RESEARCH REPORT
ANIMAL SCIENCE

Title: Investigation of the impact of increased dietary insoluble fiber through the feeding of dried distiller's grains with solubles (DDGS) on the colonic microbiota of pigs with and without *Brachyspira*-associated colitis – **NPB# 13-212**

Investigator: Eric R. Burrough, DVM, PhD

Institution: Iowa State University

Date Submitted: February 23, 2015

Industry Summary:

The diversion of an increasing portion of the U.S. corn crop to ethanol production has resulted in increased feed costs for pork producers. To mitigate this trend, many pork producers have increased their use of ethanol byproducts in commercial diets including increased feeding of distiller's dried grains with solubles (DDGS). The inclusion of DDGS raises the insoluble fiber in the ration and the impact of this diet change on the colonic flora can be considerable. The large intestine contains a dynamic microenvironment with tremendous interplay between microorganisms. Any alteration to the physical or chemical properties of the colonic contents has the potential to impact the resident bacterial population and potentially favor or inhibit the establishment of pathogenic species. Previous work has shown that the development of swine dysentery (SD) in gnotobiotic pigs is dependent upon the presence of one or more additional anaerobic bacteria concurrent with *Brachyspira hyodysenteriae*, yet it is unknown if these specific anaerobic species are more prevalent in the microbiota of pigs that develop SD naturally versus those that do not, or in those pigs that are fed diets containing increased insoluble fiber sources such as DDGS.

In the current study, colonic contents were analyzed from a previous randomized complete block experiment where one hundred 4-week-old pigs were divided into five inoculum groups (negative control, *Brachyspira intermedia*, *Brachyspira pilosicoli*, *B. hyodysenteriae* or "*B. hampsonii*") and fed one of two diets containing no (diet 1) or 30% (diet 2) DDGS. Colonic contents were collected at necropsy either 72 hours after the development of SD or at 21 days post-inoculation in control pigs and those not developing SD, and were flash frozen in liquid nitrogen and retained for the analyses described in this report. Pigs receiving diet 2 and inoculated with either *B.*

hyodysenteriae or "*B. hampsonii*" developed SD nearly twice as fast as pigs receiving diet 1. The colonic microbiome in each necropsy sample was analyzed using 16S rRNA profiling and compared for differences in richness and diversity of bacterial species. In the non-inoculated control pigs, no difference in richness (alpha diversity) was observed; however, a significant difference was observed in the beta diversity between groups ($P < 0.0001$) with a dramatic shift in the Bacteroidetes:Firmicutes ratio where higher ratios were observed in those pigs fed DDGS (diet 2). For pigs that developed SD, there was a significant difference in richness relative to those that did not regardless of diet with fewer total species observed in SD pigs ($P = 0.001$). The beta diversity was also significantly different between pigs with SD and those without where SD pigs had lower Bacteroidetes:Firmicutes ratios on average and a marked increase in relative abundance of Proteobacteria. The relative abundance of Spirochaetes was higher in pigs fed DDGS relative to pigs fed the control diet. Further investigation is warranted to better determine the specific bacterial species underlying these shifts in the colonic microbiome and the relationship of these bacteria with strongly beta-hemolytic *Brachyspira* spp. as pigs fed DDGS appear at increased risk for developing SD.

Contact information:
Eric Burrough
Iowa State University Veterinary Diagnostic Laboratory
1600 South 16th St, Ames, IA 50011
burrough@iastate.edu

Keywords:
Brachyspira hyodysenteriae; Brachyspira hampsonii; swine dysentery; bloody scours; porcine

Scientific Abstract:

In recent years, distiller's dried grains with solubles (DDGS), a source of insoluble dietary fiber, have been increasingly included in diets of swine; however, little is known regarding the impact of this feed component on *Brachyspira* infection. The structure of the colonic microbiome is likely a key component to the development of swine dysentery (SD), and dietary manipulations that alter the microbiota may favor or inhibit the establishment of a microbiome that is amenable to *Brachyspira* colonization and the development of disease. In the current study, colonic contents were analyzed from a previous experiment where one hundred 4-week-old pigs were divided into five inoculum groups (negative control, *Brachyspira intermedia*, *Brachyspira pilosicoli*, *B. hyodysenteriae* or "*B. hampsonii*") and fed one of two diets containing no (diet 1) or 30% (diet 2) DDGS. Pigs receiving diet 2 and inoculated with either *B. hyodysenteriae* or "*B. hampsonii*" developed SD nearly twice as fast as pigs receiving diet 1. The colonic microbiome in each necropsy sample was analyzed using 16S rRNA profiling and compared for differences in richness and diversity of bacterial species. In the non-inoculated control pigs, no difference in richness (alpha diversity) was observed; however, a significant difference was observed in the beta diversity between groups ($P < 0.0001$) with a significant shift in the Bacteroidetes:Firmicutes ratio where higher ratios were observed in those pigs fed diet 2. For pigs that developed SD, there was a significant difference in richness relative to those that did not regardless of diet with fewer total species observed in SD pigs. The beta diversity was also significantly different between pigs with SD and those without where SD pigs had lower Bacteroidetes:Firmicutes ratios on average and a marked increase in relative abundance of proteobacteria. The relative abundance of

spirochaetes was higher in pigs fed DDGS relative to pigs fed the control diet. Further investigation is warranted to better determine the specific bacterial species underlying these shifts in the colonic microbiome and the relationship of these bacteria with strongly beta-hemolytic *Brachyspira* spp. as pigs fed DDGS appear at increased risk for developing SD.

Introduction:

In recent years, an increasing portion of the U.S. corn crop has been diverted to ethanol production. This increase in ethanol production has resulted in increased feed costs for pork producers and feeding of byproducts in commercial swine diets has been one means to offset these costs. A primary example is the increased feeding of distiller's dried grains with solubles (DDGS); however, previous research has been somewhat conflicting on the impact of higher fiber diets, especially insoluble fiber, on the intestinal environment of pigs. Relatively little is published about the precise pathogenesis of swine dysentery (SD) although diet is considered to be an important factor in disease expression following *Brachyspira hyodysenteriae* infection.[1] In general, feeding of highly digestible diets has been associated with decreased expression of SD following *B. hyodysenteriae* infection[2] whilst feeding of rapidly fermentable fiber sources can increase disease expression.[3] Diets high in inulin have also been associated with decreased SD expression following experimental infection with *B. hyodysenteriae* and it has been postulated that this may be due to associated alterations in the colonic microbiota.[4] The structure of the colonic microbiome is likely a key component to the development of SD, and dietary manipulations that alter the microbiota may favor or inhibit the establishment of a microbiome that is amenable to *Brachyspira* colonization and the development of disease. Indeed, such a role for the microbiome in the development of SD is supported by previous work in gnotobiotic pigs where additional bacteria were required in addition to *B. hyodysenteriae* for experimental induction of SD.[5]

By the mid-1990s, SD had essentially disappeared from North American swine herds as a result of effective treatment, control, and elimination methods. Interestingly, concurrent with recent industry trends toward increased feeding of byproducts in the United States has been an increase in *B. hyodysenteriae* detection since 2007[6] and also the emergence of the proposed novel species "*Brachyspira hampsonii"* associated with mucohemorrhagic colitis and diarrhea (swine dysentery).[7] There are numerous *Brachyspira* spp. that can be isolated from the porcine colon, and these can differentiated by various laboratory means including strength of beta-hemolysis on blood agar, biochemical testing, polymerase chain reaction assays, *nox* gene sequencing, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Strength of beta-hemolysis is a sensitive indicator of the potential to induce SD[8] and serves as a useful laboratory method for preliminary identification of the common SD-associated species *B. hyodysenteriae* and *"B. hampsonii"*.

The recent availability of high-throughput genetic sequencing techniques and platforms has made microbial community profiling possible within biological samples. These techniques allow for an *in situ* comparison of the relative abundance of various microorganisms within a sample and to make comparisons between samples to investigate potential shifts associated with health and disease. Being culture independent, these metagenomics approaches allow for detection of uncommon, unculturable, and/or unknown organisms within samples and prevent selection bias that can occur when organisms are grown on various laboratory media.

Metagenomics approaches also reveal differences in richness (alpha diversity) and diversity (beta diversity) where richness reflects the number of species observed and diversity reflects the particular species observed within a set of samples.

Objectives:

1. Utilize a well-characterized porcine challenge model for *Brachyspira* infection to produce clinical disease in pigs fed either a control diet or a diet containing high levels of DDGS and collect colonic contents from the apex of the spiral colon of these pigs at necropsy to assess alterations in the colonic microbiome.
2. Perform culture independent metagenomic techniques to allow microbial community profiling and comparison between diet groups as well as between those animals that develop *Brachyspira*-associated disease and those that do not.

Materials & Methods:

1. *Colonic content samples*
   a. All content samples utilized in this study were collected during a previous experiment where one hundred 4-week-old pigs were divided into five inoculum groups (negative control, *Brachyspira intermedia*, *Brachyspira pilosicoli*, *B. hyodysenteriae* or "*B. hampsonii*") and fed one of two diets containing no (diet 1) or 30% (diet 2) DDGS.[9] Contents were collected from the apex of the spiral colon at necropsy which was performed either 72 hours after the development of SD or at 21 days post-inoculation in control pigs and those not developing SD. Contents were collected within 5 minutes of euthanasia and were flash frozen in liquid nitrogen and retained at -80°C until extracted for the analyses described in this report. As part of the previous experiment, colonic content samples from all pigs were submitted for selective anaerobic culture for *Brachyspira* spp. as described.[9]
2. *DNA Purification*
   a. Colonic contents were processed for DNA extraction using the Qiagen DNA Stool MiniKit following the manufacturer's recommendations. Following DNA purification samples were screened for DNA concentration and purity using a Nanodrop DNA Flouremeter and the Qubit fluorometer (Life Technologies, Grand Island, NY) and DNA was stored at -80°C prior to downstream processing.
3. *16S sequencing:*
   a. One hundred extracted colonic content samples were submitted to Argonne National Laboratory - Institute for Genomics and Systems Biology Next Generation Sequencing Core (http://ngs.igsb.anl.gov/) to be utilized for metagenomic analysis using amplification of the V3-V4 hypervariable region of the bacterial 16S rRNA gene. All samples were processed by the routine methodology of the core laboratory. Briefly, amplicons were synthesized using a universal 16S forward primer (515F) and 100 unique Golay barcoded reverse primers (806R) as described by Caporaso et al.[10] Appropriate positive and negative controls were included by the sequencing facility. Sample library DNA concentrations were quantified and samples were pooled with equal amounts of DNA. The pooled libraries were cleaned up with the MO-BIO UltraClean PCR Clean-Up Kit and the concentration was then diluted to 2 nM. A single flow cell lane of 300-bp paired end sequences was run on the Illumina MiSeq.
4. *Metagenomic data analysis:*

a. Forward and reverse reads from the paired end sequencing were first merged using the fastq.join script. Qiime 1.8 was then used for additional data analysis. De-multiplexing and quality filtering were then performed using the split_libraries_fastq.py script. The pick_reference_otus_through_otu_table.py script was used for operational taxonomic unit (OTU) calling and taxonomic assignment was performed based on the greengenes database.[11] Comparisons of specific OTUs within groups were made at the phylum, order, and genus level and only those reads detected in at least 25% of samples were included in the analysis.

5. *Statistical analyses*
   a. Statistical output was generated by Qiime 1.8. Prior to calculations, all libraries were adjusted to 47,000 reads to avoid potential interpretation errors due to variable sampling depth. Alpha diversity (chao1) was compared using a nonparametric two sample t-test with 999 Monte Carlo permutations. Beta diversity (Bray-Curtis dissimilarity) was compared using a two-sided student's two-sample t-test with Bonferroni correction. The frequency of detection (group significance) of specific OTU calls within groups was compared using a Kruskal-Wallis nonparametric analysis of variance with Bonferroni correction where appropriate. Bacteroidetes:Firmicutes ratios were calculated based upon the relative abundance percentages reported in Qiime and were compared using a two-sided student's two-sample t-test. Statistical significance was defined as $P < 0.05$.

Results:

1. Objective 1: Collection and categorization of colonic content samples
   a. Complete results of the animal inoculation experiment have been reported previously.[9] Briefly:
      i. All pigs were culture negative for *Brachyspira* spp. at the beginning of the experiment.
      ii. SD was only observed in pigs inoculated with either *B. hyodysenteriae* or "*B. hampsonii*" and pigs receiving diet 2 shed viable strongly beta-hemolytic spirochetes earlier and develop SD nearly twice as fast as pigs receiving diet 1; however, the overall incidence of SD was similar between groups with 70 – 100% of inoculated pigs developing disease during the course of the study. The incidence of SD and/or positive *Brachyspira* cultures at necropsy within each group are summarized in Table 1.
      iii. Diarrhea with mild mucus was occasionally noted in both *B. pilosicoli*-inoculated diet groups while no significant diarrhea was noted in either group of *B. intermedia*-inoculated pigs throughout the study period. The frequency of positive *Brachyspira* culture at necropsy is summarized in Table 1.
      iv. Sham-inoculated control pigs did not develop significant diarrhea over the course of the experiment or have a positive *Brachyspira* necropsy culture.
   b. Colonic content samples were recovered from storage at -80°C and DNA was extracted as described above. Screened samples provided DNA of sufficient concentration and purity for submission to Argonne National Laboratory.

i. Raw data generated by the reference laboratory included over 10,000,000 individual reads and all but two extracted colonic content samples (one from a pig fed diet 2 and inoculated with *B. pilosicoli* and one from a pig fed diet 1 and inoculated with *B. hyodysenteriae*) had more than 47,000 reads available for analysis.

2. Objective 2: Microbial community profiling
    a. Impact of feeding 30% DDGS on the colonic microbiota of pigs (diet 1 versus diet 2)
        i. Microbial profiles were compared for the 20 sham-inoculated control pigs.
        ii. No difference in richness (chao1) was detected ($P = 0.736$; Figure 1a); however, beta diversity was significantly different between diet groups ($P < 0.001$; Figure 1b).
        iii. In general, Bacteroidetes accounted for a higher relative percentage of reads in the Diet 2 pigs whereas Firmicutes were more abundant in pigs fed Diet 1 (Figure 1c). The Bacteroidetes:Firmicutes ratios were significantly higher in pigs fed Diet 2 (mean 3.555 ± 0.644) relative to pigs fed Diet 1 (mean 1.798 ± 0.262) ($P = 0.027$). Consistent with this, *Lactobacillus* spp. were significantly more common in the microbiota of pigs fed Diet 1 ($P = 0.016$), and bacteria in the family Rikenellaceae were more common in pigs fed Diet 2 ($P = 0.014$).
    b. Comparison of colonic microbial profiles from pigs with SD versus those without
        i. Microbial profiles were compared for all 98 pigs with sufficient sequencing data.
            1. 32 pigs with SD, 66 pigs without SD
        ii. A significant difference in richness (chao1) was detected between groups with fewer bacterial species observed in pigs with SD ($P = 0.001$; Figure 2a). Beta diversity was also significantly different ($P < 0.001$; Figure 2b).
        iii. In general, Firmicutes, Proteobacteria, and Fusobacteria were more abundant in pigs with SD with a concurrent reduction in Bacteroidetes (Figure 2c). The Bacteroidetes:Firmicutes ratios were significantly lower in pigs with SD (mean 2.388 ± 0.174) relative to pigs without (mean 3.645 ± 0.225) ($P < 0.001$). At the phylum level, Fusobacteria and Proteobacteria were significantly more abundant the microbiota of pigs with SD ($P < 0.001$). At the genus level, *Brachyspira* spp., *Campylobacter* spp., *Fusobacterium* spp., *Bacteroides* spp., and a putative novel genus in the family Paraprevotellaceae were more common in pigs with SD ($P < 0.001$, all analyses) whereas *Lactobacillus* spp. were more common in pigs without SD ($P = 0.001$).
    c. Comparison of colonic microbial profiles from pigs with SD versus those without by diet (diet 1 versus diet 2)
        i. Microbial profiles were compared for all 98 pigs with sufficient sequencing data.
            1. 32 pigs with SD (16 diet 1, 16 diet 2), 66 pigs without (33 diet 1, 33 diet 2)

ii. A significant difference in richness (chao1) was detected between diet groups with SD and those without ($P \leq 0.012$, all analyses); however, no difference in richness was observed within diet subclasses for each disease state and fewer bacterial species observed in pigs with SD (Figure 3a). Beta diversity was also significantly different ($P < 0.001$; Figure 3b).
iii. Proteobacteria and Fusobacteria were more abundant in pigs with SD regardless of diet ($P < 0.001$), Firmicutes were significantly less abundant in pigs without SD fed diet 2 ($P = 0.009$), and Spirochaetes were more abundant in these same pigs at the phylum level ($P < 0.001$; Figure 3c). A statistically significant difference was detected between one or more groups for 47 different genera. Of these, *Brachyspira* spp., *Campylobacter* spp., *Fusobacterium* spp., *Bacteroides* spp., *Flexispira* spp. and a putative novel genus in the family Paraprevotellaceae were more common in pigs with SD regardless of diet ($P < 0.001$, all analyses). *Lactobacillus* spp. were more abundant in pigs without SD and fed diet 1 ($P < 0.001$), whereas *Treponema* spp. and *Prevotella* spp. were more abundant in pigs without SD regardless of diet ($P \leq 0.004$).

d. Comparison of colonic microbial profiles of pigs colonized with *Brachyspira* spp. at necropsy versus those culture negative by diet (diet 1 versus diet 2)
i. Microbial profiles were compared for all 98 pigs with sufficient sequencing data.
1. 53 pigs with negative *Brachyspira* culture (26 diet 1, 27 diet 2), 11 pigs with weakly beta-hemolytic *Brachyspira* spp. by culture (6 diet 1, 5 diet 2), and 34 with strongly beta-hemolytic *Brachyspira* spp. by culture (17 diet 1, 17 diet 2).
a. No OTUs for *Brachyspira* were detected in the culture negative samples.
b. *Brachyspira* spp. OTUs were detected in only 1 of 11 samples culture positive for weakly beta-hemolytic *Brachyspira* spp.
c. *Brachyspira* spp. OTUs were detected in 24 of 34 samples culture positive for strongly beta-hemolytic *Brachyspira* spp. and two of the samples not detected were from the two pigs that were culture positive but did not develop SD. In all instances where OTUs were detected, the relative abundance of *Brachyspira* spp. was less than 0.035% of the total microbiota.
ii. A significant difference in richness (chao1) was only observed between pigs colonized with strongly beta -hemolytic *Brachyspira* spp. and those with negative cultures at necropsy regardless of diet ($P \leq 0.03$, all analyses) where pigs colonized with strongly beta-hemolytic spirochetes had fewer bacterial species detected (Figure 4a). Beta diversity was also significantly different between groups ($P < 0.001$).
iii. Proteobacteria and Fusobacteria were significantly more abundant in pigs colonized with strongly beta-hemolytic spirochetes regardless of diet ($P < 0.002$) and Cyanobacteria were less abundant in these same pigs. Spirochaetes were significantly different between groups

($P < 0.001$) and were most abundant in pigs fed diet 2 and either culture negative or colonized with weakly beta-hemolytic spirochetes. A statistically significant difference was detected between one or more groups for 40 different genera. Of these, *Brachyspira* spp., *Campylobacter* spp., *Fusobacterium* spp., *Bacteroides* spp., *Flexispira* spp. and a putative novel genus in the family Paraprevotellaceae were more common in pigs colonized with strongly beta-hemolytic spirochetes regardless of diet ($P \leq 0.003$, all analyses; Figure 4b). *Lactobacillus* spp. were more abundant in pigs fed diet 1 and either culture negative or colonized with weakly beta-hemolytic spirochetes ($P < 0.001$), whereas *Treponema* spp. were more abundant in pigs fed diet 2 and either culture negative or colonized with weakly beta-hemolytic spirochetes ($P \leq 0.001$).

Discussion:

A resurgence of SD has recently been observed in North American swine herds after a period in which the disease was nearly eliminated. The underlying reason(s) for this increased observance is poorly understood; however, coincident with the reemergence of *Brachyspira*-associated disease in pigs has been increased feeding of insoluble dietary fiber through the addition of DDGS in many commercial swine diets.

Currently, relatively little is published about the precise pathogenesis of SD; however, diet is considered to play a major role in disease expression.[1] The precise mechanisms by which diet composition impacts the pathophysiology of SD continue to be elusive. In a previous study, pigs fed a highly digestible diet of cooked rice developed an increased colonic content pH and decreased total volatile fatty acid concentrations and were protected from developing SD.[12] The protective mechanism of such a diet could be due to decreased fermentation in the large intestine[12] and/or changes in the microbiota with a subsequent increase in species that inhibit colonization of *B. hyodysenteriae*.[13,14] The microbiota may inhibit pathogens through competition for nutrient resources or by production of anti-microbial compounds. Alternatively, the indigenous microbiota may favor colonization and/or disease associated with a pathogen through the production of cofactors or metabolites essential to the survival or virulence of a given pathogen. Although a highly digestible diet has been associated with protection against SD in several experiments, this has not always been the case illustrating the multifactorial and complex nature of the pathophysiology of SD.[15]

The colonic contents evaluated in the present study were collected at necropsy from pigs fed either a corn-soy diet with no DDGS (diet 1) or a diet containing 30% DDGS (diet 2) in a previous study where pigs fed diet 2 and inoculated with either *B. hyodysenteriae* or "*B. hampsonii*" developed SD nearly twice as fast as pigs receiving diet 1.[9] The inclusion of 30% DDGS in the ration reflects a practical level, relative to industry practice, while adding sufficient insoluble dietary fiber to adequately test the hypothesis that such inclusion may significantly alter the colonic microbiota.[16]

The inclusion of DDGS in the diet (diet2) imparted significant changes in the microbiota of control pigs or those colonized by weakly beta-hemolytic *Brachyspira* spp. with increases in the relative abundance of Bacteroidetes and Spirochaetes and

a reduction in Firmicutes. Minimal differences in the relative abundance of bacterial taxa were observed in the microbiota of pigs colonized with weakly beta-hemolytic *Brachyspira* spp. relative to controls within each diet subclass and there were no significant difference in richness or diversity associated with the presence of these spirochetes in the microbiota. These findings and the low frequency of detection of *Brachyspira* spp. OTUs within the weakly beta-hemolytic inoculum groups despite positive necropsy cultures is consistent with a commensal status under the conditions of this experiment and sampling period.

The colonic microbiome was significantly altered in pigs with SD and/or colonization by strongly beta-hemolytic *Brachyspira* spp. regardless of diet subgroup with a marked reduction in richness and significant diversity as measured by Bray-Curtis dissimilarity. These features suggest a destabilization of the microbiome associated with infection with these highly virulent spirochetes. *Brachyspira* spp. OTUs were detected in 24 of 32 samples (75%) from pigs with SD and at a very low relative abundance. This frequency of detection is considerably higher than a previous report where only 2 of 8 (25%) fecal samples from pigs with SD had *Brachyspira* OTUs detected in their microbiota[17]; however, this previous investigation was limited to 1,000 reads per sample compared with 47,000 reads per sample in the current study and the increased frequency of detection likely reflects greater sequencing depth rather than differences in the samples themselves. Pigs with SD had a significant increase in Firmacutes and significantly lower Bacteroidetes:Firmicutes ratios. Such a reduction in this ratio has been reported in dogs with diarrhea of any cause[18] and also in a previous investigation of pigs with SD after "*B. hampsonii*" infection[17] suggesting such a shift may be primarily a reflection of a diarrheic state. Previous studies in gnotobiotic pigs have demonstrated the requirement of additional anaerobes, including *Bacteroides vulgatis* and *Fusobacterium necrophorum*, within the microbiome for the development of SD.[5,19] Consistent with this, *Fusobacterium* spp. and *Bacteroides* spp. were more abundant in colonic contents of pigs with SD regardless of diet. Additionally, *Flexispira* spp. *Campylobacter* spp., and a putative novel genus in the family Paraprevotellaceae were also more abundant in the colonic contents of pigs with SD, but it is unknown if any of these agents act synergistically with strongly hemolytic *Brachyspira* spp. and contribute to SD development or if they preferentially proliferate in the colonic environment produced during SD. The observation of more abundant *Campylobacter* spp. in pigs with SD is not unexpected given that prior to the identification of a spirochetal etiology for SD the disease was believed to be caused by *Campylobacter coli*[20] or that *C. coli* was required for SD development, [21] and a recent survey of diagnostic samples from pigs with enteric disease revealed a significant association between *Brachyspira*-associated disease and concurrent positive *Campylobacter* culture.[22] Further investigation into the potential interplay of these bacterial agents in the colonic microbiome is warranted.

In summary, the results of the present study reveal significant alterations in the colonic microbiome of pigs fed 30% DDGS (diet 2) relative to those fed a standard corn-soy diet. Spirochaetes were generally more abundant in pigs fed diet 2 regardless of inoculum suggesting this diet produces a colonic environment conducive to spirochete survival and supports the observation that SD developed faster in pigs fed 30% DDGS in a previous study.[9] Accordingly, dietary composition should be considered an important risk factor for the development of SD and dietary modification with reduction in DDGS should be considered as part of disease

elimination strategies for SD. Further investigation is warranted to elucidate the potential interrelationship between specific bacterial alterations observed in the colonic microbiome of pigs fed DDGS and strongly beta-hemolytic *Brachyspira* spp. as pigs fed DDGS appear at increased risk for developing SD.

References:

1. Hampson DJ (2012) Brachyspiral colitis. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of swine. Ames, IA: Wiley-Blackwell. pp. 680-696.
2. Siba PM, Pethick DW, Hampson DJ (1996) Pigs experimentally infected with *Serpulina hyodysenteriae*
   can be protected from developing swine dysentery by feeding them a highly digestible diet. *Epidemiol Infect* 116:207–216.
3. Pluske JR, Durmic Z, Pethick DW, et al. (1998) Confirmation of the role of rapidly fermentable carbohydrates in the expression of swine dysentery in pigs after experimental infection. *J Nutr* 128:1737-1744.
4. Hansen CF, Hernández Á., Mansfield J, et al. (2011) A high dietary concentration of inulin is necessary to reduce the incidence of swine dysentery in pigs experimentally challenged with *Brachyspira hyodysenteriae*. *Br J Nutr* 2011;106:1506-1513.
5. Harris DL, Alexander TJ, Whipp SC, et al. (1978) Swine dysentery: studies of gnotobiotic pigs inoculated with *Treponema hyodysenteriae*, *Bacteroides vulgatus*, and *Fusobacterium necrophorum*. J Am Vet Med Assoc 192: 468-471.
6. Burrough ER (2013) Swine dysentery - Re-emergence in the United States and Canada. Proceedings of the 6th International Conference on Colonic Spirochaetal Infections in Animals and Humans, Guildford, UK. pp. 55-56.
7. Chander Y, Primus A, Oliveira S, Gebhart CJ (2012) Phenotypic and molecular characterization of a novel strongly hemolytic *Brachyspira* species, provisionally designated *'Brachyspira hampsonii'*. J Vet Diagn Invest 24, 903-910.
8. Burrough ER, Strait EL, Kinyon JM, et al. (2012) Comparative virulence of clinical *Brachyspira* spp. isolates in inoculated pigs. *J Vet Diagn Invest* 24: 1025-1034.
9. Wilberts BL, Arruda PH, Kinyon JM, et al. Investigation of the impact of increased dietary insoluble fiber through the feeding of distillers dried grains with solubles (DDGS) on the incidence and severity of *Brachyspira*-associated colitis in pigs. *PLoS One* 2014;9:e114741.
10. Caporaso JG, Lauber CL, Walters WA, et al. (2012) Ultra-high throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J*;6(8):1621-1624.
11. Caporaso JG, Kuczynski J, Stombaugh J, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*;7(5): 335-336.
12. Siba PM, Pethick DW, Hampson DJ (1996) Pigs experimentally infected with *Serpulina hyodysenteriae* can be protected from developing swine dysentery by feeding them a highly digestible diet. *Epidemiol Infect* 116: 207-216.
13. Klose V, Bruckbeck R, Henikl S, et al. (2010) Identification and antimicrobial susceptibility of porcine bacteria that inhibit the growth of *Brachyspira hyodysenteriae* in vitro. *J Appl Microbiol* 108: 1271-1280.
14. Leser TD, Lindecrona RH, Jensen TK, et al. (2000) Changes in bacterial community structure in the colon of pigs fed different experimental diets and after infection with *Brachyspira hyodysenteriae*. *Appl Environ Microbiol*. 66: 3290–3296.
15. Durmic Z, Pethick DW, Mullan BP, et al. (2000) Extrusion of wheat or sorghum and/or addition of exogenous enzymes to pig diets influences the large intestinal microbiota

but does not prevent development of swine dysentery following experimental challenge. *J Appl Microbiol* 89: 678-686.
16. Urriola PE, Shurson GC, Stein HH (2010) Digestiblity of dietary fiber in distillers co-products fed to growing pigs. *J Anim Sci* 88: 2373-2381.
17. Costa MO, Chaban B, Harding J, Hill JE (2014) Characterization of the fecal microbiota of pigs before and after inoculation with "*Brachyspira hampsonii*". *PLoS One* 2014;9:e106399.Chaban B, Links MG,
18. Hill JE (2012) A molecular enrichment strategy based on cpn60 for detection of Epsiol-Proteobacteria in the dog fecal microbiome. *Microbial Ecol* 63:348-357.
19. Whipp SC, Robinson IM, Harris DL, et al. (1979) Pathogenic synergism between *Treponema hyodysenteriae* and other selected anaerobes in gnotobiotic pigs. *Infect Immun* 26:1042-1047.
20. Lussier G (1962) Vibrionic dysentery in swine in Ontario: 2. Morphological, biochemical and serological characteristics of *Vibrio coli*. *Can Vet J* 3:267-278.
21. Fernie DS, Griffin RM, Park WA (1975) The possibility that *Campylobacter* (*Vibrio*) *coli* and *Treponema hyodysenteriae* are both involved in swine dysentery. *Br Vet J* 131:335-338.
22. Burrough ER, Terhorst S, Sahin O, Zhang Q, (2013) Prevalence of *Campylobacter* spp. relative to other enteric pathogens in grow-finish pigs with diarrhea. *Anaerobe* 22:111-114.

XI. Figures

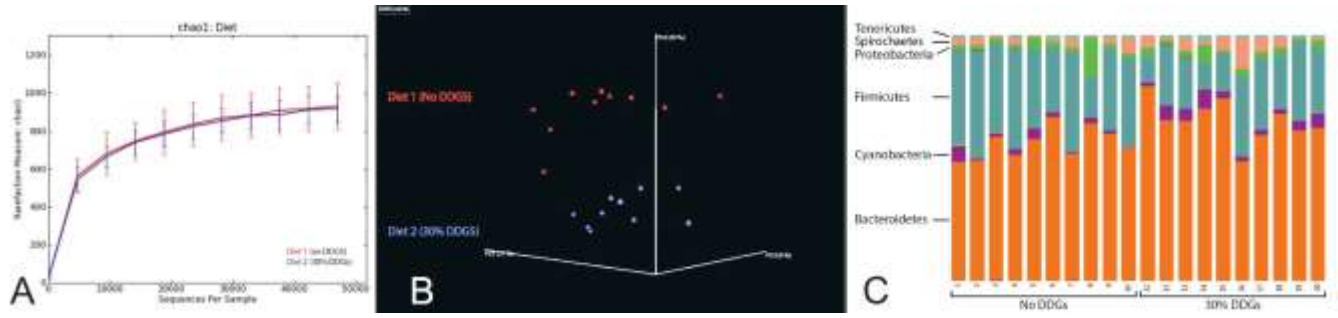

Figure 1. A) Rarefaction curve revealing no significant difference in richness of the colonic microbiota between sham-inoculated controls fed either diet 1 (no DDGS) or diet 2 (30% DDGS). B) PCoA plot demonstrating significant diversity (Bray-Curtis dissimilarity) between diet groups. C) Stacked bar charts representing proportional abundance of major phyla in the colonic microbiota of 20 sham-inoculated control pigs revealing a trend toward lower Bacteroidetes:Firmicutes ratios in pigs fed diet 1.

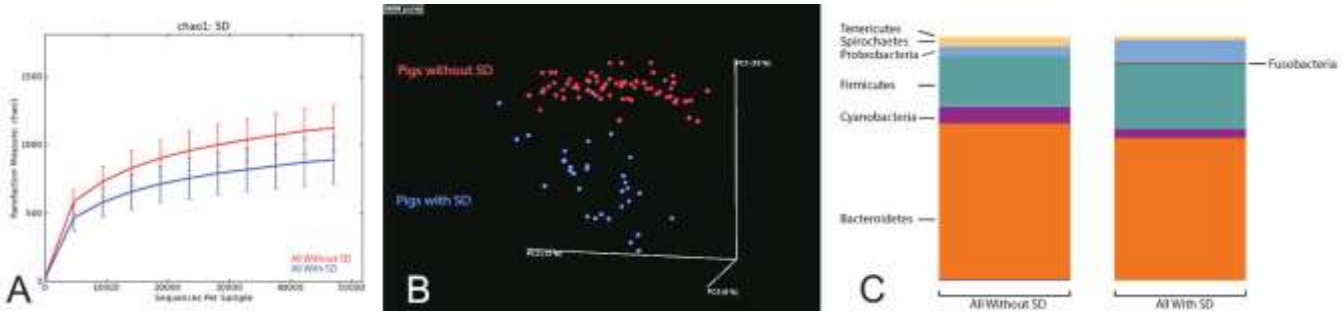

Figure 2. A) Rarefaction curve revealing a significant difference in richness of the colonic microbiota between pigs with swine dysentery (SD) and pigs without. B) PCoA plot demonstrating significant diversity (Bray-Curtis dissimilarity) between disease groups. C) Stacked bar charts representing proportional abundance of major phyla in the colonic microbiota of 98 pigs with or without SD revealing significantly more abundant Fusobacteria and Proteobacteria in pigs with SD and lower Bacteroidetes:Firmicutes ratios in pigs with SD.

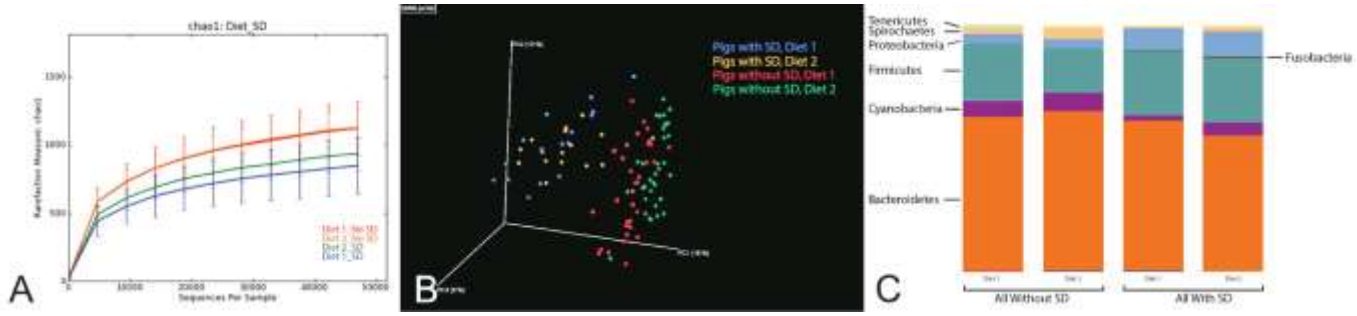

Figure 3. A) Rarefaction curve revealing a significant difference in richness of the colonic microbiota between pigs with swine dysentery (SD) and pigs without but no difference between diet subgroups. B) PCoA plot demonstrating diversity (Bray-Curtis dissimilarity) between groups. C) Stacked bar charts representing proportional abundance of major phyla in the of the colonic microbiota of 98 pigs with or without SD by diet subgroup revealing significantly more abundant Fusobacteria and Proteobacteria in pigs with SD regardless of diet, and significantly more abundant Spirochaetes in pigs without SD fed diet 2 and reduced abundance of Firmicutes in these same pigs.

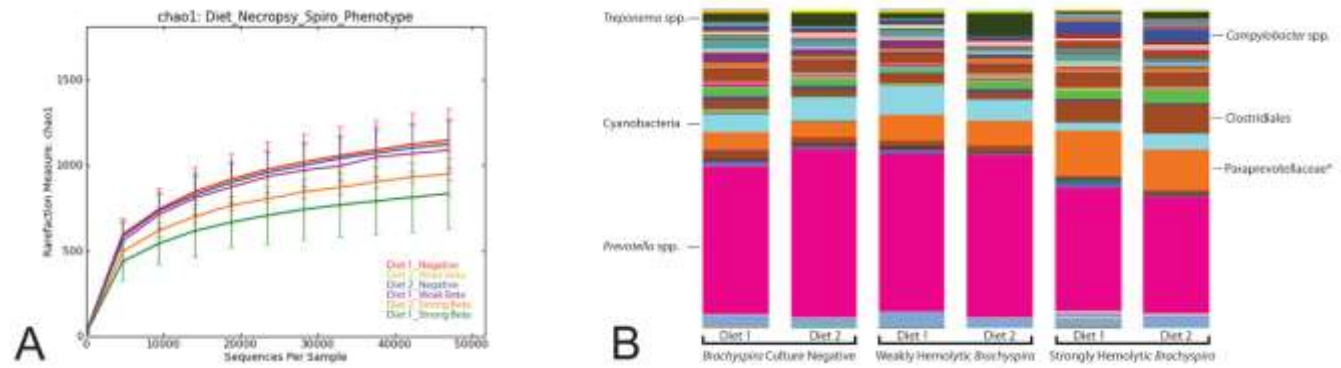

Figure 4. A) Rarefaction curve revealing a significant difference in richness of the colonic microbiota between pigs with strongly beta-hemolytic *Brachyspira* spp. recovered by culture of colonic contents at necropsy and all other disease and diet subgroups. B) Stacked bar charts representing proportional abundance of major genera in the colonic microbiota of 98 pigs grouped by *Brachyspira* culture results and diet subgroup revealing significantly more abundant *Campylobacter* spp., *Fusobacterium* spp., and a putative novel genus in the family Paraprevotellaceae in pigs colonized with strongly beta-hemolytic *Brachyspira* spp. Cyanobacteria were more abundant in *Brachyspira* culture negative pigs and those colonized with weakly beta-hemolytic species while *Treponema* spp. were more abundant in these same pigs fed diet 2.

Tables:

TABLE 1. Summary of positive *Brachyspira* culture and frequency of swine dysentery associated with 100 colonic content samples collected during a previous investigation[9] and the basis of the microbial community profiling in the present report.

	Positive Necropsy Culture*		Development of SD†	
Inoculum group	Diet 1[a]	Diet 2[b]	Diet 1	Diet 2
Sham-inoculated controls	0/10	0/10	0/10	0/10
Brachyspira hyodysenteriae	8/10	10/10	7/10	10/10
"*Brachyspira hampsonii*"	9/10	7/10	9/10	6/10
Brachyspira pilosicoli	1/10	4/10	0/10	0/10
Brachyspira intermedia	5/10	2/10	0/10	0/10

*Results reflect the number of samples with positive *Brachyspira* culture at necropsy / number inoculated

†Results reflect the number of pigs with mucohemorrhagic colitis / number inoculated

[a] Diet 1 = 0% Distiller's dried grains with solubles

[b] Diet 2 = 30% Distiller's dried grains with solubles