ALTERATION TO GASTROINTESTINAL MICROBIAL COMMUNITIES ASSOCIATED WITH INCREASING FIBER DIGESTIBILITY

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ABSTRACT

Distillers dried grains with solubles (DDGS) is a byproduct of distillation. Its increased availability has led to research into overcoming the nutritional limitations of its high non-starch polysaccharide (NSP) content for use in monogastric animal feed. The purpose of this study was to examine the effects of two factors (the inclusion of DDGS and/or inclusion of a carbohydrase mixture) on the richness and abundance of swine gastrointestinal bacteria. The carbohydrase mixture was expected to aid digestion, leading to increased nutritional availability for the host while simultaneously shifting dominant communities within the gut microbiome.

Ileal cannulated pigs (n = 8, BW = 64.3 ± 0.5 kg) were allotted to 4 dietary treatments in a replicated 4 × 4 Latin square design. Treatments resulted in changes to bacterial richness, and diets containing DDGS increased the 16S rDNA abundance of members of *Bacteroides* ($P < 0.0001$), *Ruminococcus* ($P < 0.0001$), the *Clostridium coccoides* group ($P < 0.0001$), and the *Clostridium leptum* subgroup ($P = 0.005$). Significant interactions between diet and carbohydrases were determined for total bacteria in the ileal digesta ($P = 0.01$) and feces ($P = 0.02$), *Bacteroides* ($P = 0.003$), and the *Clostrdium leptum* subgroup ($P = 0.03$). The DDGS diet with the inclusion of the carbohydrases was able to maintain, or increase the abundance of fiber degrading bacteria while theoretically increasing nutrition for the host.

ATTRIBUTION

Several people contributed to the completion of the research described in this thesis. Descriptions of their contributions are included below.

Monica A. Ponder - Ph.D. (Department of Food Science and Technology, Virginia Tech) Primary advisor and committee chair. Provided guidance for development and implementation of thesis research project.

Jeffery Escobar – Ph.D. (Novus International, Inc.) Committee member. Performed animal surgeries. Actively involved with the design and implementation of the animal trial as well as the data analysis.

Andrew Neilson – Ph.D. (Department of Food Science and Technology, Virginia Tech) Committee member. Provided feedback and support throughout project duration.

Hanbae Lee – Ph.D. (Easy Bio, Inc.) Fellow graduate student. Lead the animal trial, performed animal care, and assisted with sample collection.

Dianne Bourne (Department of Food Science and Technology, Virginia Tech) Lab research specialist. Assisted with all aerobic and anaerobic bacterial culturing.

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LIST OF ABBREVIATIONS

ADG: Average daily gain

AGP: Antibiotic growth promoters

BW: Body weight

CHO: Carbohydrate

CP: Crude protein

DDGS: Dried distillers grains with solubles

DG: Distillers grain

DGGE: Denaturing gradient gel electrophoresis

GIT: Gastrointestinal tract

ME: Metabolizable energy

NSP: Non-starch polysaccharide

PCR: Polymerase chain reaction

SBM: Soybean meal

SEM: Standard error of the mean

CHAPTER 1: INTRODUCTION AND JUSTIFICATION

Studies of the swine distal gut metagenome have shed light on the diversity and high occurrence of antibiotic resistance mechanisms employed by the microbiome (Lamendella et al., 2011). Continued development of antimicrobial resistance and transference of antibiotic resistance genes from animal to human microbiota is a worldwide concern (Mathur and Singh, 2005; Salyers et al., 2004). Antibiotic growth promoters (AGP) are still actively used in commercial swine diets, but pressure is being put on the industry to seek alternatives (Berg, 1998; Turner, 2001).

Diet modifications have been shown to yield significant changes in gastrointestinal microbiota. Mice switched from a low-fat, plant polysaccharide-rich diet to a "western" high-fat, high-sugar diet resulted in fecal microbiota alterations within 24 hours (Turnbaugh et al., 2009). In humans, notable shifts were observed in the fecal microbiota in as little as one day when their high-fat/low-fiber diet was substituted with a low-fat/high-fiber diet (Wu et al., 2011). These rapid modifications demonstrate the feasibility of using diet design to encourage the growth of health-promoting microbial species. Foods that target and modify the GIT activity, such as probiotics, prebiotics, and synbiotics have proven to be an important area of research (Salminen, 1998) and have gained substantial attention in animal nutrition due to their potential to completely replace antibiotics in feed (Forestier et al., 2001; Hopkins and Macfarlane, 2003). In pigs, altering the microbiota of the gut can result in more efficient digestion and absorption of nutrients, while also increasing resistance to infectious diseases (Kyriakis et al., 1999).

Beginning in the 1970s, ethanol plant construction lead to increased production of dried distillers grains with solubles (DDGS) and research into its use in livestock feed

(Cromwell et al., 1983; Smelski, 1973; Wahlstrom et al., 1970). DDGS is a high fiber, co-product of distillation (Stein and Shurson, 2009). It is a combination of unfermented nutrients such as oil, protein, and minerals that have increased 3-fold in comparison to the original feedstock (Liu, 2011). Because of its high non-starch polysaccharide content (NSP, 35% insoluble fiber vs. 6% soluble fiber), most of its nutrition is derived from bacterial fermentation either in the large intestine of monogastrics or the rumen of ruminant animals (Stein and Shurson, 2009). There are many studies that examine the effects of DDGS on swine nutrition, but very little information is available regarding its effect on intestinal microbiota.

The purpose of this study was to examine the effects of two exogenous factors (the inclusion of DDGS and/or inclusion of a carbohydrase mixture) that were hypothesized to alter microbial communities within a porcine model. This project was a companion study to the work performed by H. Lee et al. (2012) measuring growth, digestibility, and amino acid usage. A novel mixture of carbohydrases (i.e., mixture of cellulase, hemicellulase, β-glucanase, and xylanase) was incorporated into diets containing a high inclusion of corn DDGS (40%). The enzyme mixture was expected to aid digestion, leading to increased nutritional availability for the host while simultaneously shifting dominant communities within the gut microbiome. Most of these communities cannot be cultured through conventional techniques but are identifiable via molecular methods (Shanahan, 2002). In order to effectively characterize treatment-induced change, molecular techniques based on the 16S rDNA were used (Amann et al., 1995; Mai and Morris, 2004; Zoetendal et al., 2004). Denaturing gradient gel electrophoresis (DGGE) identified changes in bacterial richness associated with diet,

while quantitative real-time PCR (qPCR) provided an efficient measure to compare the abundance of specific bacterial groups hypothesized to change with the inclusion of the DDGS fraction.

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CHAPTER 2: LITERATURE REVIEW

Introduction

Digestion occurs through physical and enzymatic processes performed by the host. Despite extensive enzymatic hydrolysis occurring within the small intestine, a portion of dietary breakdown occurs via microbial fermentation within the large intestine (Ewing, 1994; Williams, 2001). Microbial presence within the gastrointestinal tract benefits host health by assisting in nutrient extraction, immune system and epithelium development, and defense against pathogens (Zoetendal et al., 2004). Studies of germ-free animals reveal the significant impact of commensal microbiota presence on intestinal structure and function. Germ-free animals tend to be more susceptible to infection by having reduced digestive enzyme activity, gut motility, mucosal cell turnover, muscle cell wall thickness, cytokine production and serum immunoglobulin levels, smaller Peyer's patches, and fewer intraepithelial lymphocytes, but have increased enterochromaffin cell area (O'hara and Shanahan, 2006; Shanahan, 2002).

Diet modifications have been shown to yield significant changes in gastrointestinal microbiota (Turnbaugh et al., 2009; Wu et al., 2011). There has been growing interest in the inclusion of dietary fibers in livestock feed due to their potential prebiotic effects and promotion of beneficial bacteria (Verstegen and Williams, 2002). Cereal grains are already a major component of pig diets and can be selected based on the type and amount of dietary fiber in order manipulate the microbial composition of the gut (Knudsen, 2001; Pieper et al., 2008). In pigs, modifying this composition has the

potential to yield more efficient digestion and absorption of nutrients, while also increasing resistance to infectious diseases (Kyriakis et al., 1999).

Microbial Ecology of the Swine Gut

The mammalian gut microbiota is remarkably similar among different species, and although it experiences fluctuations, the core populations remain relatively stable (Gaskins et al., 2008). Animal models are important for studying microbial niches of the gut because they allow for better control of the environment and treatments (Cilieborg et al., 2012). Pigs are commonly used as model organisms for human beings. In comparison to rodents, they have greater structural and physiological similarities of gastrointestinal organs as well as potential colonizing bacteria (Sangild, 2006). Additionally, the composition of the human and swine fecal microbiota have been shown to be ~70% similar to each other, as demonstrated through high-throughput sequencing of the bacterial 16S rRNA gene (Lamendella et al., 2011). However, unlike humans, pigs posses many bacterial species adapted for fiber fermentation, thus in some ways the pig large intestine more closely resembles a rumen than a human intestine (Lamendella et al., 2011; Ouwehand and Vaughan, 2006).

Pigs are monogastric animals, meaning their gastrointestinal tract (GIT) is comprised of a single-chambered stomach for the breakdown and processing of food (Hasheider and National, 2008). At birth, a piglet's GIT is virtually sterile; however, contact with sow feces and the environment results in rapid microbial colonization (Ducluzeau, 1985; Pluske et al., 2003). Post-weaning bacterial colonization of the gut is subject to the composition of native residents, the degree of bacteria present on ingested

feed, the fermentation of digesta, and the sow's skin and pen environment. The porcine GIT is colonized by more than 400 different microbial phylotypes (Hill, 2002; Leser, 2002) with the majority comprised of Gram-positive anaerobes with low guanine-cytosine (GC) content (Cotta et al., 2003; Whitehead and Cotta, 2001). The distal portion of the small intestine contains 7-9 log CFU/gram of fresh material of bacteria with populations increasing to 10-11 log CFU/gram of fresh material closer to the colon (De Angelis et al., 2006). Genera present most numerously in the colon include *Bacteroides*, *Selenomonas*, *Butyrivibrio*, *Lactobacillus*, *Peptostreptococus*, and *Eubacterium* (Ouwehand and Vaughan, 2006). Similar to a ruminant, the swine microbiota is also comprised of highly active cellulolytic and hemicellulolytic bacterial species, such as *Fibrobacter succinogenes* (*intesitnalis*), *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Butyrivibrio species*, *Prevotella* (*Bacteroides*) *ruminicola*, and *Clostridium herbivorans*. These populations have been shown to increase in response to the consumption of plant cell wall material (Ouwehand and Vaughan, 2006).

Microbial Community Analysis Techniques

16S Ribosomal RNA

Fecal samples are often used for studying intestinal microbial communities because of the ease of collection. Stool samples represent a combination of bacteria from shed mucosa surface and the separate, nonadherent luminal populations (Eckburg et al., 2005). The diversity of these populations is defined by species richness, which is the number of species in a given area; species evenness, which is the relative abundance of various species; and species composition, which are the actual species present within a sample (Forney et al., 2004). The 16S ribosomal RNA (rRNA) gene has been identified

as a molecule with a sequence that reflects the phylogenetic position of a given bacterial taxonomic unit. It contains highly conserved regions of base sequences with interspersed hypervariable regions that reflect evolutionary divergence, allowing for classification of sequences based on their similarity to other sequences of known bacteria within a database (Shanahan, 2002). In comparison to culturing, technologies targeting the 16S rRNA gene, such as fluorescent *in situ* hybridization (FISH), denaturing gradient gel electrophoresis, quantitative dot blot hybridization, and restriction length polymorphism are more effective at characterizing complex gut microbial communities (Amann et al., 1995; Mai and Morris, 2004; Zoetendal et al., 2004).

Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is used to examine changes in microbial communities based upon electrophoresis of small PCR amplicons through an acrylamide gel formed with the denaturing chemicals formamide and urea. The migration capability of the DNA is based on the nucleic acid sequence of the product; GC rich sequences require more energy to denature and thus travel further through the gel (Justé et al., 2008; Muyzer, 1993). Migration can be modified through the incorporation of GC rich sequences within primers to prevent complete denaturation (Muyzer, 1993; Sheffield et al., 1989). A highly stable GC-clamp, in combination with an optimal gradient, has the theoretical potential to detect differences as little as a single base pair (Ogier et al., 2002; Sheffield et al., 1989). The 16S rRNA gene-based DGGE is frequently used for characterizing microbial community changes within the GIT, however DGGE is limited by its lack of reproducibility (Powell, 2005). Heterogeneity of the *rrn* operon copy number and sequence can result in multiple bands being produced by a single bacterial

species and thus an overestimation of richness (Klappenbach et al., 2000; Makarova and Koonin, 2007). Species with closely related sequences may co-migrate, forming a single band and resulting in an underestimation of richness (Simpson et al., 1999). Limitations can be overcome by band excision, further amplification, cloning and sequencing in order to identify individual species without the need for a culturing step (Shanahan, 2002). DGGE is only capable of obtaining qualitative information about population composition (Muyzer, 1993), so it is often run in combination with a quantitative methodology (Justé et al., 2008).

Real-Time Polymerase Chain Reaction

Real-time PCR is the monitoring of fluorescent signals produced by polymerase chain reactions over a range of cycles. Quantitative real-time PCR (qRT-PCR) is a technique that uses the conversion of these fluorescent signals into a numerical value (Dorak, 2006), and has shown to be a powerful tool for quantifying bacterial abundance in the complex environment of the gut (Xiang et al., 2011). qRT-PCR has proven more reliable than other methods such as single-strand conformation polymorphism analysis, temperature gradient gel electrophoresis, and fluorescence in situ hybridization (Fang et al., 2002; Nadkarni et al., 2002). It enables the detection of a PCR product without the necessity of post-PCR processing and allows for accurate template quantification over a broad range (Bustin et al., 2005). The initial concentration of the DNA is linked to a precise threshold cycle, defined as the cycle number at which fluorescence increases above the background level. Target DNA is quantified using a calibration curve that relates threshold cycle to the exact template concentration (Lievens et al., 2005; Mackay, 2004). qRT-PCR results are available immediately after amplification, thus reducing

potential contamination that may occur while loading agarose gels as well as saving time (Hein et al., 2006; Schmittgen, 2001). Results are easily reproducible and highly sensitive (Nakatsu et al., 2000; Smith and Osborn, 2009).

Functional Foods

Metagenomics has revolutionized microbiology through the use of culture-independent techniques and characterization of microbial communities present in complex ecosystems (Simon and Daniel, 2011). Studies of the swine distal gut metagenome have shed light on the diversity and high occurrence of antibiotic resistance genes. Lamendella et al. (2011) found that nearly 6% of assigned metagenomic reads were involved in antibiotic resistance, and more than 12% were classified as genes associated with multi-drug resistance. These genes act as potential sources for the transfer of drug resistance to other bacteria, including potential human pathogens. Continued development and transference of antibiotic resistance genes is a worldwide concern (Mathur and Singh, 2005; Salyers et al., 2004). Presently, antibiotic growth promoters (AGP) are actively used in commercial swine diets, but pressure is being put on the industry to seek alternatives (Berg, 1998; Turner, 2001).

"Functional food," is a term used to describe foods with suspected or proven health benefits that are able to surpass basic health nutritional requirements (Roberfroid, 1999). Diet supplements that modify the activity of the GIT have proven to be an important area for research and development. (Salminen, 1998). In particular, probiotics and prebiotics have gained substantial attention in swine nutrition due to their potential to completely replace antibiotics in feed (Forestier et al., 2001; Hopkins and Macfarlane

2003). A probiotic is typically defined as a live microbe, that when administered in adequate amounts, benefits host health. Strains of *Lactobacilli* and *Bifidobacteria* are the bacterial groups most frequently investigated for use as probiotics (Roberfroid, 2000). A prebiotic is a non-digestible, but fermentable food substance that benefits host health by selectively stimulating the growth or activity of specific bacteria within the colon (Gibson et al., 2004; Quigley, 2012). Synbiotics are a mixture of probiotics and prebiotics (Gibson and Roberfroid, 1995).

Colonic bacteria are strict anaerobes, deriving their energy from fermentation. The two major fermentative substrates obtained through the diet are non-digestible carbohydrates and proteins that escaped digestion in the small intestine. Non-digestible carbohydrates include resistant starches, non-starch polysaccharides (NSP), dietary fibers, and non-digestible oligosaccharides originating from plants (Lupton, 2004; Topping and Clifton, 2001). Not all non-digestible carbohydrates and dietary fibers are prebiotics. In order to be classified as a prebiotic, ingredients must be: resistant to gastric acidity, not be hydrolyzed by mammalian enzymes, not be absorbed in the upper gastrointestinal tract, be fermented by the intestinal microbiota, and selectively stimulate either the growth or activity of potentially beneficial bacteria (Gibson and Roberfroid, 2008).

Fiber is a broad term used to describe a wide range of components with varying physiochemical properties and physiological effects (Leterme et al., 2000). Dietary fiber includes cellulosic and non-cellulosic polysaccharides such as pectins, hemicelluloses, mucilages, and gums. These polymers primarily consist of glucose, galactose, arabinose, xylose, and galacturonic acids (Cummings and Branch, 1986; Englyst et al., 1987). Total

fiber digestibility varies considerably based on the nature of the fiber and the animal species. Fiber digestibility in chickens is less than 10%; however pigs have been shown to digest fibers as well as sheep and may use it to contribute up to 30% of their maintenance energy needs (Ouwehand and Vaughan, 2006). Fiber is now becoming a popular inclusion in pig diets due to its potential prebiotic effects (Verstegen and Williams, 2002).

Cereal grains such as barley and oat are potential sources of prebiotic carbohydrates, especially mixed-linked β-glucan and resistant starch (Topping, 2007). In Europe, cereals provide more than half the feed protein in pig diets (Leterme et al., 2000). Cereals such as barley and oats are composed predominately of carbohydrates, which are a chief component of pig diets and because they are enzymatically indigestible, serve as the main substrate for bacterial fermentation (Knudsen and Hansen, 1991). Dietary carbohydrate composition can alter swine gastrointestinal microbiota based on substrate preference of bacteria (Castillo et al., 2007). Different varieties of non-starch polysaccharides (NSP) in a swine diet can manipulate microbiota based on reduced digestibility of nutrients within the small intestine (Dikeman and Fahey, 2006), which can result in either prevention or stimulation of pathogen growth (Hopwood et al., 2002). Metzler-Zebeli et al. (2010) tested the impact of NSPs with varying fermentabilities and starch concentrations on swine intestinal microbiota. High-viscosity digesta impaired intestinal contractions, which inhibited mixing of gut contents and thus the ability of bacteria to locate new substrates for breakdown. In comparison to its low-viscosity counterpart, high-viscosity carboxymethylcellulose was poorly fermented, resulting in significant reductions in fecal short chain fatty acid concentrations; however, species

richness was increased. In a similar study, Pieper et al. (2008) attempted to manipulate the composition of swine gut bacteria through the use of different sources of dietary fiber. Findings indicated that the addition of β-glucan as a purified supplement to common hulled barley had little effect on species richness and resulted in a slight increase in the presence of ileal lactobacilli when compared to the hulled barley control. In contrast, piglets fed a diet consisting of hulless barley with high levels of the naturally occurring mixed-linked β-glucan decreased microbial species richness, and significantly reduced lactobacilli numbers in the small intestine. Reasons for this change were unclear, but it was hypothesized to be a result of the favoring of bacterial strains that display β-glucanase activity. The results of this study shed light on the importance of physical form and possible interactions with other nutrients in the matrix of grain, as well as, animal-related factors such as gastric emptying rate or altered production of bile acids that can also play a role.

Dried Distillers Grains with Solubles (DDGS)

Distillers grains (DG) are a cereal co-product of the distillation process that occurs in both fuel-ethanol plants and breweries producing beverage ethanol. Starch in grain flour is converted into ethanol and carbon dioxide, while the protein, lipids, fiber, minerals, and vitamins remain chemically unchanged, but increase concentration. These components form a co-product, commonly known as distillers grains with solubles (DDGS) (Liu, 2011). DDGS is low in starch and high in non-starch polysaccharides (NSP, 35% insoluble fiber vs. 6% soluble fiber) (Stein and Shurson, 2009). Beginning in the 1970s, construction of ethanol plants resulted in increased DDGS production and led

to research into its use in animal feed (Cromwell et al., 1983; Smelski, 1973; Wahlstrom et al., 1970). Approximately two-thirds of the starting material is converted into ethanol and carbon dioxide during dry-grind processing, thus concentrations of unfermented nutrients such as oil, protein, and minerals, increase by 3-fold in comparison to the original feedstock. This makes DDGS a rich source of protein, amino acids, phosphorus, and other nutrients desired in animal feed (Liu, 2011). Cromwell et al. (1983) showed that up to 40% of a corn diet could be replaced by DDGS before there was a decrease in average daily gain (ADG) in grower-finisher pigs.

Diets of DDGS are likely to cause an increase in *Bifidobacteria* and *Lactobacilli*, which are capable of utilizing isolated cereal β-glucans found in the NSP fraction, depending on the degree of polymerization (Jonsson and Hemmingsson, 1991). Pieper et al. (2008) found that pigs fed high-β-glucan barleys had almost non-detectable levels of *Enterobacteria* and *Streptococci* in the ileum. This could potentially be beneficial, especially for susceptible animals such as piglets. Using colonic bacteria isolated from pigs, Jha et al. (2011) was able to show that fermentation of DDGS resulted in higher levels of total SCFA production/g CHO fermented when compared to other common feed ingredients due to its greater composition of indigestible protein available for bacterial fermentation.

Pigs are predominately fed a corn-based diet, which is why in the United States there is a direct correlation between the geographical distribution of swine and the acreage of corn (Holden and Ensminger, 2006). Typical commercial swine diets are composed of 63.5% corn, 28% soybean meal, 5.2% fat, 1.9% deflourinated phosphate, 0.9% vitamins, 0.35% NaCl, 0.25% $CaCO_3$ and 0.05% $CuSO_4$ (Katouli and Wallgren,

2005). At 22.7%, DDGS is high in total non-starch polysaccharide (NSP) content (Ward et al., 2008), especially when compared to the 6.1% of corn and 12.6% of soybean meal (Cowieson and Adeola, 2005). Recommendations for DDGS inclusion levels for swine are: 20-30% for nursery to finishing pigs, 20-30% for lactating sows, and 40-50% for gestating sows (Shurson and Noll, 2005; Stein, 2007). Vertebrates lack production of carbohydrases to digest NSP, and limited fermentation occurs prior to the small intestine, except in ruminants, so most of the nutrition is derived from bacterial fermentation in the hindgut.

Butyrate

Short chain fatty acids (SCFAs) are produced through fermentation of luminal, non-digestible dietary fibers and are predominately comprised of butyrate, acetate, and propionate (Hamer et al., 2008). Increasing the number of butyrate-producing bacteria within the large intestine has gained interest due to their potential role in colonic health (Pryde et al., 2002; Wachtershauser and Stein, 2000). Already known as an epithelial cell energy source, butyrate is now being studied for its potential anti-carcinogenic and anti-inflammatory effects, as well as its involvement in the intestinal barrier and role in satiety and oxidative stress (Hamer et al., 2008).

Butyrate-producing ability has considerable variability based on environmental factors such as pH and diet (Duncan et al., 2007; Schwiertz et al., 2002; Topping and Clifton, 2001; Walker et al., 2005). Fermentation of dietary fiber is frequently associated with production of butyrate, but fermentation efficiency can be altered by chemical structure such as solubility and degree of polymerization. Soluble fibers are highly

fermentable and thus more likely to generate higher quantities of SCFAs within the colon (Hamer et al., 2008). Larger concentrations of butyric, propionic, and acetic acid were obtained from an *in vitro* fecal fermentation of guar gum, a water-soluble dietary fiber, compared to a fecal culture without additional prebiotic. In addition, larger concentrations of propionic and butyric acid were obtained when compared to a fecal culture supplemented with inulin, an alternative prebiotic fiber (Ohashi et al., 2012). Interestingly, no statistical differences were seen in abundance of the *Clostridium leptum* subgroup, which is known to contain high numbers of butyrate-producing and/or fibrolytic bacteria (Pryde et al., 2002; Robert and Bernalier-Donadille, 2003). Targeting 16S rDNA to identify butyrate producers has proven difficult because both butyrate and non-butyrate producers are found within the same phylogenetic clusters belong to *Clostridium* clusters I, III, IV, XI, XIVa, XV, and XVI (Louis et al., 2007). Thus, the novel approach of looking at the expression of butyrate production pathway genes has proved valuable.

In the final step of butyrate synthesis from butyryl-CoA, two alternative pathways have been identified. In the first, phosphotransbutyrylase and butyrate kinase convert butyryl-CoA to butyrate with butyrl-phosphate forming as an intermediate. Alternatively, a butyryl-CoA:acetate CoA-transferase transfers the CoA component to external acetate, leading to the formation of acetyl-CoA and butyrate (Duncan et al., 2002; Walter et al., 1993). Louis et al. (2004) found that out of 38 anaerobic, butyrate-producing human fecal isolates, only 4 strains possessed detectable butyrate kinase activity. This indicates that the butyrate kinase operon is not widespread among human butyrate-producing bacteria and that a majority of human colonic butyrate-producers use butyryl-CoA transferase for

the last step of butyrate formation. Similarly, in a study of the impact on NSP on butyrate production, the main route of butyrate formation in the hindgut of pigs was also the butyrl-CoA transferase pathway. A direct correlation was found between this pathway and the ileal flow of dry matter into the large intestine. In contrast, butyrate kinase was negatively correlated with the ileal flow of fermentable substrate (Metzler-Zebeli et al., 2010).

Conclusions

Modulating the gastrointestinal microbial communities through the administration of a prebiotic compound has the potential to promote host health. Although very similar to humans, pigs possess a gut microbiome more adapted for the metabolism of fibrous compounds. Examining the fluctuations of specific community members during the consumption of prebiotics as well as an enzyme to promote fiber breakdown will provide insight into the porcine intestinal tract and how it reacts to diet alterations.

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CHAPTER 3: SWINE GUT BACTERIAL COMMUNITY SHIFTS ASSOICATED WITH A HIGH CORN DDGS DIET SUPPLMENTED WITH A NOVEL MIXTURE OF CARBOHYDRASES

INTRODUCTION

The intestinal microbiota and its metabolic activities are important factors for the health and performance of growing pigs (Lewis, 2001). Knowledge of dietary influences on microbial activity and their possible implications on gut health is an important criterion for feed ingredient selection (Niba et al., 2009). Addition of prebiotics, non-digestible ingredients that are fermented by intestinal bacteria, can benefit the host health by selectively stimulating the growth or activity of specific bacteria within the hindgut (Gibson et al., 2004; Quigley, 2012). Prebiotics include non-starch polysaccharides (NSPs), resistant starch, and non-digestible oligosaccharides (Bauer et al., 2006).

Dried distillers grain with solubles (DDGS) is a byproduct of corn distillation that contains a high fraction of NSPs (Stein and Shurson, 2009). Ethanol plants constructed in the 1970s lead to increased production of DDGS and thus research into its use in animal feed (Cromwell et al., 1983; Smelski, 1973; Wahlstrom et al., 1970). Non-starch polysaccharide inclusion in pig diets has been shown to modulate intestinal microbiota composition (Metzler-Zebeli et al., 2010); however, partial degradation of these fractions by exogenous enzymes may improve the nutritional value for the pig (Ao et al., 2010; Högberg and Lindberg, 2004).

The aim of the present study was to examine the effects of two exogenous factors that were hypothesized to alter microbial communities within a porcine model. A novel mixture of carbohydrases (i.e., mixture of cellulase, hemicellulase, β-glucanase, and xylanase) was incorporated into diets containing a high inclusion of DDGS (40%). The

enzyme mixture was expected to aid digestion, leading to increased nutritional availability for the host while simultaneously shifting dominant communities within the pig gut microbiome. Denaturing gradient gel electrophoresis (DGGE) identified changes in bacterial richness based on diet, while quantitative real-time PCR (qPCR) was used to compare the abundance of specific bacterial groups hypothesized to change with the inclusion of the DDGS fraction.

MATERIALS AND METHODS

Animals, housing, and diets

All experimental procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. A total of 8 Duroc x (Landrace x Yorkshire) pigs (4 barrows/4 gilts) were obtained from the Swine Center at Virginia Tech (Blacksburg, VA) at approximately 93 d of age (BW = 47.0 ± 1.5 kg). Pigs were fitted with a simple T-cannula at the distal ileum, approximately 5 cm cranial to the ileo-cecal junction. A detailed description of cannula preparation, surgical procedure and postsurgical care was previously given by Wubben et al. (2001). One ml of ceftiofur-HCl (Excenel RTU, 50 mg ceftiofur/ml, Pfizer Animal Health, Fort Dodge, IA) was administered per 10 kg BW i.m. for 3 days following surgery. Neomycin sulfate (NeoMed 325, 0.5 g neomycin/g, Bimeda, Inc., Oakbrook terrace, IL) and carprofen (Rimadyl, 75 mg carprofen/tbl, Pfizer Animal Health, Fort Dodge, IA) were ground and mixed into feed for 6 days post-surgery at 2 g and 300 mg per day, respectively. Pigs were allowed to recover for 3 weeks in individual pens (1.5 × 1.5 m) equipped with a feeder and nipple waterer. Feed allotments were increased in 200 g increments over 7 days until a daily intake of 1.4 kg was attained. Skin sutures were removed 13 days after surgery. After a 3 week recovery

period, the average BW of the pigs increased to 51.0 ± 1.4 kg. Following the recovery period, feed allotments were calculated as 3 times the estimated energy requirement for maintenance (i.e., 106 kcal ME/kg $BW^{0.75}$; NRC, 1998), divided into 2 equal meals given at 0800 and 1600. The facility was independently ventilated with 100% clean air (i.e., no recirculation) under negative pressure. Temperature (24°C) and lighting (18-hours light:6-hours dark with lights on at 0600) were controlled by automated systems (Lee et al., 2012).

Sample Collection

On day 191 of age, pigs (BW = 64.3 ± 0.5 kg) were moved into metabolic cages (0.6 × 1.4 m) and allotted to 4 dietary treatments (Table 3.2) in 4 × 4 Latin Square design involving 4 periods with 2 replicates. Each period consisted of a washout, adaptation, digesta, and fecal stage (Table 3.1). Pigs were rotated among four diets, a control and three experimental diets (Table 3.2). The 7 day washout stage was used to equalize the microbial community between periods by feedings pigs a predominately corn-based diet similar to those seen in commercial production (Lewis, 2001). After washout, pigs were randomly assigned to 1 of the 4 experimental diets and samples were collected.

During the Adaptation and Fecal stages of the study, fecal samples were collected daily, no more than 12 hours after defecation. Samples were then stored in a -80°C freezer until processed. Following a 5 day adaptation period, digesta was collected for 2 days from 0800 to 1800 into plastic bags attached to the cannula with a rubber band. Subsamples of digesta (0.5 ml) were taken from the plastic bags when they were changed either when full or after 30 min to minimize bacterial fermentation. Samples were

immediately mixed with Ambion® RNAlater in a 1:2 ratio (Life Technologies Corporation, Carlsbad, CA**)** (Popova et al., 2010) and frozen in a -80°C freezer until processed.

Community Profiling

Fecal DNA Extractions

Fecal samples collected on treatment days 4-5 from each animal on each diet were pooled (n=32) and used to represent the Adaptation stage. Fecal samples collected on treatment days 8-9 from each animal on each diet treatment, were pooled (n=31) and used to represent the Fecal stage. Samples were homogenized in a 1:10 ratio using 0.01% peptone water (Sigma-Aldrich, St. Louis, MO) and a lab blender (Bag Mixer, Interscience, Weymouth, MA). DNA extractions were performed using the Mo Bio UltraClean® Fecal DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) per manufacturer's instructions.

Digesta DNA/RNA Extractions

Extractions from pooled digesta collected on treatment days 6-7 were performed using the Qiagen AllPrep DNA/RNA Micro Kit (Qiagen, Valencia, CA), incorporating additional chemical and mechanical lysis steps to better target microorganisms in the gut. Each sample consisted of 0.5 ml of pooled digesta in RNAlater that was placed in a 1.5 ml microcentrifuge tube and centrifuged for 10 min at 10,000 × g. The supernatant was then removed and 50 µl of DEPC treated tris-EDTA (TE) buffer was added. Tubes were vortexed vigorously for 10 sec and 5 µl of mutanolysin (1 U/µl) (Sigma-Aldrich, St. Louis, MO) was added. Samples were incubated for 30 min shaking (125 rpm) at 37°C in

an Innova® 42 (Eppendorf, Enfield, CT) incubator with vortexing every 5 min throughout incubation. After mutanolysin lysis, 20 µl of Qiagen Proteinase K (20 mg/ml) (Qiagen, Valencia, CA) was added and incubated for 30 min shaking (125 rpm) at 37°C with vortexing every 5 min throughout incubation. After incubation, 700 µl of Qiagen RLT Plus (Qiagen, Valencia, CA) was added. Samples were vigorously vortexed for 10 sec and the lysate transferred into a fresh, 1.5 ml freestanding Screw-Cap Microcentrifuge Tubes (VWR®, Radnor, PA) containing a 100 mg mixture of 0.5 mm, 0.1 mm and 2.3mm disruption beads (Research Products International, Mount Prospect, IL). Cells were mechanically disrupted in a Mini-Beadbeater-1 (Biospec Products Bartlesville, OK) for 5 min at max speed. Samples were then centrifuged for 10 sec at 10,000 × g in a Sorvall Legend Micro 21 Centrifuge (Thermo Scientific® Marietta, OH). Supernatants were transferred to Qiagen AllPrep DNA spin columns placed in 2-ml collection tubes. Samples were centrifuged for 30 sec at 8,000 × g. The AllPrep DNA spin columns were then placed in new 2-ml collection tubes and stored at 4°C for later DNA purification, while the flow-through was used for RNA purification. The remainder of extraction was done based on manufacturer's instructions.

DGGE Product Amplification

Amplification of the bacterial 16S rRNA gene was performed using genomic fecal DNA to generate a 200-bp product with the reverse primer 16S_518R (5'-ATT ACC GCG GCT GCT GG-3') and the forward primer 16S_357 (5'-CCT ACG GGA GGC AGC AG-3'). Each 50 µl reaction was performed using 25 µl of HotStart-IT FideliTaq PCR Mater Mix (2X) (Affymetrix, Santa Clara, CA), 2.5 µl of 10% dimethylsulfoxide (DMSO), 0.25 µM of each primer, and 100 ng of DNA. The PCR

protocol consisted of 20 cycles of 94°C for 2 min and amplification at 65°C for 1 min (with temperature decreasing by 0.5°C after every 1 cycle), then elongation at 72°C for 3 minutes. This was followed by 15 cycles of 94°C for 2 min, amplification at 55°C for 1 min, elongation for 3 min at 72°C, and a final 10 min elongation step at 72°C. The PCR product was then cleaned using a Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA) per manufacturer's instructions and re-amplified using the same PCR protocol; however, a modified forward primer, 16S_357_GCclamp, containing an additional 40 nucleotide GC-clamp on the 5' end was used (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') in order to form a 250-bp product. Amplification was carried out with an iQ5™ Optical system Real Time PCR detection system (Bio-Rad, Hercules, CA). Products were confirmed by electrophoresis through a 1% agarose gel (Fisher-Scientific, Atlanta, GA).

Denaturing Gradient Gel Electrophoresis (DGGE)

A total of 24 µl of product (5 µl PCR product, 15 µl TE, and 4 µl of loading dye) was run on an 8% polyacrylamide gel in a 30-60% denaturant gradient of urea and formamide [100% denaturant corresponds to 7 M urea plus 40% (vol/vol) of deionized formamide] using the Bio-Rad DCode™ Universal Detection System (Bio-Rad, Hercules, CA). Gels were submerged in 1X TAE and run at constant voltage of 65 V at a temperature of 60°C for 15.5 h. DNA bands were visualized by staining with ethidium bromide (10 µg/ml) and photographed using a Molecular Imager GelDoc XR (Bio-Rad, Hercules, CA). Banding patterns were analyzed with FPQuest gel analysis software (Bio-Rad) and clustered using the unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) to produce dendrograms for the different

treatments. Each sample was run in duplicate per gel and gels were run in duplicate (Figure 3.1).

Bacterial Culturing for Standard Curves

To construct standard curves for real time PCR analysis, we utilized bacteria previously isolated from gastrointestinal communities obtained through the American Type Tissue Culture Collection (Manassas, VA). Specific culturing instructions are outlined in Table 3.3.

Quantitative Real-Time PCR (qPCR)

DNA for standard curves was extracted from bacteria in Table 3.3 using the Qiagen Puregene Yeast/Bact. Kit B (Qiagen, Valencia, CA) per manufacturer's instructions. DNA was serially diluted 10-fold from 100 ng/µl to 0.001 ng/µl. Each 25 µl reaction consisted of 12.5 µl of HotStart-IT™SYBR®Green qPCR Master Mix 2X (USB® 75762 Cleveland, OH, USA), 0.5 µl of fluorescein (USB® 75767 Cleveland, OH, USA), 0.4 µM of both forward and reverse primers, and 10 ng of template DNA (except for the Total Bacteria primer set which consisted of 0.6 µM of both forward and reverse primers and 1ng of template DNA). Primers and their respective annealing temperatures are listed in Table 3.4. The PCR conditions consisted of a HotStart Binding Protein inactivation for 2 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec, a variable annealing temperature for 30 sec (Table 3.4) and 72°C extension for 60 sec (acquire real-time data at this step). Each concentration in the standard curve was done in triplicate, while samples were run with 4 replicates on single plates. Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal

originated from specific PCR product. Amplification was carried out with an iQ5™ Optical system Real Time PCR detection system (Bio-Rad, Hercules, CA).

Quantitative Reverse Transcriptase Real-Time PCR (qRT-PCR)

RNA was used to analyze metabolically active bacterial communities. For standard curves, RNA was extracted from *Escherichia coli* ATCC 43890 (Table 3.3) using the Qiagen Puregene Yeast/Bact. Kit B (Qiagen, Valencia, CA) per manufacturer's instructions and serially diluted 10-fold from 10 ng/µl to 0.01ng/µl. Each 25 µl reaction contained 1 ng of total RNA, 0.2 µl of M-MLV RT (reverse transcriptase), 0.2 µl of RNase Inhibitor, 12.5 µl of HotStart-IT™SYBR®Green qPCR Master Mix 2X (USB® 75770 Cleveland, OH, USA), 0.5 µl of fluorescein (USB® 75767 Cleveland, OH, USA), and 0.6 µM of forward and reverse primers (Table 3.4). PCR conditions consisted of 1 cycle 50°C for 10 minutes for reverse transcription of RNA, 1 cycle 95°C for 2 minutes for activation of HotStart-IT polymerase and reverse transcriptase inactivation, followed by, 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 60 sec (acquire real-time data at this step). Each concentration in the standard curve was done in triplicate, while samples were run with 4 replicates on single plates. Melting curve analysis of the PCR products was conducted to distinguish specific PCR products from non-specific. Amplification was carried out with an iQ5™ Optical system Real Time PCR detection system (Bio-Rad, Hercules, CA).

Statistical Analysis

Statistical analyses were performed using SAS (version 9.2, SAS Institute, Cary, NC) statistical software. The *proc mixed* procedure of SAS was used to determine the

main effect of dietary and NSPase, as well as their interaction in a replicated 4 × 4 Latin square design using pig as a random variable (Kaps and Lamberson, 2004). Differences between least square means were tested using the *pdiff* option in SAS. Samples were considered significantly different when p-values < 0.05.

RESULTS

Denaturing Gradient Gel Electrophoresis (DGGE)

Changes in bacterial community composition were indicated by band presence/absence. DGGE profiles of fecal samples from the same animal were most similar to each other regardless of diet with similarity indices ranging from 68-90% (Figure 3.2). There was a 49% similarity amongst all analyzed fecal samples, indicating that the animal was the largest source of variation (Figure 3.2). With a 70% similarity index among all treatments and time points, pig 1 appeared to be the least susceptible to change (Figures 3.2). Between the final two days of Adaptation (days 4 and 5 of treatment) and the final two days of Fecal (days 8 and 9 of treatment) there was a 6% decrease in similarity in banding pattern among all analyzed fecal samples (Figures 3.3 and 3.4) indicating the bacterial community is adapting to the new diet and the community has not stabilized.

Ileal Bacterial Community

The abundance of total bacterial DNA in ileal digesta was quantified using qPCR amplification of the 16S rDNA copies per genome and compared to the total numbers of active bacteria determined by qRT-PCR of 16S rRNA genes (Table 3.5). Inclusion of

DDGS in the diet reduced the abundance of bacteria ($P < 0.0001$), however despite this reduction in 16S rDNA there was no statistically significant reduction in active members of the community (16S rRNA) (Table 3.5). Inclusion of NSPase in the diets had no effect on total bacteria ($P = 0.34$) but slightly reduced the numbers of metabolically active bacteria ($P = 0.02$). An interaction between diet type and NSPase inclusion was noted for total bacteria ($P = 0.01$) yet resulted in minimal effect on log copies/g of 16S rRNA genes ($P = 0.15$). Interestingly, the DDGS diet containing NSPase resulted in an increase in total bacteria, but the control diet containing NSPase slightly reduced total digesta populations ($P = 0.01$).

Fecal Bacterial Communities

Inclusion of DDGS in diets was associated with statistically significant increases in the abundance of fecal 16S rDNA copies for members of *Bacteroides*, *Ruminococcus*, the *Clostridium coccoides* group, and the *Clostrdium leptum* subgroup (Table 3.6). In comparison with the control diet, *Ruminococcus* copy numbers increased by ~1 log copies/g feces in DDGS-containing diets, while increases in the numbers of *Bacteroides* and the *Clostridium coccoides* group were smaller, but still statistically significant. Inclusion of DDGS in the diets of pigs did not alter the log copies/g of total bacteria ($P = 0.30$) and *Firmicutes* ($P = 0.26$) in feces. Inclusion of fiber-degrading enzymes (i.e., NSPase) reduced ($P = 0.05$) total bacteria numbers and tended to reduce ($P = 0.11$) numbers of the *Clostrdium leptum* subgroup regardless of diet. More importantly, interactions between diet type and NSPase supplementation were detected for total

bacteria ($P = 0.02$), *Bacteroides* ($P = 0.03$), the *Clostrdium leptum* subgroup ($P = 0.003$), *Firmicutes* ($P = 0.07$), and the *Clostridium coccoides* group ($P = 0.09$) (Table 3.6).

DISCUSSION

Concerns related to the development of antibiotic resistance have lead European nations to implement bans on the use of growth-promoting antibiotics, and the practice in the USA is under increasing regulatory and political scrutiny (Allen et al., 2013). Foods that target and modify the activity of endogenous gut bacteria have gained substantial attention in swine nutrition due to their potential to replace antibiotic growth promoters in feed (Forestier et al., 2001; Hopkins and Macfarlane, 2003). In particular, the prebiotic effects of dietary fiber are currently being explored (Jha et al., 2011; Lin et al., 2011; Metzler-Zebeli et al., 2010; Verstegen and Williams, 2002). Dried distillers grain with solubles is high in both total non-starch polysaccharides (NSP) (Ward et al., 2008) and crude fat content, which results in higher metabolizable energy (ME) similar to that of corn (Shurson et al., 2004). Despite the extensive research into its use in animal feed (Kim et al., 2008; Lee et al., 2012; Stein and Shurson, 2009), very little information is available regarding the effects of DDGS on intestinal microbiota.

In the present study, we examined the effects of two factors (the inclusion of DDGS and/or inclusion of a carbohydrase mixture) on the bacterial richness and abundance of swine gastrointestinal bacteria. Washout diets (composed of corn-soybean meal) were applied to minimize the carryover effects of dietary treatments on the bacterial population of the GIT (Moya et al., 2009). As part of a companion study,

observed changes of the bacterial community in response to the dietary treatments (data not shown), minimum washout periods of 7 days were required (Lee et al., 2012).

NSP inclusion in pig diets has been shown to modulate intestinal microbiota composition (Metzler-Zebeli et al., 2010). Accessibility of dietary nutrients proved critical for bacterial growth. Increased digesta viscosity with inclusion of high-viscosity NSP impaired intestinal contractions, thereby preventing mixing of digesta and thus reduced access to a new substrate (Metzler-Zebeli et al., 2010); however, partial degradation of these fractions by exogenous enzymes may improve the nutritional value for the pig (Ao et al., 2010; Högberg and Lindberg, 2004).

In our study, decreased viscosity of the corn control diet through the inclusion of NSPase reduced ileal 16S rDNA copies/g of digesta; however DDGS inclusion was able to maintain total numbers of 16S rRNA gene copies despite the addition of NSPase (Figure 3.5). This indicates that DDGS made available additional nutrients for bacterial use within the small intestine. Fermentation of the DDGS fiber fraction was likely to promote growth of specific bacterial species (Pieper et al., 2008) , increasing their activity and thus numbers of copies of the 16S rRNA gene. In a similar study, inclusion of carboxymethylcellulose, a diet most closely resembling our corn control (high-viscosity, low fermentabilty), was associated with a increased susceptibility of pigs to overgrowth of pathogenic *E. coli* (Metzler-Zebeli et al., 2010), suggesting that our experimental diets may prove beneficial for the health of the animal.

The *Clostrdium leptum* subgroup is known to contain high numbers of butyrate-producing and fibrolytic bacteria (Pryde et al., 2002; Robert and Bernalier-Donadille, 2003). We hypothesized that the inclusion of DDGS would increase populations of fiber

degrading genera, chiefly the *C. leptum* subgroup. NSPase-induced reductions in the *C. leptum* subgroup were overcome by the inclusion of DDGS (Figure 3.6). In a companion study performed by H. Lee (Lee et al., 2012), results suggested a possibility for improved nutrient utilization in pigs, when carbohydrases are supplemented to a diet with high corn DDGS content. This indicates that supplementing the DDGS diet with NSPase was able to sustain the number of the fibrolytic, *C. leptum* bacterial subgroup while simultaneously improving host nutrient utilization.

Supplementation of the experimental diets with NSPase resulted in a significant reduction in total bacteria. However, there was also a significant interaction between diet type and NSPase supplementation for total bacteria, *Firmicutes*, *Bacteroides*, *C. coccoides* group and *C. leptum* subgroup indicating an important relationship between fiber degradation by supplemental enzymatic activity and changes in the microbiome (Figure 3.6). Furthermore, inclusion of NSPase in the DDGS diet either prevented or minimized the reduction in bacterial rDNA copy numbers caused by the addition of DDGS to the diet. These results suggest that addition of NSPase to diets containing high levels of fiber can contribute to the return the microbiota to a "normal" state.

In an *in vitro* study examining the influence of dietary fiber, the effect of partially hydrolyzed guar gum (PHGG) on microbial composition was analyzed. *C. leptum* abundance was not altered; however, butyric acid production increased by 5.8 μmol/ml within a 24-hour period in comparison to a non-inoculated control. The authors hypothesized that the *Rosburia*/*Eubacterium recatale* group played not only an important role in butyric acid production but also the stimulation of the *C. coccoides* group (Ohashi et al., 2012). Although our study did not quantify the *Rosburia*/*E. recatale* group, these

results might explain the significant increase in the *C. coccoides* group when DDGS was included in diets.

Perez et al. (2010) studied the effect of DDGS diet supplementation on digesta characteristics and intestinal microbiota of pigs. The authors found that the inclusion of DDGS increased bacterial richness, represented by an increased number of bands within the cecum and colon. In our study, changes to bacterial richness were indicated by number of bands and band positions, which were then clustered based on similarity of DGGE profiles and used to generate information regarding microbial community shifts associated with diet. Although treatments produced changes in microbial profiles there were no distinct clustering patterns. Susceptibility to change varied among animals. Changes in dietary ingredient composition (i.e., inclusion of DDGS) resulted in significant alterations to most of the studied bacterial populations without changing the total number of bacteria in feces. Each DGGE band represents a single bacterial amplicon, in theory a single bacterial species, but in reality amplicons of similar GC content can co-migrate and a single band may in fact represent multiple bacterial species (Simpson et al., 1999).

Perez et al. (2009) noted the largest number of bands and thus greatest bacterial richness occurred in intestinal mucosa microbiota of young pigs fed diets supplemented with 5% DDGS. However, concentrations greater than 5% coincided with reduced diversity. Our experimental diets were composed of 40% DDGS, which is nutritionally sufficient (Shurson and Noll, 2005; Stein, 2007), but likely resulted in extreme fermentation and thus the promotion of fewer bacterial species to become dominant (Pieper et al., 2008). Length of treatment may have also played a factor. There was only a

6% decrease in similarity indices between all analyzed fecal samples during the final two days of Adaptation (days 4 and 5 of treatment) and the final two days of treatment (Fecal; days 8 and 9 of treatment); however, the clustering was radically different between the two (Figures 3.3 and 3.4).

Our analysis was unable to take into account band intensity due to high variability between gels, which may have led to an underestimation of change. For example, although the Pig 1 Control+NSPase banding pattern appears distinct from the other three treatments (Figure 3.1), it still clustered very closely together (Figure 3.3 and Figure 3.4) because many of the bands are still present, just at much lower concentrations. These varying intensities may have resulted from either the heterogeneity of *rrn* operon copy number and sequence among bacterial species leading to multiple bacterial species producing the same banding pattern (Klappenbach et al., 2000; Makarova and Koonin, 2007) or closely related species possessing near identical sequences which an co-migrate to the same position and lead to an underestimation of diversity (Simpson et al., 1999). These limitations emphasize the importance of combination with another technique such as sequencing or real-time PCR.

IMPLICATIONS

Experimental diets were able to alter both microbial species richness and abundance within the porcine gastrointestinal tract. Despite the reduction in total bacteria within the ileum, the abundance of metabolically active bacteria remained constant. The increase in the *C. leptum* subgroup indicates increased presence of potential butyrate-producing bacteria. The ability of NSPase supplementation to induce changes in bacterial

populations according the ingredient composition and/or fiber content of the diet merits further investigation.

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TABLES

Table 3.1. Experimental design breakdown for each period.

Stage	Treatment Days	Sample	
Adaptation	1-5	Feces	Acclimation to experimental diet
Digesta	6-7	Digesta	Observation of microbial shifts in the small intestine
Fecal	8-9	Feces	Observation of microbial shifts in the entire GIT

A 7-day washout diet was fed to the pigs between each period in order to equalize microbial communities between treatments.

Table 3.2. Diet formulation and calculated nutrient composition of experimental diets, as fed basis

Item	CORN	CORN + Enzymes	DDGS	DDGS + Enzymes
Ingredients, %				
Corn	76.46	76.26	45.08	44.88
Soybean meal (47.5%)	20.00	20.00	13.20	13.20
Corn DDGS	- - -	-	40.00	40.00
Limestone	1.00	1.00	1.20	1.20
Soy oil	1.00	1.00	- - -	- - -
Dicalcium phosphate	0.80	0.80	- - -	- - -
Salt	0.22	0.22	- - -	- - -
Vitamin premix[1]	0.08	0.08	0.08	0.08
Mineral premix[2]	0.04	0.04	0.04	0.04
Chromic oxide	0.40	0.40	0.40	0.40
Enzyme[3]	- - -	0.20	- - -	0.20
Calculated nutrients				
ME, kcal/kg	3,375	3,368	3,110	3,103
CP, %	15.85	15.83	20.61	20.6
Ca, %	0.59	0.59	0.57	0.57
P, %[4]	0.23	0.23	0.25	0.25
Lysine, %[5]	0.8	0.8	0.66	0.66
Methionine, %[5]	0.26	0.26	0.32	0.31
Threonine, %[5]	0.59	0.59	0.62	0.62
Arginine, %[5]	0.98	0.98	0.97	0.97

[1] Provided the following per kg of diet: Vitamin A, 3,227 IU; Vitamin D3, 445 IU; Vitamin E, 19 IU; Vitamin K, 3 mg; biotin, 0.14 mg; choline, 242 mg; folic acid, 0.61 mg, niacin, 14 mg; D-pantothenic acid, 9 mg; riboflavin, 3 mg; and B12, 0.01 mg (from 77320014, ADM, Decatur, IL)

[2] Provided the following as mg/kg of diet: Zn, 144 mg from ZnSO4; Fe, 144 mg from FeSO4; Mn, 44 mg from MnSO4; Cu, 9 mg CuSO4; and Se, 0.2 mg from Na2SeO3 (from 77069014, ADM, Decatur, IL)

[3] Carbohydrase mixture provided per kg of diet: cellulase, 20,360 unit (from Trichoderma longibrachiatum); hemicellulase, 3,284 unit (from Aspergillus niger); ß-glucanase, 99 unit (from Trichoderma longibrachiatum); and xylanase, 35,466 unit (from Trichoderma longibrachiatum); all carbohydrase from Bio-Cat Inc., Troy, VA

[4] Available phosphorus

[5] Standardized ileal digestibility values

Table 3.3. Culturing conditions for real-time PCR bacterial standards.

Bacterial Stain	Media	Growth Conditions
Escherichia coli ATCC 43890	Tryptic Soy broth (TSB; Difco, Sparks, MD)	Inoculated 5ml TSB from freezer stock using a sterile loop. Grew overnight, shaking at 37°C.
Lactobacillus rhamnosus ATCC 53103	Chopped meat broth (Anaerobe Systems, CA), MRS Broth (Difco, Sparks, MD)	Thawed freezer stock. Used filtered eye dropper to transfer approximately 5 drops of stock to 7ml chopped meat broth. Incubated overnight at 37°C. Using filtered eye dropper, transfered approximately 5 drops of culture to ~50ml MRS broth. Grew overnight at 37°C.
Bacteroides fragilis VPI 13785	Chopped meat broth (Anaerobe Systems, CA), MRS Broth (Difco, Sparks, MD)	Thawed freezer stock. Used filtered eye dropper to transfer approximately 5 drops of stock to 7ml chopped meat broth. Incubated overnight at 37°C. Using filtered eye dropper, transfered approximately 5 drops of culture to ~50ml MRS broth. Grew overnight at 7°C.
Blautia producta ATCC 27340	Chopped meat broth (Anaerobe Systems, CA), MRS Broth (Difco, Sparks, MD)	Resuspended lyophil in chopped meat broth, then used a filtered eye dropper to separated resuspended culture into two 7ml tubes of chopped meat. Incubated overnight at 37°C. Using filtered eye dropper, transferred approximately 5 drops of culture to ~50ml MRS broth. Grew overnight at 37°C.
Faecalibacterium prausnitzii ATCC 27766	MTGE Anaerobic enrichment broth (Anaerobe Systems, CA)	Used filtered eye dropper to separate hawed freezer into two 7ml tubes of MTGE. Incubated overnight at 37°C.

Table 3.4. Primer sets used to quantify abundance of key members of swine microbial community using real-time PCR.

Target Bacterial Group	Primer Sequence	Annealing Temperature °C	Reference
Total Bacteria[1]	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	60	Lane, 1991; Muyzer et al., 1993
Firmicutes[2]	GGAGYATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	60	Guo et al., 2008
Bacteroides[3]	GAGAGGAAGGTCCCCCAC CGCTACTTGGCTGGTTCAG	60	Layton et al., 2006
Ruminococcus[4]	GGTGGCAAAGCCATTCGGT GTTACGGGACGGTCAGAG	64	Malinen et al., 2003
Clostridium coccoides Group[4]	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	58	Matsuki et al., 2002
Clostridium leptum Subgroup[5]	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	58	Matsuki et al., 2004

DNA/RNA extracted from [1]*Escherichia coli* ATCC 43890, [2]*Lactobacillus rhamnosus* ATCC 53103, [3]*Bacteroides fragilis* VPI 13785, [4]*Blautia producta* ATCC 27340, and [5]*Faecalibacterium prausnitzii* ATCC 27766 and served as standard for real-time PCR.

Table 3.5. Effects of DDGS and NSPase on total bacterial abundance within ileal digesta (log 16S rDNA and rRNA copies/g of digesta).

	Diet							
	Control		**DDGS**					
	NSPase		**NSPase**			***P*-value**		
	No	**Yes**	**No**	**Yes**	**SEM**	**Diet**	**NSPase**	**Diet × NSPase**
Total Bacteria (DNA)	10.27	10.17	9.88	9.93	0.06	0.0001	0.34	0.01
Total Active Bacteria (RNA)	10.39	10.24	10.37	10.34	0.06	0.30	0.02	0.15

Table 3.6. Effects of DDGS and NSPase on specific bacterial populations within feces (log 16S rDNA copies/g of feces).

	Diet							
	Control		DDGS					
	NSPase		NSPase			*P*-value		
	No	Yes	No	Yes	SEM	Diet	NSPase	Diet × NSPase
Total Bacteria	10.05	9.63	9.92	9.96	0.23	0.30	0.05	0.02
Firmicutes	9.21	8.84	9.12	9.22	0.28	0.26	0.29	0.07
Bacteroides	8.68	8.24	8.88	9.05	0.26	0.0001	0.19	0.003
Ruminococcus	5.94	5.73	6.84	6.95	0.34	0.0001	0.74	0.31
Clostridium coccoides Group	9.17	8.76	9.70	9.89	0.35	0.001	0.53	0.09
Clostridium leptum Subgroup	8.45	7.84	8.55	8.65	0.33	0.005	0.11	0.03

FIGURES

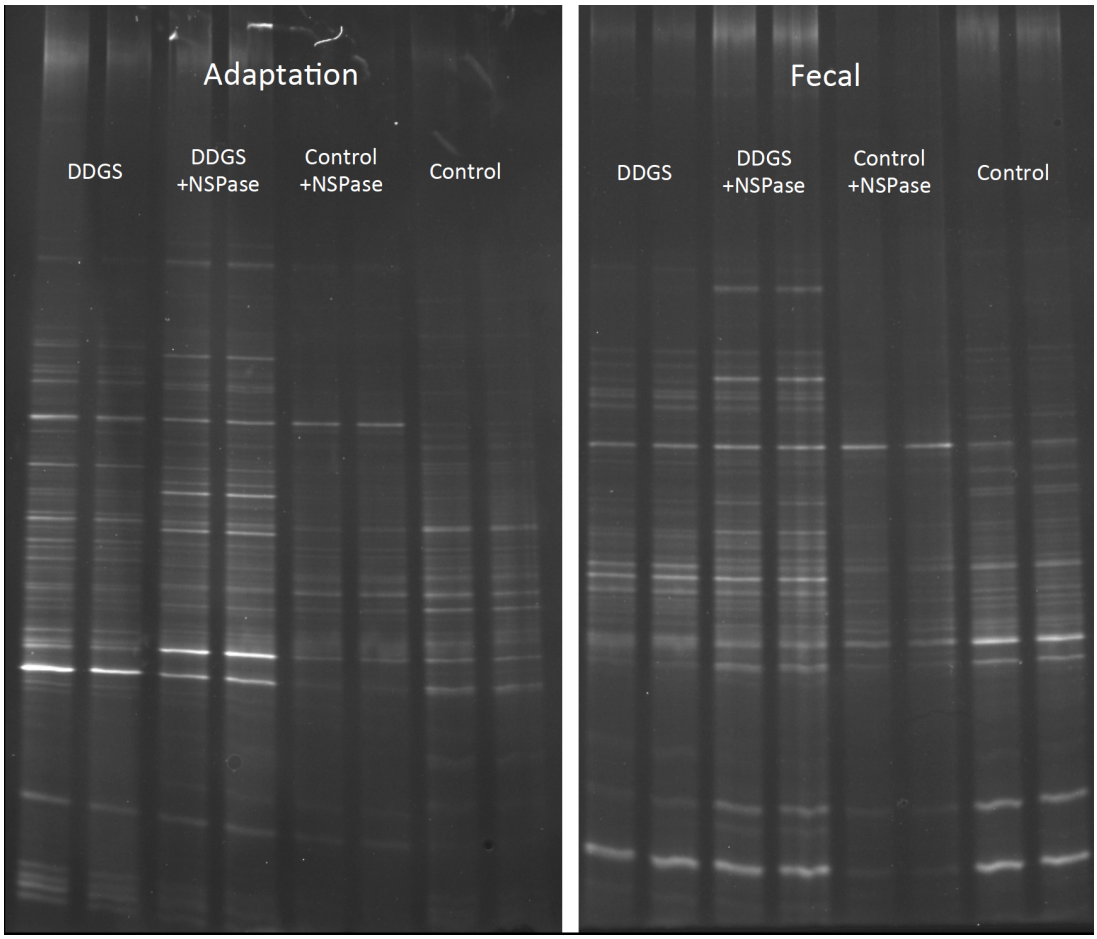

Figure 3.1. Denaturing Gradient Gel Electrophoresis (DGGE) diet based changes in microbial species richness for all Pig 1 treatments.

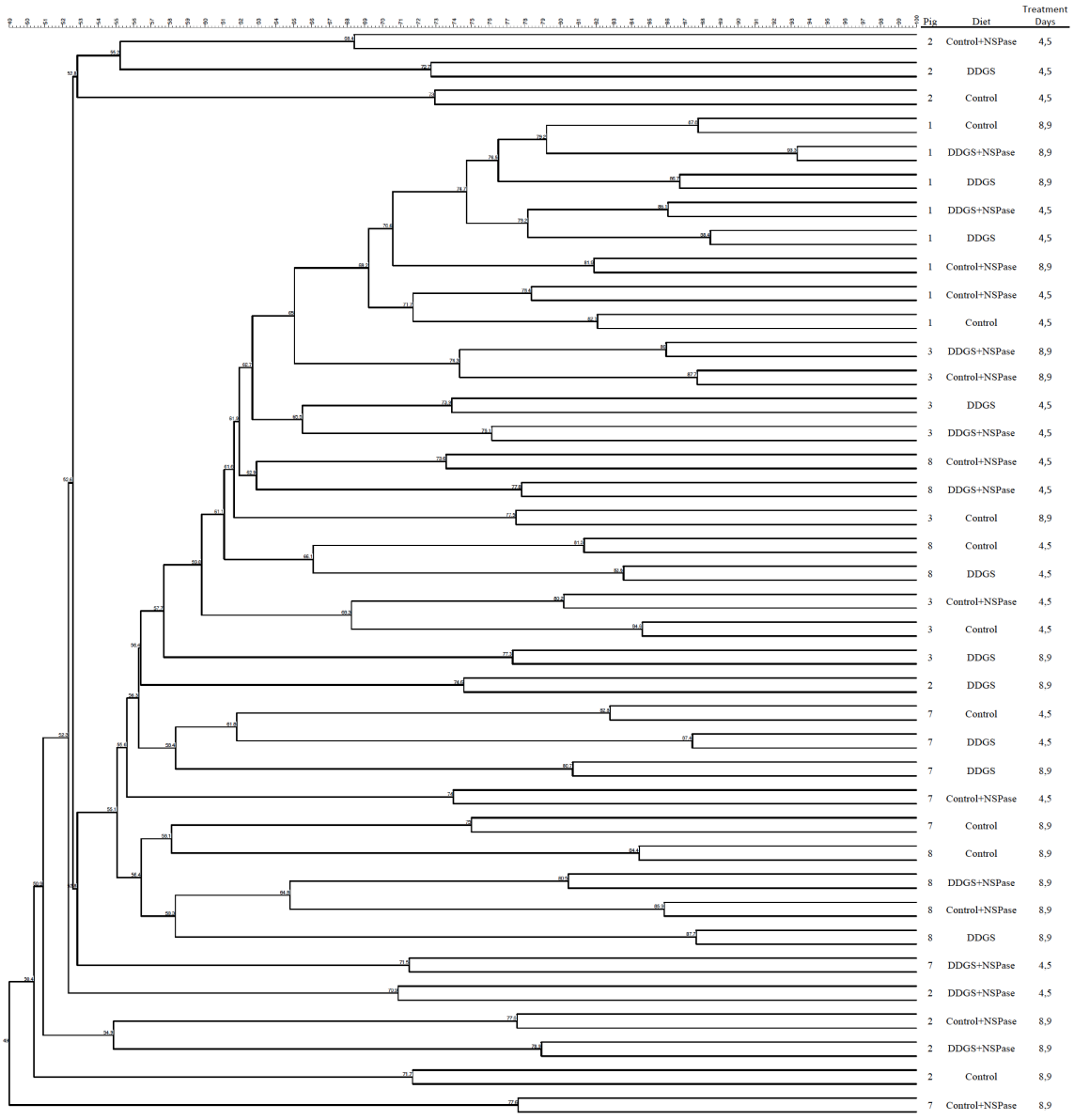

Figure 3.2. Adaption and Fecal: Effects of DDGS and NSPase on fecal species richness from treatment days 4 and 5, and day 8 and 9, demonstrated through Denaturing Gradient Gel Electrophoresis (DGGE).

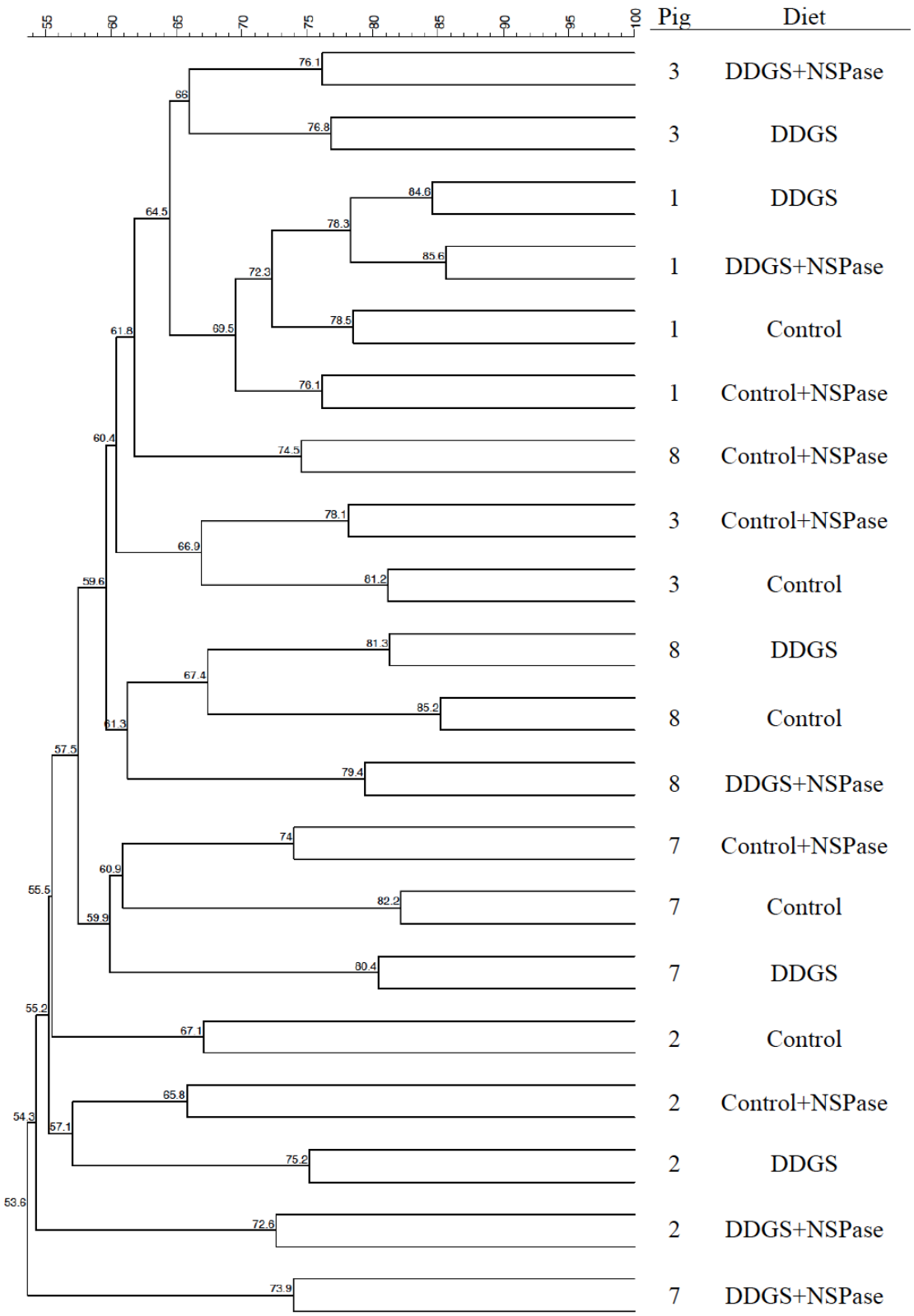

Figure 3.3. Adaption: Effects of DDGS and NSPase on fecal species richness from treatment days 4 and 5 demonstrated through Denaturing Gradient Gel Electrophoresis (DGGE).

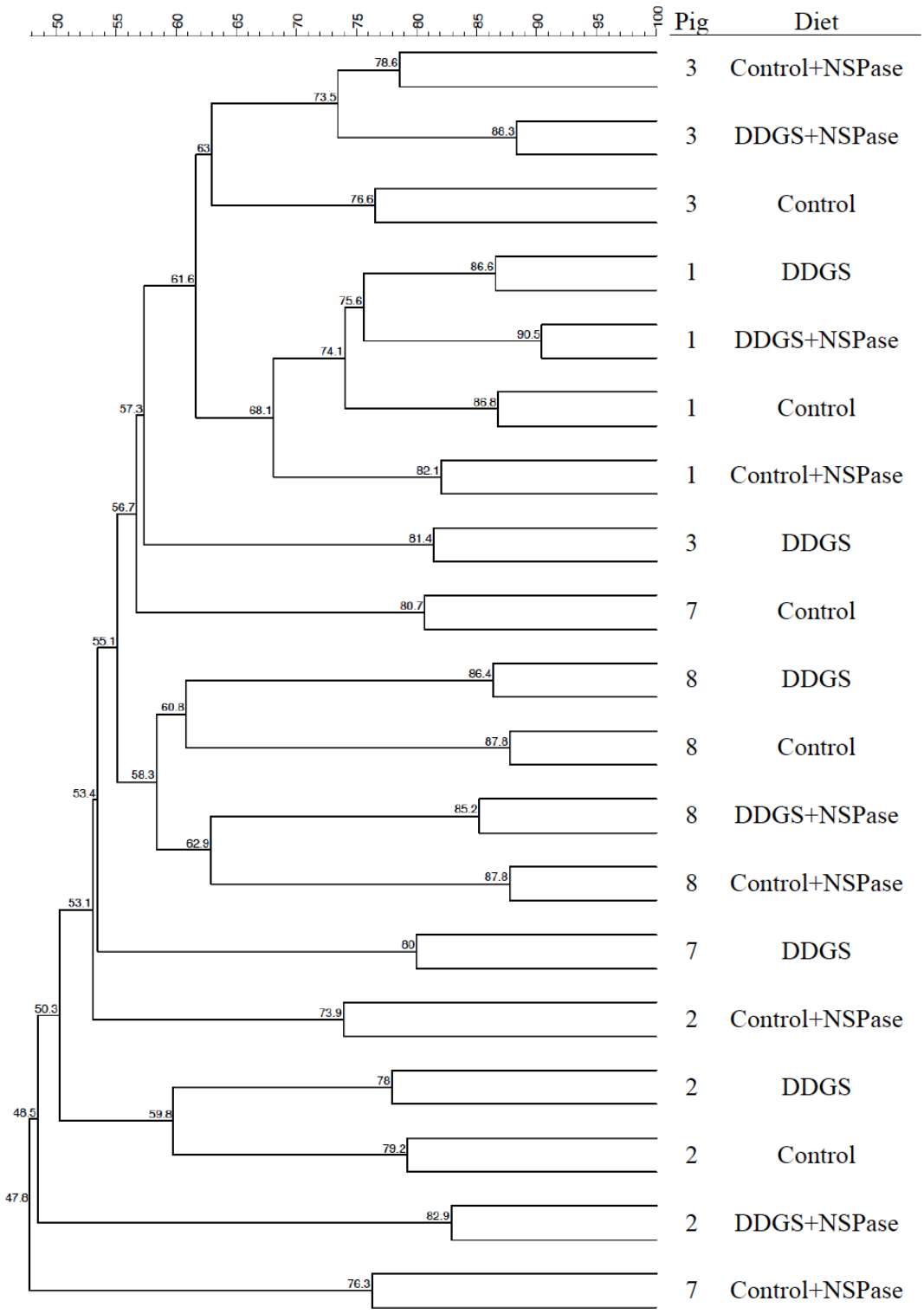

Figure 3.4. Fecal: Effects of DDGS and NSPase on fecal species richness from treatment days 8 and 9 demonstrated through Denaturing Gradient Gel Electrophoresis (DGGE). Pig 7 DDGS+NSPase was not included however its inclusion in other analysis did not affect overall percent similarity.