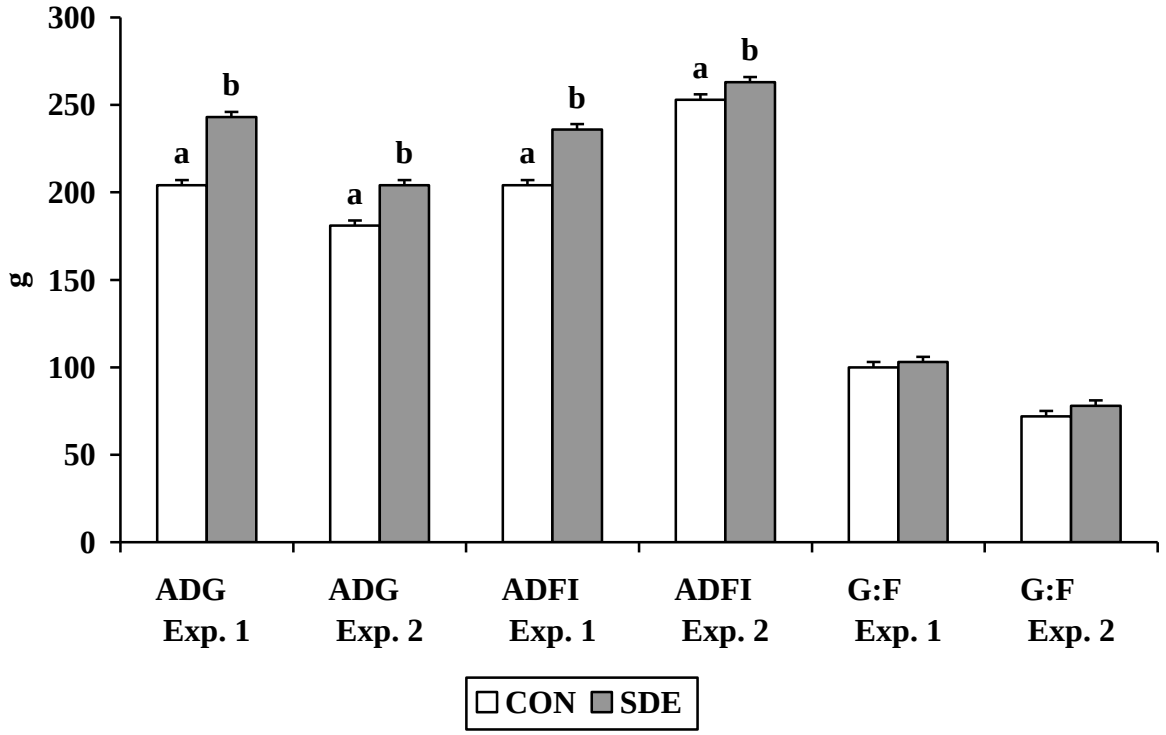

Figure 7.1. Effect of spray-dried egg on ADG and ADFI (Exp. 1 and 2). [ab]Means with different letters differ between dietary treatments ($P < 0.05$). CON is control diet and SDE is 5% spray-dried egg diet. There were 14 replicates in each experiment. The unit is g for ADG, g for ADFI, and g/100g for G:F.

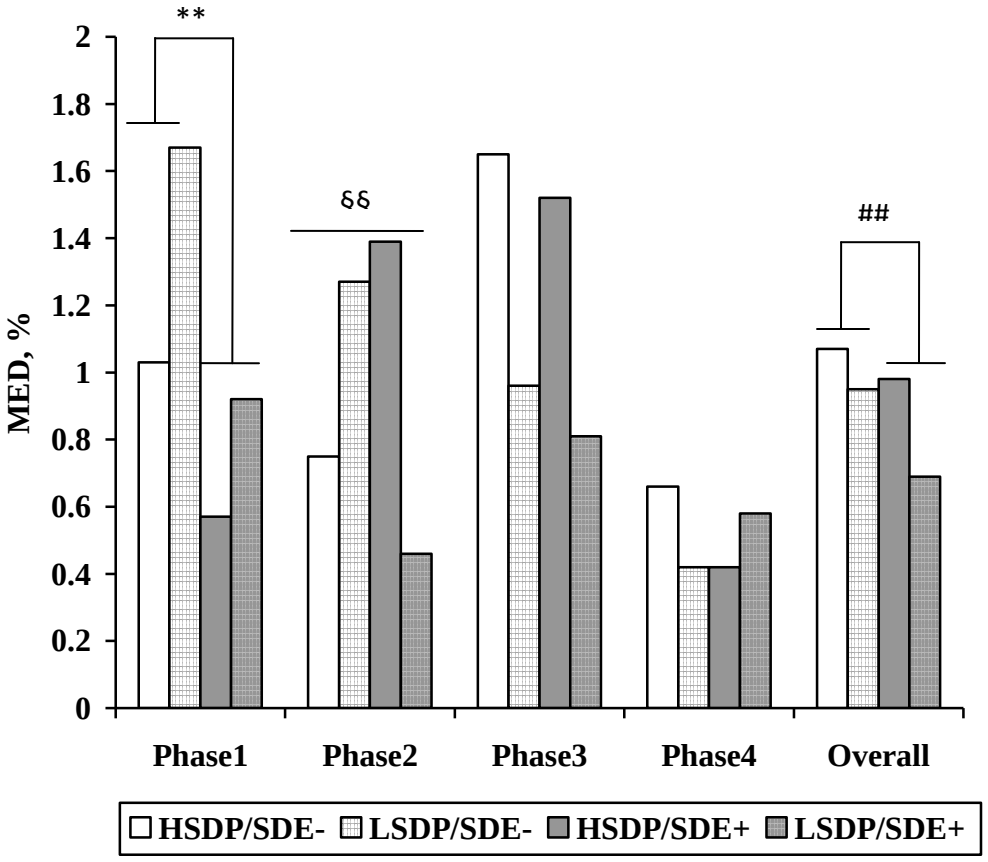

Figure 7.2. Effect of spray-dried egg on frequency of medical treatment per pen and day (MED) (Exp. 3). **Indicates difference between CON and SDE ($P < 0.05$). §§Indicates an interaction between SDE and SDP level ($P < 0.05$). ##Indicates difference between CON and SDE ($P = 0.062$). Phase 1 (wk 1), phase 2 (wk 2), phase 3 (wk 3 and 4), phase 4 (wk 5 and 6), and Overall (wk 1 to 6). HSDP is high concentration of spray-dried plasma and LSDP is low concentration of spray-dried plasma. CON is control diet and SDE is spray-dried egg diet. There were 12 replicates (12 pens/treatment). Data were analyzed by chi-square test.

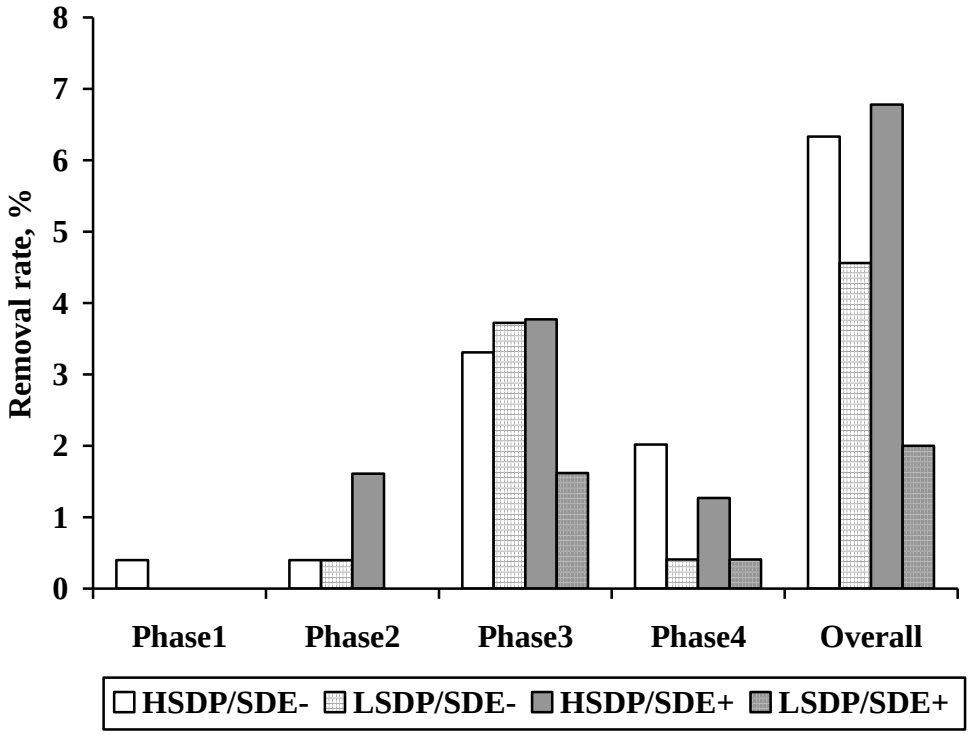

Figure 7.3. Effect of spray-dried egg on removal rate including mortality and morbidity (Exp. 3). Phase 1 (wk 1), phase 2 (wk 2), phase 3 (wk 3 and 4), phase 4 (wk 5 and 6), and Overall (wk 1 to 6). HSDP is high concentration of spray-dried plasma and LSDP is low concentration of spray-dried plasma. CON is control diet and SDE is spray-dried egg diet. There were 12 replicates (12 pens/treatment). There were no SDE, SDP, and interaction effects at any phases and overall ($P > 0.05$). Data were analyzed by chi-square test.

CHAPTER 8

EFFECTS OF DIETARY ENZYMES ON ILEAL MICROBIAL ECOLOGY OF PIGS FED DIETS CONTAINING DISTILLERS DRIED GRAINS WITH SOLUBLES

ABSTRACT

A study was conducted to evaluate effects of enzymes on ileal microbial ecology of pigs fed diets containing distillers dried grains with solubles (DDGS). Pigs (n = 8 barrows; 27.2 ± 1.66 kg BW) were surgically equipped with a T-cannula in the distal ileum. Each dietary treatment was fed to each of the pigs. All diets included 20% DDGS and the treatments were: 1) control diet (CON), 2) CON + 0.1% phytase (1000 units phytase/kg diet), 3) CON + 0.1% xylanase (1000 units xylanase/kg diet), 4) CON + 0.05% phytase (500 units phytase/kg diet) + 0.05% xylanase (500 units xylanase/kg diet), and 4 other enzyme combinations, but the effects of only treatments 1) through 4) on ileal microbial ecology of pigs were evaluated. Pigs were allowed *ad libitum* access to feed and water. Ileal digesta were collected on d 6 and 7 of each 7-day. Denaturing gradient gel electrophoresis (DGGE) was used to estimate the species diversity of the bacterial population (the number of bands) and quantitative measures of the similarity of population structures (banding pattern expressed by Sorenson′s pairwise similarity coefficients (Cs)) among pigs within (INTRA) and between treatments (INTER). Bands of interest were extracted from the DGGE gel and sequencing analysis was conducted to identify microbial species. There was no effect on the number of bands (diversity of the microbial populations) when pigs were fed the enzyme treatments. The INTRA Cs values were lower when pigs were

fed the xylanase treatments (45.4 vs. 51.3; $P < 0.05$) than when pigs were not fed the xylanase treatments. This suggests that the xylanase treatments may modulate ileal microbial populations, resulting in less homogenous microbiota among pigs. However, this pattern was not found in pigs fed the phytase treatments. The INTER Cs values were not affected by either phytase or xylanase treatment. In a few cases, specific bands were present in most pigs fed the CON, but absent from most pigs fed either phytase or xylanase treatments, or the converse. *Lactobacillus avarius* and *Burkholderia cepacia* appeared in pigs fed the phytase treatment, members of the genus *Serratia* and *Burkholderia* in pigs fed the xylanase treatment, and members of the genus *Pseudomonas and Serratia* in pigs fed the CON. In conclusion, both phytase and xylanase enzymes may modify ileal microbial populations of pigs fed diets containing 20% DDGS.

Key words: denaturing gradient gel electrophoresis (DGGE), distillers dried grains with solubles (DDGS), enzymes, microbial ecology, pigs, sequencing

INTRODUCTION

In swine production, several enzyme products (carbohydrases, proteases, phytases, etc.) as well as their combinations are commercially available to improve digestion of nutrients (Grieshop et al., 2001; Crenshaw, 2001). In addition, the products of enzymes may alter factors of the intestinal environment such as pH, passage rate, viscosity, etc. (Kiarie et al., 2007; Vahjen et al., 2007; Emiola et al., 2009) as well as microbial populations in the digestive tract (Durmic et al., 2000; Hardy, 2002; Pluske et al., 2002).

Several studies showed that enzymes improve growth performance (Mavromichalis et al., 2000; Pan et al., 2002; Olukosi et al., 2007), feed efficiency (Kim et al., 2003; Barrera et al.,

2004), and nutrient digestibility (Gdala et al., 1997; Omogbenigun et al., 2004; Kim et al., 2008) of pigs. They may also contribute to improvement of pig health as indicated by increased populations of beneficial microbes (Pan et al., 2002; Kiarie et al., 2007; Vahjen et al., 2007) or by inhibition of activation of pathogenic *E. coli* receptors in the mucosal and epithelial cells of the digestive tract by proteases (Chandler et al., 1994; Jin and Zhao, 2000).

Distillers dried grains with solubles (**DDGS**) are commonly used in swine diets, but it contains higher fiber, especially insoluble fiber, than corn (Stein and Shurson, 2009) and thus enzymes are used in swine diets containing DDGS to improve growth performance or nutrient digestibility of pigs. However, there is little information whether enzymes can also change microbial populations in the digestive tract of pigs fed the diets containing fibrous corn byproducts, especially DDGS. Therefore, the objective of this study was to evaluate the effects of enzymes (phytase, xylanase, and their combination) on ileal microbial ecology of pigs fed diets containing 20% DDGS via denaturing gradient gel electrophoresis (**DGGE**).

MATERIALS AND METHODS

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. The experiment was conducted in the Swine Research Center at the University of Illinois, Urbana.

Animals, Diets, Housing, Experimental Design, and Sample Collection

Eight growing pigs (barrows; 27.2 ± 1.66 kg BW; Line 337 boars x C 22 sows (PIC, Hendersonville, TN)) were surgically equipped with T-cannulas in the distal ileum using procedures adapted from Stein et al. (1998). Following the surgery, pigs were housed individually in 1.4 m x 0.72 m metabolism crates of an environmentally controlled room and had

ad libitum access to feeder and water. Pigs were allowed a 15-d recovery period after the surgery and were fed a standard diet during this period. Following the recovery period, pigs were allotted to 8 experimental diets during 8 wk periods in a Latin square design. All 8 of the diets were used in a companion experiment (Urriola et al., Unpublished), but only 4 diets were used in the present experiment.

Diets containing 20% DDGS with addition of 0.1% enzymes (phytase, xylanase, and their combination) were formulated to meet or exceed the nutrient requirements for growing (35 to 70 kg) pigs (Table 8.1; NRC, 1998). The 4 experimental treatments were the control diet (CON), CON + 0.1% phytase (1000 units phytase/kg diet; Danisco Animal Nutrition, Marlborough, Wiltshire, UK), CON + 0.1% xylanase (1000 units xylanase/kg diet; Danisco Animal Nutrition, Marlborough, Wiltshire, UK), and CON + 0.5% phytase (500 units phytase/kg diet) + 0.5% xylanase (500 units xylanase/kg diet).

Pigs were fed a daily quantity of the assigned diet that supplied 3 times the estimated maintenance requirement for energy (i.e., 106 kcal ME/$kg^{0.75}$; NRC, 1998). The daily feed allotments were divided into 2 equal meals and fed at 0800 and 1700. Pigs were fed dietary treatments during 8 periods of 7 days each. Ileal digesta were collected in plastic bags (Stein et al., 1999) for 8h on d6 and 7 of each period. Bags were removed when they were filled with ileal digesta, or at least once every 30 min, and immediately stored at -20ºC. Ileal digesta samples were thawed and mixed within animal and collection period, and sub-samples were taken and stored at -20ºC for microbial analyses.

Genomic DNA extraction and PCR-DGGE Analysis.

Genomic DNA was isolated from approximately 250 mg of all of ileal digesta and fecal samples using a commercially available kit (MO BIO UltraPowerSoil™ DNA isolation Kit; MO

BIO Laboratories, Inc., Solana Beach, CA). The isolated DNA samples were standardized to 20 μg DNA/ml and PCR amplification was performed by using a PTC-100^{TM} Peltier Thermal Cycler (MJ Research, Inc., Boston, MA). The DNA was amplified using primers specific for the conserved sequences flanking the variable V3 region of 16S rDNA (341F: 5´CACGGGGGGGCCTACGGGAGGCAGCAG 3´ + 5´ 40 nucleotide GC clamp and 534R: 5´ ATTACCGCGGTGCTGG 3´) (Muyzer et al., 1998; Collier et al, 2003). Touchdown PCR was performed to reduce spurious PCR products (Muyzer and Smalla, 1998). After PCR amplification, the PCR products were verified using 1% agarose gel electrophoresis, followed by ethidium bromide staining and capturing the image under an ultraviolet (UV) light (Alpha Imager^{TM} IS-2200, Alpha Innotech Corp., San Leandro, CA).

After visual confirmation of PCR products, DGGE was performed using a DGGE-4801 Multiple Gel Caster (C.B.S. Scientific Company, Inc., Del Mar, CA). The PCR fragments were separated using a linear 35 to 60% denaturing gradient (100% denaturant is equivalent to 7 mol urea/L and 40% deionized formamide) formed in 8% polyacrylamide gels using the GM-500 Gradient Maker (C.B.S. Scientific Company, Inc., Del Mar, CA). Sample baterial V3 16S PCR products (10 μl) were loaded in each lane and bacterial standard ladders representing known bacterial strains were loaded to allow standardization of band migration and gel curvature among different gels (Simpson et al., 1999). The reference ladders consisted of the following species, listed in order from the top of the gel to the bottom: *Bacteroides vulgatus*, *Escherichia coli*, *Bacteroides fragilis*, *Porphyromonas sp.*, *Clostridium perfringens*, *Lactobacillus casei*, and *Enterococcus sp.*. After electrophoresis was performed at 60ºC at 150V for 7 h in 0.5X Tris-acetate-EDTA running buffer, gels were stored in 40% fixative (40% reagent grade methanol, 10% reagent grade acetic acid, 10% BioRad Fixative Enhancer Concentrate, and 40% deionized

water) overnight. After fixation, gels were silver-stained and scanned using the BioRad GS-710 calibrated imaging densitometer (BioRad). Gel images were captured to estimate microbial richness and diversity.

Captured gel images were analyzed using the GelCompar II (version 4.5) software (Applied Maths, Inc., Austin, TX). This software was used to determine the number of bands produced by samples from each pig. A detectable band is created by a species that makes up approximately 1% or more of the total bacterial population (Muyzer et al., 1993). The software also calculates Sorenson's pairwise similarity coefficients (**Cs**) by comparing banding patterns among pigs within treatments (**INTRA**) and between treatments (**INTER**) as quantitative measures of the similarity of population structures (Simpson et al., 1999), and produces a dendrogram based on the Cs values. A Cs value of 100 indicates the two samples being compared have exactly the same bands and a Cs value of 0 indicates the two samples share no bands.

The number of bands indicates microbial diversity as the number of dominant microbial species, except that multiple species may coincidentally occupy the same band. A low INTRA Cs value indicates the microbiota among pigs within a same treatment is not similar. Both measurements may be considered to stability of the microbiota.

A low INTER Cs value indicates the microbiota among pigs between any two different treatments is not similar. Thus, average INTRA Cs values for the two treatments was used as the standard and compared with average INTER Cs value between the two treatments. If the INTER Cs value is lower than the INTRA Cs value, it indicates that the microbial populations are different between the two treatments and there is a treatment effect. If the INTER Cs value is

equal to or higher than the INTRA Cs value, it indicates that the microbial populations are similar between the two treatments and there is no treatment effect.

Patterns of the DGGE bands were compared between CON and antibiotics treatments and then instances in which a band, representing one or more bacterial species, appeared or disappeared when one of the antibiotics was introduced into the diet, were identified.

Cloning and Sequencing Analysis.

Bands of interest were extracted from the DGGE polyacrylamide gel using a modified PureLink Quick Gel Extraction Kit (Invitrogen Corporation, CA, USA) following the manufacturer's procedure. DNA was re-amplified by PCR using primers 341F (no GC-clamp) and 534R. The PCR products were run on agarose gel to check for their purity. Cloning of Taq polymerase-amplified PCR products was performed by TOPO TA Cloning kit (Invitrogen Corporation, CA, USA) following the manufacturer's procedure. Sequencing was performed using primers M13Forward and M13Reverse (TOPO TA Cloning kit, Invitrogen Corporation, CA, USA) at the UIUC Core Sequencing Facility in Edward R. Madigan Laboratory. All 16S rDNA sequences were subject to nucleotide basic local alignment search tool (BLAST) search against Genbank (http://www.ncbi.nlm.nih.gov).

Statistical Analyses

Data were analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The experimental unit was the pig. For the number of bands and the INTRA Cs values, the statistical model included effects of two dietary treatments (phytase and xylanase), and their interaction as fixed effects and pig and period as random effects. Each INTER Cs value was compared by specific contrast to the 2 pertinent INTRA Cs values.

RESULTS

There were no effects of enzyme treatments on the number of bands (diversity of the microbial populations) when pigs were fed the enzyme treatments (Figure 8.1). In addition, the enzyme treatments did not produce marked clusters in the dendrogram (Figure 8.2). However, the INTRA Cs values were lower when pigs were fed the xylanase treatments ($P < 0.05$) than when pigs were not fed the xylanase treatments (Figure 8.3). This suggests that the xylanase treatment modulated ileal microbial populations, resulting in less homogenous ileal microbiota among pigs within the treatments. The INTER Cs values were not affected by either phytase or xylanase treatment (Figure 8.4).

In a few cases, specific bands were present in most pigs fed the CON, but absent from most pigs fed either phytase or xylanase treatments, or the converse (Table 8.2). The sequences of a DGGE band present in pigs fed the phytase treatment matched *Lactobacillus avarius* and *Burkholderia cepacia* with 99% and 100% similarities, respectively (Table 8.2). The sequences of a DGGE band present in pigs fed the xylanase treatment matched members of the genus *Serratia* and *Burkholderia* with 100% similarities (Table 8.2). In addition, the sequences of a DGGE band present in pigs fed the control diet matched members of the genus *Pseudomonas and Serratia* with 99% similarity (Table 8.2).

DISCUSSION

The present experiment indicates that enzyme treatments may change microbial populations in the ileum of pigs fed diets containing 20% DDGS, which is in agreement with reports by Garry et al. (2007), Vahjen et al. (2007), and Reilly et al. (2010). The xylanase treatments decreased the INTRA Cs values, perhaps because the enzyme shifted microbial

populations more rapidly in some pigs than in others, resulting in less homogenous ileal microbiota among pigs within treatments, but this result is not in agreement with data showing that xylanase does not affect INTRA Cs values (Gao et al., 2008). This pattern is also different from the pattern that carbadox and/or other antibiotics make pigs' microbiota more similar to each other (homogenous) after the initial disruption during the feeding of the antibiotics (Collier et al., 2003; Miguel et al., 2006; Song et al., 2009). It may indicate enzymes affect the microbial populations differently compared with antibiotics. In addition, the enzyme effects were not large enough to be detected by the INTRA or INTER Cs values in the present experiment, perhaps because of natural individual variations of animals (Gong et al., 2005; Richard et al., 2005) or the short period of the enzymes treatments. However, Santos et al. (2008) showed xylanase increased the total number of DGGE bands and INTER Cs values and did not affect INTRA Cs values. On the other hand, there is little information about phytase effects on microbial populations by DGGE analysis.

In addition, both enzymes eliminated some species of microbes. Enzymes can break down the structures of nutrients which pigs cannot digest and thus help to improve digestion of the nutrients (Grieshop et al., 2001; Crenshaw, 2001). Due to the enzyme property, some microbes can use the substances produced by breaking down the structures of nutrients and may become dominant in the digestive tract of pigs and then these microbes may compete against others by various means and markedly reduce the populations of those other species (Santos et al., 2008), resulting in changes of their microbial populations (Hardy, 2002; Pluske et al., 2002; Santos et al., 2008). Of the species identified as changing with treatment, the most significant appears to be the appearance of a *Lactobacillus* species, generally considered beneficial, when the phytase treatment was fed to pigs. This result is in agreement with the results showing

increased populations of *lactobacillus* and *bifidobacteria* by addition of enzymes in pig diets (Garry et al., 2007; Reilly et al., 2010).

The connection between digestibility of pigs and modulation of microbiota by enzymes has not been fully understood (Zijlstra et al., 2010), but the modulation of microbial populations by enzymes in the present experiment may affect improvement of apparent ileal digestibility of AA, the results from the companion experiment (Urriola et al., Unpublished).

In conclusion, both phytase and xylanase enzymes may change ileal microbial ecology of pigs fed diets containing 20% DDGS, as indicated by less homogenous ileal microbiota and dominant beneficial microbes in the ileum. In addition, it may contribute to improvement of apparent ileal digestibility of AA.

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Table 8.1. Ingredient composition of the control diet containing 20% distillers dried grains with solubles (DDGS) (as-fed basis)

Item	Control
Ingredient, %	
Corn	55.10
Soybean meal, 48%	23.00
DDGS	20.00
Limestone	1.05
Titanium dioxide	0.10
Salt	0.40
Vitamin-micro mineral premix[1]	0.30
Enzyme[2]	0.10
Calculated energy and nutrient levels	
ME, mcal ME/kg	3.33
Crude protein, %	21.00
Lysine, %	0.99
Calcium, %	0.60
Phosphorus, %	0.52
Available phosphorus, %	0.20

Table 8.1. (cont.)

[1]Provided per kilogram of diet: vitamin A, 11,121 IU as vitamin A acetate; vitamin D_3, 2,204 IU as D-activated animal sterol; vitamin E, 66 IU as alpha tocopherol acetate; vitamin K_3, 1.41 mg as menadione dimethylpyrimidinol bisulphate; thiamin, 0.24 mg as thiamine mononitrate; riboflavin, 6.58 mg; pyridoxine, 0.24 mg as pyridoxine hydrochloride; vitamin B_{12}, 0.031 mg; D-pantothenic acid, 23.5 mg as calcium pantothenate; niacin, 44mg; folic acid, 1.58 mg; biotin, 0.44 mg; choline, 0.924 mg as choline chloride; Cu, 10 mg as copper sulfate; Fe, 125 mg as iron sulfate; I, 1.26 mg as potassium iodate; Mn, 60 mg as manganese sulfate; Se, 0.30 mg as sodium selenite; Zn, 126 mg as zinc oxide.

[2]Enzymes (phytase, xylanase, and a combination between phytase and xylanase) were provided by Danisco Animal Nutrition, Marlborough, Wiltshire, UK.

Table 8.2. Effects of enzymes on the pattern of DGGE bands in ileal digesta and species identification of the specific DGGE bands by sequencing analysis

	Band No.[1]	**6**	**9**	**14**	**63**
Treatment[2]	**Similarity, %**[3]	***Number of pigs (of 8) showing the band***[4]			
Control		0	4	6	1
Phytase		4	1	2	2
Xylanase		1	0	2	5
Combination		1	1	2	1
Species	≥ 99	*Burkholderia cepacia*	*Pseudomonas sp.*	*Serratia sp.*	*Burkholderia sp.*
		Lactobacillus avarius	*Serratia sp.*	-	Serratia sp.

[1]Band number is for the specific band number in DGGE gel image.

[2]Control = control diet containing 20% DDGS; Phytase = 0.1% phytase treatment; Xylanase = 0.1% xylanase treatment; Combination = combination treatment between 0.05% phytase and 0.05% xylanase.

[3]Similarity is % for the sequences of a DGGE band to match the sequences of microbes by nucleotide basic local alignment search tool (BLAST) search against Genbank.

[4]About 70 bands were identified in DGGE gel image. The number for each treatment indicates the number of pigs that had the specific band of total 8 pigs in each treatment.

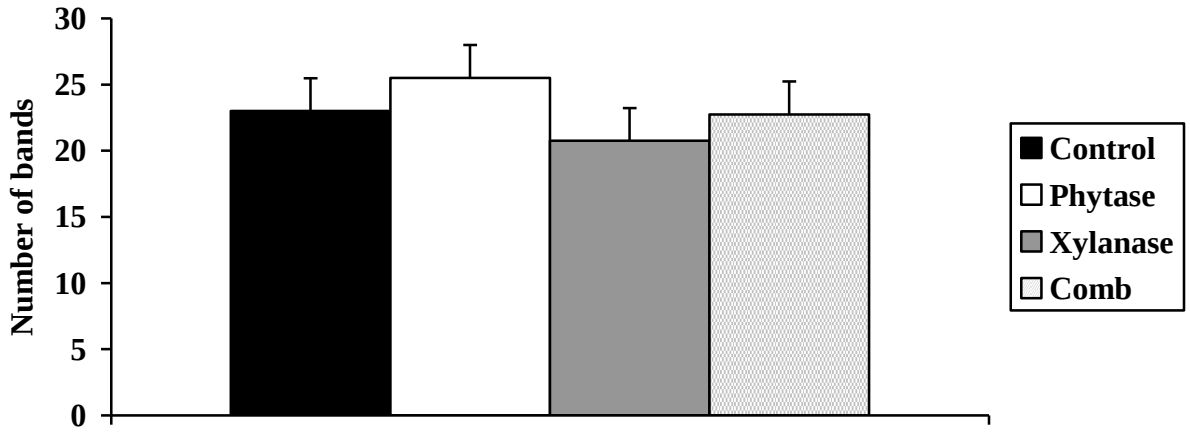

Figure 8.1. Effect of enzymes on the number of bands in ileum of pigs by DGGE analysis. Control is for control diet containing 20% DDGS, Phytase is for 0.1% phytase treatment, Xylanase is for 0.1% xylanase treatment, and Comb is for combination treatment between 0.05% phytase and 0.05% xylanase. There were no enzyme effects on the number of bands ($P > 0.05$).

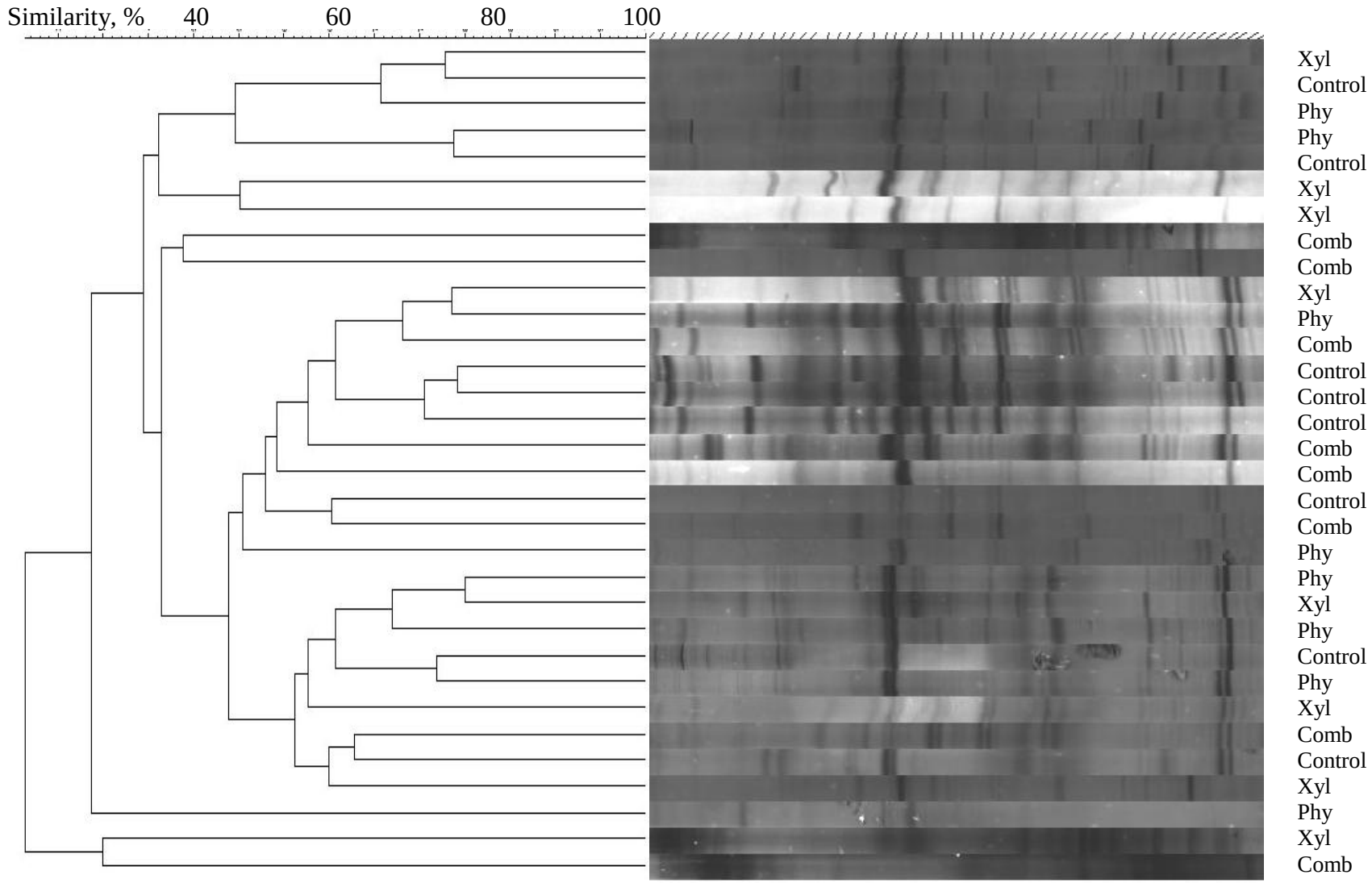

Figure 8.2. Effect of enzymes on ileal microbial ecology (dendrogram). Control is for control diet containing 20% DDGS, Phytase is

Figure 8.2. (cont.)

for 0.1% phytase treatment, Xylanase is for 0.1% xylanase treatment, and Comb is for combination treatment between 0.05% phytase and 0.05% xylanase.

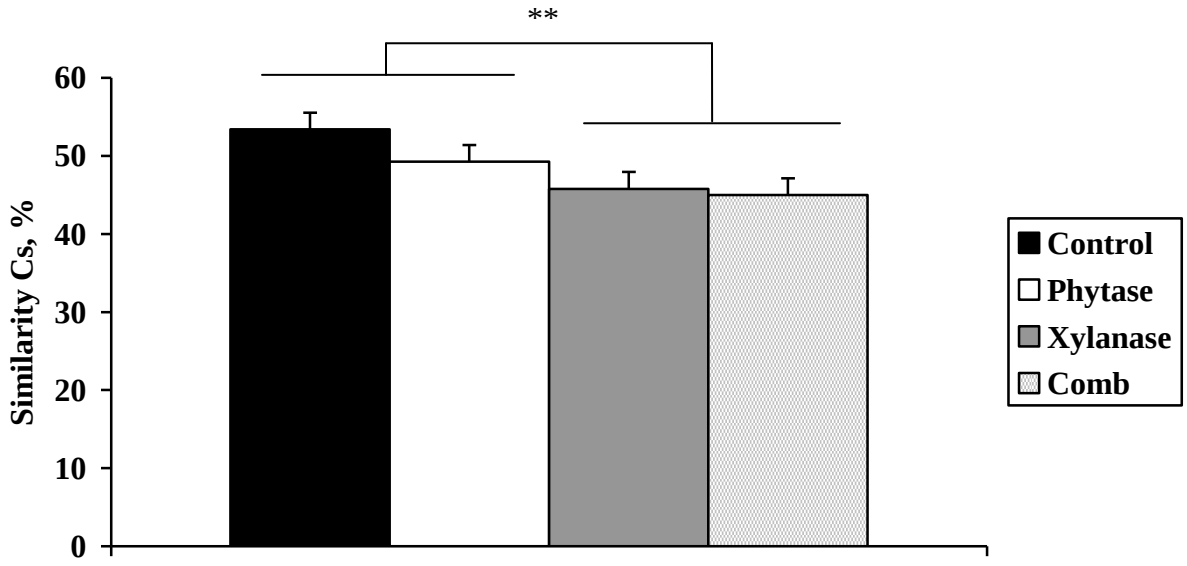

Figure 8.3. Effect of enzymes on the intratreatment (INTRA) Cs values in ileum of pigs by DGGE analysis. ** Xylanase reduced ($P < 0.05$) INTRA Cs values. Control is for control diet containing 20% DDGS, Phytase is for 0.1% phytase treatment, Xylanase is for 0.1% xylanase treatment, and Comb is for combination treatment between 0.05% phytase and 0.05% xylanase.

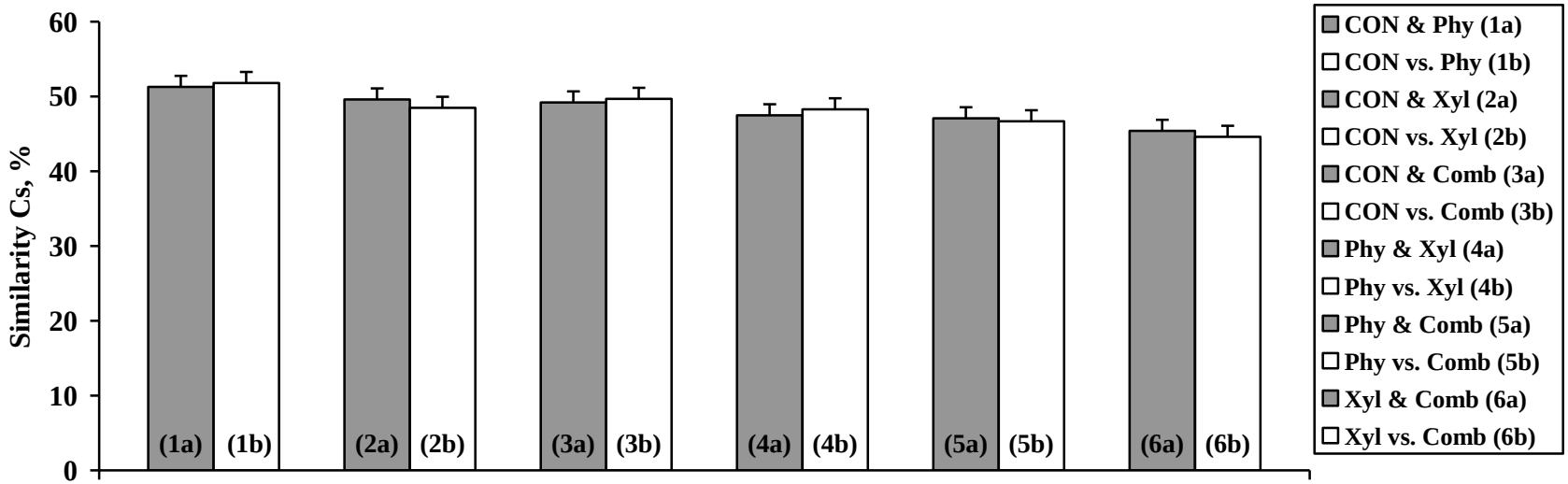

Figure 8.4. Effect of enzymes on the intertreatment (INTER) Cs values in ileum of pigs by DGGE analysis. Control is for control diet containing 20% DDGS, Phytase is for 0.1% phytase treatment, Xylanase is for 0.1% xylanase treatment, and Comb is for combination treatment between 0.05% phytase and 0.05% xylanase. (1) Comparison of similarity Cs values between average INTRA Cs values for CON and Phy (CON & Phy) and average INTER Cs value between CON and Phy (CON vs. Phy). (2) Comparison of similarity Cs values between average INTRA Cs values for CON and Xyl (CON & Xyl) and average INTER Cs value between CON and Xyl (CON vs. Xyl). (3) Comparison of similarity Cs values between average INTRA Cs values for CON and Comb (CON & Comb) and average INTER Cs value between CON and Comb (CON vs. Comb). (4) Comparison of similarity Cs values between average INTRA Cs values for Phy and Xyl (Phy & Xyl) and average INTER Cs value between Phy and Xyl (Phy vs. Xyl). (5) Comparison of similarity

Figure 8.4. (cont.)

Cs values between average INTRA Cs values for Phy and Comb (Phy & Comb) and average INTER Cs value between Phy and Comb (Phy vs. Comb). (6) Comparison of similarity Cs values between average INTRA Cs values for Xyl and Comb (Xyl & Comb) and average INTER Cs value between Xyl and Comb (Xyl vs. Comb). No differences were detected ($P > 0.05$

CHAPTER 9

GENERAL SUMMARY, DISCUSSION, AND CONCLUSION

Dietary factors, such as feed ingredients, feed additives, feed formulation practices, or feeding methods, are believed to be able to improve pig health as well as productive performance by modulation of microbial populations in the digestive tract and/or immune system and thus it is suggested that some dietary factors may be important components in pig health management programs along with practical health management practices. Therefore, the overall objective of these experiments was to evaluate whether some dietary factors can be useful in the presence or absence of antibiotics as they potentially improve pig health and/or growth performance by modulating microbial populations in the digestive tract and/or immune system of pigs.

It was needed to know how dietary antibiotics affect improvement of pig performance and health because it has not been fully understood although there are potential mechanisms of antibiotics. Thus, the first experiment evaluated whether or how dietary antibiotics modulate microbial populations in the digestive tract of pigs. Both virginiamycin and carbadox modified microbial populations in the digestive tract of pigs by eliminating some species of ileal microbes. These alterations of microbial populations may be an evidence how antibiotics improve growth performance of pigs.

More broadly, the potential economic benefits from using antibiotics for all phases of growing pigs as well as the breeding herd are reported to be significant. These economic benefits derive from increased growth and reproductive performance, and from decreased morbidity and

mortality. The swine industry is under pressure to reduce antibiotic use because of legitimate concern that use of antibiotics in animals may contribute to antibiotic resistance in pathogens that complicates treatment of sick people, although the magnitude of that contribution to the problem is not clear.

As compared with the effects of antibiotics, five studies addressed potential dietary factors, spray-dried plasma, clay, spray-dried egg, and enzymes, on pig health and growth performance. The second experiment evaluated whether dietary spray-dried plasma (SDP) improves pregnancy rate after transport stress using mated female mice as a model for stressed sows. The SDP markedly improved pregnancy rate after transportation stress. The result may support a potential role of SDP in improvement of sow farrowing rate, especially when stress causes inflammation.

The third experiment evaluated whether dietary SDP moderates inflammation and ameliorates impairment of reproduction caused by lipopolysaccharide (LPS) using pregnant mice as a model for inflammation in sows. The SDP attenuated inflammatory immune responses to LPS administration and markedly improved growth rate before and after acute inflammation, but it appears not to affect late-term pregnancy loss or fetal death after acute inflammation. The results may suggest that SDP can improve sow health and reproduction as it regulates inflammation.

However, these beneficial effects of SDP need verification in several practical conditions, such as using sows instead of mice, lower levels near 1% of SDP in sow diets, commercial conditions, etc., for their further application. In the swine industry now, SDP is widely used in nursery pig diets because of its consistent and clear beneficial effects as well as cost-effectiveness. If the potential beneficial effects of SDP in sow diets are shown in the practical

conditions, they may also contribute to potential economic benefits like those for nursery pigs and those from use of antibiotics.

The fourth experiment evaluated whether dietary clays reduce diarrhea of weaned pigs experimentally infected with a pathogenic *Escherichia coli*. The clays tested (smectite, kaolinite, zeolite, and their combinations) alleviated diarrhea of weaned pigs experimentally infected by a pathogenic *E. coli*, as indicated by reduction of diarrhea score, frequency of diarrhea, and populations of pathogenic *E. coli*, but did not affect growth rate. These results may suggest that clays can be a solution to reduce mortality of weaned pigs by post-weaning diarrhea which is one of the biggest problems in swine production, resulting in improvement of swine productivity. If a pig early in the nursery period is worth $30, and if adding clay to the diet reduced the mortality rate by 1 percentage unit, the economic benefit of the reduced mortality would average $0.30/pig placed. During the first 2 weeks or so after weaning, the pig may consume about 5 kg of diet on average. If clay were added to the diet at the level of 0.3% of the diet, it would require 0.015 kg of clay per pig. The breakeven cost of clay would then be $20/kg. The actual cost of clay is much lower than that. Therefore, the potential effect of clays may contribute to potential economic benefits when they are used during the first 2 weeks after weaning or even in the face of an outbreak of post-weaning diarrhea in commercial conditions.

The fifth experiment evaluated whether dietary spray-dried egg (SDE) can improve growth performance or health of weaned pigs. The SDE had no negative effect on growth rate and reduced frequency of medical treatments, but had some negative effects on G:F and did not affect mortality. These results may suggest that SDE can be an efficacious protein source in nursery pig diets by its nutrient contributions and physiological benefits, resulting in improvement of growth performance and health of weaned pigs. If the cost of medical treatment

were $0.20 per pig during the first week after weaning, the feeding SDE could reduce about $0.01 per pig of the cost of medical treatment based on the data from the commercial farm. If a pig consumed about 2 kg of the diet during the first week after weaning, and if 6% SDE ($1.37 per kg) replaced 3.38% unit of poultry byproduct meal ($0.74 per kg), 2% unit of soy protein concentrate ($1.14 per kg), and 1.66% unit of soybean oil ($1.04 per kg), the feeding SDE could increase the diet cost $0.03 per pig. Therefore, the potential effects of SDE may not contribute to potential economic benefits because feeding SDE increases the diet cost more than it reduces the cost of medical treatment. These estimates are sensitive to ingredient prices, but SDE is unlikely to change profit levels much.

The sixth experiment evaluated whether dietary enzymes modulate ileal microbial populations of pigs fed diets containing distillers grains with solubles (DDGS). Both phytase and xylanase enzymes changed ileal microbial populations of pigs fed DDGS. The most significant appeared to be the appearance of a *Lactobacillus* species, generally considered beneficial, when the phytase treatments were fed to pigs. These results may suggest that enzymes can contribute to pig intestinal health and further growth performance as it alters microbial populations. Due to the weak support for potential beneficial effects of enzymes beyond the improvement of the digestibility, potential economic benefits of enzymes cannot be drawn from these data.

In overall conclusion, based on the evidence of above potential benefits of dietary factors compared with the antibiotics effects, some dietary factors can be a kind of alternatives for antibiotics as they potentially improve pig health and/or growth performance by modulating microbial populations in the digestive tract and/or immune system of pigs. Therefore, it is suggested that some dietary factors may be important components in pig health management programs. However, one concern has to be considered that these potential health and economic

benefits may not derive from use of antibiotics and some dietary factors in the swine farms maintain good pig health conditions.

AUTHOR'S BIOGRAPHY

Minho Song was born in Seoul, South Korea. He received his Bachelor's degree in Applied Animal Sciences at the Korea University, Seoul, South Korea, 2003. After the completion of his Bachelor's degree, he joined the swine nutrition group in Animal Sciences at the University of Minnesota, Minneapolis-St. Paul, MN, Fall 2004, and received his Master's degree under the guidance of Dr. Samuel K. Baidoo, Spring 2007, focusing on effects of distillers dried grains with solubles on growth and reproductive performances, energy and nitrogen digestibility, and milk composition of lactating sows. The title of the Master's thesis was "Dietary effects of distillers dried grains with solubles on performance of lactating sows".

After the completion of his Master's degree, he joined the swine nutrition group in Animal Sciences at the University of Illinois, Urbana-Champaign, IL, Summer 2007, in pursuit of Ph.D. degree under the excellent guidance and advice of Dr. James E. Pettigrew, focusing on swine nutrition and health including evaluation of feed ingredients as well as dietary effects on pig health and growth performance by nutritional, microbiological, and/or immunological approaches. During the Ph.D. program, he made over 12 research grant proposals (6 grants funded) as a co-investigator, 7 research manuscripts and 9 abstracts with oral or poster presentations as a first author as well as 3 research manuscripts and 9 abstracts as a co-author, and 1 research article, received 1 graduate research paper award (3rd in Ph.D. poster competition), and was recognized as Graduate Fellow and Gamma Sigma Delta.

Minho Song married Younju Lee in 2004. He has a daughter, Yeonwoo, now and will have a son, Youngkwang as an assumed name, in July 2011.