Pre and post-infection microbiome associations with weight gain in pigs co-infected with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2)

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Abstract

Evidence has shown that the gastrointestinal microbiome plays an important role in response to infectious disease. Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) are two of the most important pathogens affecting the swine industry worldwide. Co-infections are common on a global scale, resulting in pork production losses through reducing weight gain and causing respiratory disease in growing pigs. Our initial microbiome work demonstrated that the fecal microbiome was associated with clinical outcome of pigs 70 days post-infection (dpi). However, it remained uncertain if microbiome characteristics could predispose response to viral challenge. The purpose of this study was to determine if microbiome characteristics present at the time of viral challenge were associated with outcome after co-infection. Using the Lawrence Livermore Microbial Detection Array, we profiled the microbiome in feces on 0 dpi from pigs identified as having high or low growth rates after co-infection. High growth rate pigs had less severe interstitial pneumonia, reduced PRRSV replication, and a significant increase in average daily weight gain throughout the study. At the level of the fecal microbiome, high growth rate pigs had increased microbial diversity on both a family and species level. Shifts in the microbiome composition of the high growth rate pigs included reduced *Methanobacteriaceae* species, increased *Ruminococcaceae* species, and increased *Streptococcaceae* species when compared to low growth rate pigs. Our results indicate that both microbiome diversity and composition prior to virus exposure may play a role in the subsequent response of pigs to PRRSV/PCV2 co-infection. We followed this study by investigating the microbiome characteristics that are present after co-infection and the role of

the microbiome in subclinical infections. Microbiome analysis at 3 and 6 weeks post-infection showed no significant difference between high and low growth rate pigs.

The results from both exploring the impact that the initial microbiome has on outcome as well as examining the trends in the microbiome during the post-infection period demonstrate that microbiome pre-infection composition may play a larger role in the outcome of subclinical disease in pigs than microbiome composition during viremia or after viral clearance.

Table of Contents

List of Figures........ vii

List of Tables viii

Acknowledgements ix

1. Introduction 1

2. Increased microbiome diversity at the time of infection is associated with improved growth rates of pigs after co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2)........ 6

Introduction 7

Materials and Methods 10

Results 18

Discussion........ 24

3. Microbiome trends after co-infection indicate that improved growth rates of pigs infected with porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) are determined by microbiome composition prior to challenge........ 30

Introduction 31

Materials and Methods ....
34
Results ....
39
Discussion...
48
4.Conclusion ....
54
5.Figures and Tables ....
55
References ....
70

List of Figures

Figure 1. Average daily gain (ADG) distribution between 0 and 35 days post-infection (dpi).....55
Figure 2. Weight gain in pigs with high and low growth rates after co-infection with PRRSV and PCV2.. 56
Figure 3. Microscopic lung lesions in pigs with high and low growth rates after co-infection with PRRSV and PCV2. 57
Figure 4. Microscopic lymphoid depletion in pigs with high and low growth rates after co-infection with PRRSV and PCV2…………………………………………………...... 57
Figure 5. PRRSV viremia of high and low growth rate pigs..58
Figure 6. PCV2 viremia of high and low growth rate pigs…………………………...……59
Figure 7. Microbiome family composition in pigs with high and low growth rates after co-infection with PRRSV and PCV2. 60
Figure 8 Number of species detected within each microbial family…………………………….61
Figure 9. Species composition of the Streptococcaceae family in pigs with high and low growth rates after co-infection with PRRSV and PCV2..62
Figure 10. Fecal microbiome diversity in pigs with high and low growth rates after co-infection with PCV2 and PRRSV…………………………………………………...........…………63
Figure 11. 21 and 41 dpi microbiome composition of pigs with high and low growth rates after co-infection with PRRSV and PCV2...65
Figure 12. Microbiome diversity in pigs with high and low growth rates after co-infection with PRRSV and PCV2 21 dpi and 42 dpi…………………………………………………...........66

List of Tables

Table 1 Total PRRSV and PCV2 virus replication between 0-42 dpi reported as area under the viremia curve (AUC). 55

Table 2 Trends in specific bacterial species from 0 dpi to 42 dpi.. 67

Table 3 Trends in specific bacterial species from 0 dpi to 21 dpi 68

Table 4 Trends in specific bacterial species from 21 dpi to 42 dpi 69

Table 5 Trends in specific viral species from 0 dpi to 42 dpi.. 69

Table 6 Trends in specific viral species from 0 dpi to 21 dpi 69

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1. Introduction

Microbiome research is an emerging field of research relevant to many fields including microbiology, human and animal biomedical research, production medicine, as well as virology. The term microbiome signifies the "ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" (Lederberg 2001). The microbiome both covers the microbes found in the specific space as well as the genes that are encoded by these microbes (Baquero and Nombela 2012). In fact, humans are roughly composed of greater than 10 times more residential microbes than bodily cells (Savage 1977). There are four general sites of microbial colonization in the human body which can be extrapolated to mammals which are the mouth, gut, vagina (or genitourinary tract), and skin (Relman 2002).

In humans and animals, the gastrointestinal microbiome has been shown to affect a variety of different human phenotypes. The microbiome has an operational role in processing energy from diet as well as maintaining digestive function regulating fat storage, and regulating metabolism (Bäckhed et al. 2004, Nieuwdorp et al. 2014). The effects the microbiome has had on metabolism has led to studies focusing on the role of microbiome in weight gain and obesity. In humans, it was found that there is an increase in the proportion of *Bacteroidetes* in obese patients consuming a calorie restricted diet which correlated with weight loss (Ley et al. 2006b). In a comparison between anorexic patients, obese patients, and patients with a normal BMI, it was found that an increase in the *Firmicutes* and a decrease in *Bacteroidetes* correlated with the more obese patients in the study (Armougom et al. 2009). In contrast, another study found that an increase in the proportion of *Bacteroidetes* was associated with weight gain and obesity while an increase in *Methanobrevibacter* was associated with weight loss (Schwiertz et al. 2010).

In addition to affecting phenotype, the microbiome has been shown to play a role in understanding clinical presentations and responses of systemic diseases. The gut microbiome has been shown to affect the protective mucus layer of the intestinal tract, improving immunity against potential pathogens (Nieuwdorp et al. 2014). There have been a multitude of correlations between various diseases and microbiome. For example, an increase in the number of *Clostridium* species has been correlated with late-onset autism (Finegold et al. 2002) with some children showing reduction in stereotyped behaviors when treated with antibiotics targeting *Clostridial* species (Bolte 1998). Decreased diversity of the gut microbiome has been associated with patients afflicted with myalgic encephalomyelitis/chronic fatigue syndrome (Giloteaux et al. 2016). Patients with type-2 diabetes show an increase in the abundance of species in the *Enterobacteriaceae* family while butyrate-producing bacteria are depleted (Wang and Jia 2016). These are just a few of the many correlations between microbiome and clinical disease. Many new revelations are presently being uncovered through the study of the microbiome, however, the role of the microbiome in a host's susceptibility to disease as well as the host's response to pathogens remains poorly understood and further research involving this aspect of microbiome research is needed. After combing through and understanding the current literature regarding the microbiome, we began to explore avenues concerning the microbiome in swine and the role the microbiome plays in swine affected with 2 primary porcine pathogens.

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) are globally endemic viruses that affect the swine production. Both viruses are common in young, growing animals, and both viruses are implicated in polymicrobial disease syndromes, most specifically porcine respiratory disease complex and porcine circovirus

associated disease (PCVAD) (Brockmeier, Halbur, and Thacker 2002, Segales 2012, Trible and R.R. Rowland 2012). Furthermore, co-infections with both viruses has the capacity to enhance the disease processes, however, most pigs simultaneously affected with both viruses carry subclinical infections (Niederwerder, Bawa, Serao, et al. 2015).

PRRSV is a small, enveloped, single-stranded RNA virus in the family *Arteriviridae* (Benfield, Nelson, Collins, Harris, Goyal, Robison, T. Christianson, et al. 1992). PRRSV has the highest rate of mutation for known RNA viruses, allowing it to escape previous immunity to heterologous strains (Murtaugh et al. 2010). In addition to its rapid ability to mutate, PRRSV causes decreased weight gain and respiratory disease in growing swine resulting in it being the most costly disease of swine production worldwide (Chand, Trible, and Rowland 2012a). Modified live vaccines are the most common method used to control the spread of PRRSV (Renukaradhya et al. 2015). However, vaccines are considered inadequate for disease control. PCV2 is a single-stranded DNA virus in the family *Circoviridae* which is associated with PCVAD which involves muscle wasting, enteric disease, and respiratory disease (Segales 2012). One of the hallmarks of PCV2 infections is lymphocyte depletion in lymphoid tissues (Opriessnig and Langohr 2013). Although co-infections between PCV2 and PRRSV can exacerbate clinical disease, the majority of pigs support subclinical infections which still has implications involving poor weight gain (Niederwerder, Bawa, Serao, et al. 2015).

As mentioned previously, both PRRSV and PCV2 can cause wasting syndromes in young swine which can negatively impact pork production on a global level. New methods to both detect and characterize endemic and emerging animal diseases as well as their relationship with

the microbiome will allow us to understand how to better control polymicrobial diseases such as PRRSV and PCV2.

Pathogen microbial detection arrays are among the technologies that can be implemented to detect emerging and foreign animal diseases in addition to diagnostic clinical application. These microbial detection arrays are essentially glass or silicon chips with oligonucleotides (probes) containing specific DNA sequences attached to their surface. These probes target both conserved and unique sequences making these arrays effective in recognizing microbes in selected samples. Select samples are prepared and labeled with a fluorescent marker and then hybridized to the array chip. RNA viruses can also be recognized in the form of cDNA after performing a reverse transcriptase experiment. As of June 2013, the Lawrence Livermore Microbial Detection Array (LLMDA), targets all vertebrate infecting microbes including 3,856 viruses, 3,855 bacteria, 254 archaebacteria, 100 fungi, and 36 protozoa (Jaing et al. 2015a).

In 2015, Jaing et al. established that pathogen microarrays, in specific, the LLMDA can dependably be utilized to detect both PRRSV and PCV2 in swine serum and tonsil samples as well as recognize other microbes present in the samples. This has proved pivotal in determining both known and unknown pathogens in porcine diagnostic samples. In this thesis, the application of the microarray technique in determining known and unknown pathogens in swine is applied to fecal samples collected from pigs in the following studies in order to map individual swine microbiomes.

In 2015, Niederwerder et al. applied the LLNL microarray to evaluate differences in the microbiomes of pigs co-infected with PCV2 and PRRSV when comparing animals with best and worst clinical outcomes. Ninety-five pigs were challenged with PRRSV and PCV2 and

individual animals' body weights were recorded on -14, -7, 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 days post-infection (dpi). Blood samples were also collected to measure viremia throughout the experiment. The 10 best clinical outcome pigs were selected based on having the best average daily gain from 0 to 63 dpi with a complete lack of overt clinical signs, while the 10 worst clinical outcome pigs were selected as having the lowest average daily gain from 0 to 63 dpi with at least 10 days of moderate to severe clinical disease. Microarray analysis was performed on serum, feces, and lung tissue from 70 dpi. *E. coli* was detected was detected in significantly greater proportion in best outcome pigs compared to worst outcome pigs, indicating there may be important differences in microbiome. No significant differences in microbiome were detected in the lung tissue. Increased fecal microbiome diversity was recognized with improved outcome. This paper by Niederwerder et al. and the paper by Jaing et al. provided the foundation of the research highlighted in this thesis.

Based on these two initial papers, two major objectives were outlined. The first objective was to determine what pre-infection microbiome characteristics predispose outcome in subclinical infections as well as to determine the role of the microbiome in subclinical infections. The second objection was to determine if these changes in the microbiome prior to viral challenge are consistent during the post-infection period.

2. Increased microbiome diversity at the time of infection is associated with improved growth rates of pigs after co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2)

Introduction

Pork is the most widely consumed protein around the world and global pork production is forecast to reach a record of 111 million tons in 2017 (USDA 2016). Worldwide, porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) are two of the most significant pathogens affecting the swine industry, costing billions in lost production over the last 3 decades. PRRSV is a single-stranded RNA virus in the family *Arteriviridae* (Benfield, Nelson, Collins, Harris, Goyal, Robison, Christianson, et al. 1992) which causes reductions in weight gain and respiratory disease in growing pigs. It is currently considered the most costly disease of swine production worldwide (Chand, Trible, and Rowland 2012a). PCV2 is a single-stranded DNA virus in the family *Circoviridae* and is associated with a group of disease syndromes termed porcine circovirus associated disease (PCVAD), which includes muscle wasting, respiratory disease, and enteric disease (Segales 2012). Both viruses result in systemic infections, cause primary lung pathology, and modulate the immune response. For example, PRRSV suppresses innate immunity through antagonizing type I interferon production (Chen et al. 2010, Han and Yoo 2014) and PCV2 depletes lymphocytes in lymphoid tissues (Opriessnig and Langohr 2013). Co-infection with the two viruses enhance disease when compared to single infections alone and can result in a wide range of overt clinical signs; however, overall morbidity is typically less than 30% (Niederwerder, Bawa, Serao, et al. 2015), leaving the majority of pigs to support subclinical infections.

Playing an essential role in both nutrient availability and immunity (Honda and Littman 2016, Turnbaugh et al. 2006), the microbiome, or collection of microorganisms within the gastrointestinal tract, has been associated with outcome during systemic viral infections in

both humans and mouse models. Microbiome associations have been found with viruses primarily affecting the respiratory tract, such as respiratory syncytial virus and influenza virus (Fujimura et al. 2014, Ichinohe et al. 2011), as well as viruses considered immunomodulatory, such as human immunodeficiency virus (Mudd and Brenchley 2016). Disease progression, airway inflammation, immune response, and morbidity can all have associations with microbiome composition and diversity during these viral infections.

The role of the microbiome and its impact on the response to systemic infections in swine is an emerging area of study and the focus of our work. To investigate the porcine microbiome, we utilized the Lawrence Livermore Microbial Detection Array (LLMDA), which was developed to detect all known microbes for which whole genome sequences are available. Current as of June 2013, this technology allows for the detection of 8,101 microbes. The LLMDA has been used in the detection of known and unknown microbes in various porcine samples, including feces, serum, lung, oral fluids, lymph node suspension and tonsil (Niederwerder et al. 2016a, Jaing et al. 2015a).

In a recent study, we investigated the associations between the microbiome and clinical outcome in pigs following PRRSV/PCV2 co-infection (Niederwerder et al. 2016a). Best and worst clinical outcome pigs were selected based on the presence and severity of clinical disease as well as weight gain after co-infection. The fecal microbiomes of best clinical outcome pigs were characterized by increased diversity and increased prevalence of *Escherichia coli* 70 days after co-infection. While this initial study confirmed a potential role for the microbiome in outcome following PRRSV/PCV2 co-infection, important gaps in our knowledge remained, such

as microbiome characteristics that predispose outcome and the role of the microbiome in subclinical infections.

In the current study, we investigated the early microbiome properties that predispose high and low growth rates after co-infection in subclinically affected pigs. Our results demonstrate that both microbiome diversity and composition prior to infection play a role in weight gain following PRRSV/PCV2 co-infection.

Materials and Methods

Animals and housing: All use and experimentation incorporating animals and viruses were done in accordance with the Federation of Animal Science Societies (FASS) Guide for the Care and Use of Agricultural Animals in Research and Teaching, the USDA Animal Welfare Act and Animal Welfare Regulations, and approved by the Kansas State University Institutional Animal Care and Use Committees and Institutional Biosafety Committees. This study was conducted as part of a project to evaluate the role of host genetics in determining the outcome following co-infection with PRRSV and PCV2; a subset of pigs from this project were included in the current study. Three week old barrows (n = 50; average age 23.5 ± 2.6 days) were obtained at weaning from a high health commercial herd negative for PRRSV. While the pigs were derived from a sow herd previously vaccinated with a PCV2 capsid subunit vaccine, the piglets were not vaccinated for PCV2 and were utilized without regards to maternal antibody. All pigs were housed in a single environmentally controlled room at the Kansas State University Large Animal Research Center under BSL-2 conditions. The piglets were randomly distributed and housed in groups of 8-10 pigs per 13.4 m^2 pen. All pigs were given a period of approximately 4 weeks to acclimate to their new environment prior to co-infection. Pigs were given access to food and water *ad libitum*.

Viruses: The PRRSV and PCV2b viral isolates used to prepare the inoculum for this study were originally derived from the lymph node of a pig with severe postweaning multisystemic wasting syndrome (PMWS) as previously described (Trible et al. 2012). PRRSV (isolate KS62; GenBank accession no. KM035803) was isolated by propagation on MARC-145 cells. Since

wild-type PCV2b (GenBank accession no. JQ692110) does not propagate to high levels in cell culture, we took advantage of the heat stability of the virus to prepare a lymph node suspension enriched for PCV2 as previously described (Niederwerder et al. 2016a, Niederwerder, Bawa, Serao, et al. 2015). The isolated PRRSV was recombined with the heat-treated PCV2 homogenate to co-infect cesarean-derived, colostrum-deprived (CD/CD) pigs. A combination lung/lymph node homogenate was prepared from the CD/CD pigs, and PRRSV and PCV2 were isolated from the homogenate by the methods described. Analysis of the inoculum yielded negative results for most heat-stable agents, but was positive for two viruses ubiquitous to swine, including Torque teno sus virus (TTSuV) and porcine endogenous retrovirus (PERVs) (Jaing et al. 2015a).

PCV2b was titrated on swine testicle (ST) cells. Briefly, serial 10-fold dilutions of PCV2 challenge stock were plated in quadruplicate onto rapidly dividing ST cells in a 96-well tissue culture plate (BD Falcon). Dilutions were prepared in Eagle's minimal essential medium (EMEM; Sigma-Aldrich) supplemented with 7% fetal bovine serum (FBS; Sigma-Aldrich) and 50 µg/mL of gentamicin (Lonza). Following a 3-day incubation at 37˚C in 5% CO_2, cells were fixed and permeabilized with 80% acetone. Cells were then stained with fluorescein isothiocyanate (FITC)-labeled porcine anti-PCV (Veterinary Medical Research and Development, Inc.). Infected cells were visualized using an inverted fluorescent microscope and the 50% tissue culture infectious dose ($TCID_{50}$/mL) was calculated using the method of Reed and Muench (Reed and Muench 1938).

MARC-145 cells were used for the titration of PRRSV. Briefly, virus was serially diluted 1:10 in minimal essential medium (MEM; Corning) supplemented with 7% FBS (Sigma-

Aldrich), penicillin-streptomycin (Pen Strep; 80 Units/mL and 80 µg/mL, respectively; Gibco), 3 µg/mL amphotericin B (Fungizone; Gibco), and 25 mM HEPES (Life Technologies). The dilutions were then added in quadruplicate to confluent MARC-145 cells in a 96-well tissue culture plate (BD Falcon). Following a 4-day incubation at 37˚C in 5% CO_2, cells were examined for PRRSV-induced cytopathic effects. The $TCID_{50}$/mL was calculated using the method of Reed and Muench (Reed and Muench 1938).

Experimental design and sample collection: At approximately 8 weeks of age (average age 54.5 ± 2.6 days), all 50 pigs were infected with PRRSV and PCV2b. The viruses were recombined to yield a 2-mL dose consisting of $10^{3.6}$ $TCID_{50}$ PCV2b and 10^5 $TCID_{50}$ PRRSV in MEM. The 2-mL dose was split, with 1 mL being delivered intranasally and 1 mL being delivered intramuscularly. Body weights of individual pigs were collected upon arrival and on 0, 7, 14, 21, 28, 35, and 42 days post-infection (dpi). Blood samples were collected from all pigs on 0, 4, 7, 11, 14, 21, 28, 35, and 42 dpi. Fecal samples were collected from all 50 pigs during the week prior to co-infection. At 35 dpi, 20 pigs were selected to represent high growth rate pigs (n = 10) and low growth rate pigs (n = 10). To select these two groups, the average daily gain (ADG) was calculated between 0 and 35 dpi as the change in weight over the change in time and reported in kg. Pigs in the high growth rate group had the highest ADG and pigs in the low growth rate group had the lowest ADG. The two groups were balanced according to initial weight on 0 dpi. Any pig that had overt clinical disease requiring veterinary medical treatment (as described below) was excluded from selection. At 42 dpi, all 20 pigs were humanely

euthanized in accordance with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals and complete necropsies were performed.

Clinical and pathologic evaluation: All pigs were assessed daily for the presence of clinical signs associated with PRRSV/PCV2 co-infection, such as dyspnea, coughing, nasal discharge, aural cyanosis, open mouth breathing, decreased body condition, muscle wasting, lethargy, depression, joint effusion, lameness, and pallor or jaundice. Pigs were visually examined by a veterinarian or veterinary assistant on each day of the study period. Under the direction of a veterinarian, appropriate treatments were administered to pigs with moderate to severe clinical disease. Examples of clinical presentations where treatment was administered included 1) dyspnea, 2) mucoid rhinorrhea, 3) lameness with joint effusion, 4) pallor or jaundice with muscle wasting, and 5) lethargy or depression with pyrexia. Clinically affected pigs were administered parenteral antibiotics, such as ceftiofur hydrochloride, oxytetracycline, or enrofloxacin. Any pig with overt clinical disease and a rectal temperature of ≥ 104˚F was administered parenteral flunixin meglumine, a nonsteroidal anti-inflammatory drug. For those pigs with pyrexia lasting longer than 4 days, a 2 day wash-out period was prescribed prior to the administration of oral meloxicam. Any pig with documented clinical disease requiring veterinary medical treatment was excluded from the high and low growth rate groups for this study.

At 42 dpi, all pigs were humanely euthanized with intravenous pentobarbital sodium. A board certified veterinary pathologist, blinded to the source of the pigs, performed complete necropsies and histopathology. Tissues collected included lung (1 section from each lobe) and tracheobronchial lymph node. Tissues were fixed in 10% neutral buffered formalin for at least 7

days, routinely processed in an automated tissue processor, embedded in paraffin, and stained with hematoxylin and eosin (H&E stain). Microscopic lung lesions were scored using a 0-4 system as previously described (Niederwerder et al. 2016a, Niederwerder, Bawa, Serao, et al. 2015). Scores were assigned as follows: 0, no significant lung lesions; 1, mild multifocal interstitial pneumonia with <50% lung lobe involvement; 2, mild to moderate multifocal interstitial pneumonia with 50-75% lung lobe involvement; 3, moderate to severe multifocal interstitial pneumonia with 50-75% lung lobe involvement; 4, severe diffuse interstitial pneumonia with >75% lung lobe involvement. Degree of lymphoid depletion was scored using a 0-3 system as previously described (Niederwerder et al. 2016a). Scores were assigned as follows: 0, no lymphoid depletion; 1, mild or small amount of lymphoid depletion; 2, moderate or intermediate amount of lymphoid depletion; 3, severe or large extent of lymphoid depletion.

Measurement of PRRSV and PCV2 viremia: Viral DNA and RNA was extracted simultaneously from 50 µL of serum using Ambion's MagMAX 96 Viral Isolation Kit (Applied Biosystems) in accordance with the manufacturer's instructions. PRRS viral RNA was quantified using EZ-PRRSV MPX 4.0 Real Time RT-PCR Target-Specific Reagents (Tetracore) according to the manufacturer's instructions. For consistency, each plate contained Tetracore Quantification Standards and Control Sets for use with EZ-PRRSV MPX 4.0 RT-PCR Reagents. All PCR reactions were carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) in a 96-well format using the recommended cycling parameters. The PCR assay results were reported as log_{10} PRRSV RNA starting quantity (copy number) per 50 µL reaction volume.

PCV2 DNA was quantified using SsoAdvanced Universal SYBR green supermix (Bio-Rad) as previously described (Niederwerder, Bawa, Serao, et al. 2015, Niederwerder et al. 2016a). Briefly, forward and reverse PCR primers were 5'-AATGCAGAGGCGTGATTGGA-3' and 5'-CCAGTATGTGGTTTCCGGGT-3', respectively, and were used at a final concentration of 300 μM. Standard curves and positive and negative controls were included on each plate. Plasmid DNA was used for the PCV2 standard curve and positive control template. DNA inserted into the plasmid was obtained from a field strain of PCV2 (PCV2b 321/393). The PCV2 PCR was carried out on a CFX96 Touch Real-Time PCR Detection System using the following settings: activation at 98°C for 2 minutes, followed by 40 cycles of denaturing at 98°C for 5 seconds and annealing/extension at 60°C for 10 seconds. The PCR assay results were reported as $\log_{10}$ PCV2 DNA starting quantity (copy number) per 20 μL reaction volume.

Microarray Analysis of Fecal Samples: The Lawrence Livermore Microbial Detection Array (LLMDA) developed at the Lawrence Livermore National Laboratory was designed to detect all sequenced microbes. The version 7 of the LLMDA in the 4plex 180K probe format was used to analyze the microbiome in fecal samples from this study. This version of the array targets all vertebrate infecting microbes including 3,856 viruses, 3,855 bacteria, 254 archaebacteria, 100 fungi, and 36 protozoa. The LLMDA oligonucleotide probes are around 60 nucleotides in length and were designed to detect all sequenced microbial families with a large number of probes per sequence (average of 30 probes) to improve sensitivity in the evaluation of microbial nucleic acids in a variety of samples. The high-density oligo LLMDA microarray and statistical analysis method have been extensively tested in numerous studies for viral and bacterial detection in pure

or complex environmental and clinical samples (Jaing et al. 2015a, Gardner et al. 2010a, Rosenstierne et al. 2014a, Niederwerder et al. 2016a).

DNA and RNA from fecal samples were extracted using the PowerViral™ Environmental RNA/DNA Isolation Kit (MO BIO, San Diego, CA) in accordance to the manufacturer's instructions. Briefly, for each sample approximately 0.25 g of feces was added to 600 µl of PV1 in a bead beating tube included in the PowerViral™ kit. Samples were homogenized and lysed using the vortex adaptor for the MO BIO Vortex-Genie 2 for 10 minutes at maximum speed. Samples were further purified using the manufacturer's standard protocols. All samples were eluted into 100 µl of RNase-Free water. The purified nucleic acids were quantified using the Life Technologies Qubit fluorimeter. For each sample, 10 µl of the extracted DNA and RNA was amplified using the random amplification procedure as previously described (Rosenstierne et al. 2014a). The amplified cDNA and DNA was purified with the Qiaquick PCR purification columns (Qiagen) and quantified using the Qubit fluorometer. Between 400-500 ng of amplified cDNA and DNA were fluorescently labeled using a one-coloring labeling kit (Roche NimbleGen, Madison, WI) following the manufacturer's instructions.

Comparative genomic hybridization (CGH) mix was prepared in the following manner using the Agilent Technologies Oligo aCGH/ChIP-on-Chip Hybridization kit (Santa Clara, CA). For each sample, 10 µg of fluorescently labeled DNA was mixed with CGH mastermix, denatured at 95˚C for 3 min, and incubated at 65˚C until the arrays were ready to load. The LLMDA version 7 4x180K format microarray (Agilent Technologies Inc., Santa Clara, CA) was utilized for this experiment. Each sample was loaded onto the array and hybridized for 40 hr at 65˚C in a microarray rotator oven (Agilent Technologies Inc., Santa Clara, CA) set to a speed of

20. Microarrays were then washed using the standard manufacturer's protocol with Oligo aCGH/ChIP-on-chip Wash Buffer 1 and Oligo aCGH/ChIP-on-chip Wash Buffer 2 (Agilent Technologies Inc., Santa Clara, CA). The arrays were each washed in CGH Wash 1 for 5 min at room temperature, followed by CGH Wash 2 for 1 min at 37˚C. Arrays were then streamed with nitrogen gas to remove particulate matter from the surface. Using the MS200 microarray scanner (Roche NimbleGen, Madison, WI), all arrays were scanned at a resolution of 2 μM.

Microarray data was generated from the microbe sequences at ≥99% threshold using the CLiMax method developed at Lawrence Livermore National Laboratory (Gardner et al. 2010a). The log likelihood for each of the positive targets is estimated from the BLAST similarity scores of the array feature and target sequences, together with the feature sequence complexity and other covariates derived from BLAST results.

Statistical Analysis: All statistical analyses were performed using GraphPad Prism 7.01 software (La Jolla, CA). Mean weekly weights, ADG, and viremia were compared between groups using the unpaired t-test. Microscopic lung and lymph node lesion scores were compared between groups using the Mann-Whitney U test. Microbiome diversity and number of species within family and phylum were compared between groups using the Mann-Whitney U test. Proportions of each growth rate group with individual species and families detected were compared using Fisher's exact test.

Results

Weight gain of high and low growth rate groups diverged after co-infection: Of the 50 pigs, 10 pigs had moderate to severe clinical disease that required veterinary medical treatment (20% overall morbidity). No mortalities occurred during the 42 dpi. Thus, 40 pigs qualified for selection of the high and low growth rate groups; these pigs supported subclinical infections or had mild and transient clinical disease that did not require veterinary intervention or antibiotic treatment. ADG between 0 and 35 dpi was used as the criteria for the selection of the two groups of pigs in this study (Fig. 1A). Mean ADG for the 10 high growth rate pigs was 0.903 ± 0.043 kg, with a range between 0.836 kg and 0.962 kg. This compares to a mean ADG for the 10 low growth rate pigs of 0.755 ± 0.075 kg, with a range between 0.596 kg and 0.827 kg. ADG values between the two groups did not overlap. Mean ADG difference between the groups was statistically significant ($p < 0.0001$, unpaired t-test). Mean weights upon arrival after weaning were 5.3 ± 0.6 kg and 5.7 ± 0.9 kg for the low and high growth rate groups, respectively ($p = 0.26$, unpaired t-test). Mean weights continued to be similar during the acclimation period and on the day of co-infection; 19.2 ± 2.2 kg for the low growth rate group and 19.5 ± 1.9 kg for the high growth rate group on 0 dpi ($p = 0.75$, unpaired t-test; Fig 1B). No significant difference was detected between the mean weights of the two groups prior to virus infection.

After infection, the mean weights between the two groups began to diverge, initially noting a trend for absolute weight differences on 21 and 28 dpi (see Fig. 1B). On 21 dpi, mean weights for the low and high growth rate groups were 33.1 ± 4.3 kg and 36.1 ± 2.6 kg, respectively ($p = 0.077$, unpaired t-test). By 28 dpi, mean weights had diverged by approximately 4.4 kg; low growth rate pigs weighed an average of 38.7 ± 4.7 kg and high growth

rate pigs weighed an average of 43.1 ± 6.1 kg ($p = 0.09$, unpaired t-test). It was not until 35 and 42 dpi that mean weights between the high and low growth rate groups were significantly different ($p = 0.001$ and $p = 0.028$, respectively). Final weights on 42 dpi were 52.5 ± 5.3 kg and 57.1 ± 2.9 kg for the low and high growth rate groups, respectively.

High growth rate pigs had reduced PRRSV and PCV2 replication: PRRSV and PCV2 viremia were measured at 9 time points during the course of the study. Individual days as well as total virus replication were compared between the two groups. Overall, PRRS virus replication in the low growth rate group had a more rapid increase and a more prolonged decay (Fig. 1C). On 4 dpi, mean viral loads for the low and high growth rate groups were 4.1 and 3.6 $\log_{10}$ copies/PCR reaction, respectively ($p = 0.03$, unpaired t-test). Both groups had peak PRRS virus replication on 11 dpi. On 14 dpi, high growth rate pigs had a significant decline in PRRSV viremia; viral loads for low and high growth rate pigs were 4.4 and 2.8 $\log_{10}$ copies/PCR reaction, respectively (0.004, unpaired t-test). A trend for reduced PRRS virus replication in the high growth rate pigs continued on 21 dpi (0.05, unpaired t-test).

PCV2 viremia followed a similar trend to PRRSV, with low growth rate pigs having a more rapid rise in viral load followed by a more gradual decline (Fig. 1D). Significant differences between the two groups were noted on days 11 and 14 post-infection. On 11 dpi, mean PCV2 loads for the low and high growth rate groups were 1.3 and 0.4 $\log_{10}$ copies/PCR reaction, respectively ($p = 0.03$, unpaired t-test). On 14 dpi, the low growth rate group continued to have an increase in mean virus load of approximately 0.73 $\log_{10}$ copies/PCR reaction ($p = 0.019$, unpaired t-test). This trend continued at 21 dpi; however, group differences were not

statistically significant (p = 0.086, unpaired t-test). Overall, virus replication followed a similar pattern in the two groups, peaking at 28 dpi and initiating a decay in PCV2 at 35 dpi (Fig. 1D).

Total virus replication over the 42-day study was determined by calculating the area under the viremia curve (AUC) as previously described (Boddicker et al. 2012, Niederwerder et al. 2016a). The PRRSV AUC ranges for the low and high growth rate groups were 83.3-115.6 and 56.0-87.7, respectively, with only a single value overlapping between the two groups. Mean PRRS viral load for the low and high growth rate groups was 98.6 ± 8.5 and 78.3 ± 9.9, respectively. This difference was statistically significant (p = 0.0001, unpaired t-test). For PCV2, there was significant variation and a broad range of total virus load in each of the groups; 0-94.9 and 0-77.5 for the low and high growth rate pigs, respectively. Mean PCV2 AUC values were higher in low growth rate pigs; 57.6 ± 28.5 and 35.6 ± 22.6 for the low and high growth rate groups, respectively. However, this difference was not statistically significant (p = 0.07, unpaired t-test). Taken together, this data further supports the role for pathogen load in determining the outcome after PRRSV/PCV2 co-infection.

High growth rate pigs had decreased lung lesion severity, but similar lymphoid lesions: Histopathologic lesions of tissues collected on 42 dpi were scored by a board certified pathologist blinded to the selection of pig groups. The degree of interstitial pneumonia was scored between 0-4 based on microscopic evaluation of lung tissue from each lung lobe (Niederwerder et al. 2016a, Niederwerder, Bawa, Serao, et al. 2015). Mean lung lesion scores were 2.3 and 1.2 for low and high growth rate groups, respectively (p = 0.05, Mann-Whitney U test; see Fig. 2A). The majority of high growth rate pigs (80%) had normal lung or mild

multifocal interstitial pneumonia with <50% lung lobe involvement. Representative pictures of microscopic lung lesions are shown in Fig. 2B-C. These data show that even when pigs are lacking in the presentation of overt clinical signs of respiratory disease, significant interstitial pneumonia may still be present.

Lymph nodes were examined for lymphocytic infiltration and for the presence of germinal centers as lymphoid depletion is essentially a pathognomonic lesion of PCVAD. Representative pictures of microscopic lymphoid lesions are shown in Fig. 2E-F. Almost all pigs had some degree of lymphoid depletion, with only a single high growth rate pig having normal microscopic lymph node appearance (Fig. 2D). Although mean scores were slightly increased for low growth rate pigs (1.9 compared to 1.6 for high growth rate pigs), no significant difference was detected between the two groups ($p = 0.6$, Mann-Whitney U test).

The microbiomes of high growth rate pigs had increased diversity, *Ruminococcaceae*, and *Streptococcaceae*, but reduced *Methanobacteriaceae*: The presence of microbial families and species was determined by the Microbial Detection Array. Overall, a total of 29 microbial families (Fig. 3A) and 112 microbial species (data not shown) were detected by the microarray. Microbial families included 6 viral families, 1 archaea family, and 22 eubacterial families. Both DNA and RNA viruses were detected, including the families of *Reoviridae*, *Picornaviridae*, *Astroviridae*, *Circoviridae*, *Parvoviridae*, and *Pospiviroidae* (see Fig. 3A). Bacterial families were detected from several phyla, including Firmicutes (10/22), Proteobacteria (6/11), Bacteroidetes (2/11), Actinobacteria (1/22), Tenericutes (1/22), Chlamydiae (1/22), and Spirochaetae (1/22).

Each of the 29 microbial families were detected at similar prevalence rates in both the high and low growth rate groups (Fig. 3A). Both groups had a 100% prevalence rate of the bacterial families *Lactobacillaceae*, *Lachnospiraceae*, *Prevotellaceae*, and *Mycoplasmataceae*. In addition, the majority of pigs in both groups had the microbial families of *Astroviridae*, *Methanobacteriaceae*, *Vibrionaceae*, *Veillonellaceae*, *Ruminococcaceae*, and *Streptococcaceae* detected. No significant difference was detected in the proportion of the two groups with each family detected ($p > 0.05$, Fisher's exact test).

To further investigate microbiome differences between the two groups, the number of species detected within each family was compared (Fig. 3B). The greatest number of species were detected in the *Lactobacillaceae* family, with a mean of 5.1 and 5.8 species detected in the low and high growth rate groups, respectively; however, this difference was not statistically significant ($p = 0.44$, Mann-Whitney U test). Three families had notable differences in the number of species detected between the two groups. First, there was a trend towards greater species diversity of the high growth rate pigs in the *Streptococcaceae* family; 4.2 species compared to 2.8 species detected in the low growth rate group ($p = 0.0998$, Mann-Whitney U test). Overall, 12 *Streptococcus* species were detected in the feces of these pigs (Fig. 3C). Second, there was a significant decrease in the species diversity of the *Methanobacteriaceae* family in the high growth rate group. An average of 1.4 *Methanobacteriaceae* species were detected in the high growth rate pigs versus an average of 2.5 species detected in the low growth rate pigs ($p = 0.0086$, Mann-Whitney U test). Third, an increased number of *Ruminococcaceae* species were detected in the high growth rate group; 1.5 species versus 1.1 species in the low growth rate group ($p = 0.0573$, Mann-Whitney U test). This data suggests that microbiome

composition at the time of virus exposure may play a role in determining outcome during co-infection. Most interestingly, species within the *Streptococcaceae* and *Ruminococcaceae* families may be beneficial to growth after co-infection whereas species within the *Methanobacteriaceae* family may be detrimental to growth after co-infection.

The prevalence rate of each of the 112 individual species were also compared between the two groups (data not shown). Only a single species was detected at a significantly different rate between the high and low growth rate groups. *Streptococcus equi* was detected at a significantly higher rate in high growth rate pigs (see Fig. 3C); 10 and 70% of the low and high growth rate pigs, respectively, had *Strep. equi* detected ($p = 0.0198$, Fisher's exact test). This data suggests that the presence of *Streptococcus equi* may be beneficial to weight gain during viral infection.

Although we are unable to calculate the relative abundance of bacteria in the Firmicutes and Bacteroidetes phyla using the LLMDA, we were able to evaluate the overall number of species detected within each phylum and compare these numbers between the low and high growth rate groups (data not shown). In general, the high growth rate pigs had increased numbers of Firmicutes species detected; 19.4 ± 1.4 species vs 15.8 ± 1.0 species for the low growth rate group ($p = 0.08$, Mann-Whitney U test). High growing pigs also tended to have decreased species numbers detected in the Bacteroidetes phylum; 3.6 ± 0.3 species compared to 4.1 ± 0.4 species in the low growing pigs ($p = 0.34$, Mann-Whitney U test). Although the differences in the current study were not statistically significant, it is interesting to consider the similarities with characteristics of the obese human microbiome, where increased Firmicutes

bacteria coupled with reduced Bacteroidetes bacteria are associated with obesity (Ley et al. 2006a).

Microbiome diversity was determined by calculating the number of families and species detected in the feces of each pig (Fig. 4). The mean number of families detected in the low growth rate group was 13.1 with a range of 9-16 compared to a mean of 15.1 with a range of 10-18 for the high growth rate group. The difference in family diversity between the two groups was significant ($p = 0.0385$, Mann-Whitney U test). Species diversity between the two groups followed a similar trend. The mean number of species detected in the low growth rate group was 32.7 with a range of 29-37 whereas the high growth rate group had a mean of 36.9 species with a range of 29-42. The difference in species diversity was also significant ($p = 0.0328$, Mann-Whitney U test). Overall, high growth rate pigs had greater microbiome diversity, on both a family and species level, than low growth rate pigs. This data suggests that increased microbiome diversity may contribute to improved weight gain in pigs after co-infection.

Discussion

The current study builds on our previous work describing the microbiome profiles of best and worst clinical outcome pigs 10 weeks after co-infection with PRRSV and PCV2 (Niederwerder et al., 2016). The study described herein sought to investigate the microbiome characteristics prior to co-infection that may predict or predispose outcome. Second, in the current study, we included only those pigs which had not developed clinical signs warranting veterinary intervention or antibiotic treatment.

Prior to profiling the microbiome, we also investigated several other pathogenic characteristics of PRRSV/PCV2 co-infection. Perhaps the most interesting was the significant increase in virus replication of low growth rate pigs. This was surprising given the subclinical nature of disease and again emphasizes the importance of pathogen load in determining even subclinical outcomes of viral infections. Moreover, the low growth rate pigs had more severe lung lesions, with 2 low growth rate pigs having severe diffuse interstitial pneumonia. This is important to consider in a population of infected pigs, as poor growth, even in the absence of dyspnea, coughing, or nasal discharge, may be indicative of severe pulmonary pathology. Furthermore, this underscores the significant impact of subclinical infections on animal health and welfare.

At the level of the fecal microbiome, the LLMDA detected 29 microbial families and 112 microbial species, including viruses, bacteria, and archaebacteria in samples from pigs. In this study, we observed an increase in the Firmicutes:Bacteroidetes species ratio in the microbiomes of high growth rate pigs (5.6 compared to 4.2 for the low growth rate group; $p = 0.05$, Mann-Whitney U test). The association between the relative abundance of Firmicutes to Bacteroidetes can be found in studies of human obesity. The microbiome of obese individuals has been described as having an enhanced ability to extract energy from food (Turnbaugh et al. 2006) and is typically characterized by an increase in the abundance of the Firmicutes phylum coupled with a decrease in the abundance of the Bacteroidetes phylum (Ley et al. 2006a). Similar to humans, obese pigs have also been described as having decreased members of the Bacteroidetes phylum when compared to lean pigs (Guo et al. 2008) and have a positive correlation between weight gain and the Firmicutes phylum (Pedersen, Andersen, Molbak, et al. 2013). We found similar

correlations in the current study between high and low growth rate pigs, supporting a role for Firmicutes and Bacteroidetes bacteria in growth during viral infection; however, limitations to quantifying the relative abundance of microbial populations with the LLMDA should be considered.

Perhaps the most intriguing microbiome characteristic present in low growth rate pigs prior to infection was the decrease in microbiome diversity (see Fig. 4). This finding was consistent with our previous work, where pigs with the worst clinical outcome had reduced microbiome diversity at 70 dpi compared to pigs with the best clinical outcome (Niederwerder et al. 2016a). Reduced microbiome diversity is associated with the development of several non-infectious diseases of the respiratory tract, such as allergy and asthma (Tulic, Piche, and Verhasselt 2016, Abrahamsson et al. 2014, Bisgaard et al. 2011), as well as the severity of infectious respiratory disease following experimental inoculation. For example, Schachtschneider et al. (2013) evaluated the effects of increasing microbiome diversity through fecal microbiota transplantation on the response of pigs to *Mycoplasma hyopneumoniae* infection. Pigs with increased microbiome diversity had a more rapid antibody response, decreased gross lung lesions, and a significant reduction in coughing compared to their non-transplanted littermates (Schachtschneider et al. 2013). Similar effects have been seen in mice models of human respiratory pathogens. For example, Schuijt et al. (2016) compared antibiotic-treated mice with reduced microbiome diversity to mice with endogenous microflora in response to *Streptococcus pneumoniae* infection. Mice with increased microbiome diversity had reduced *S. pneumoniae* present in lung tissue, enhanced alveolar macrophage phagocytosis, lower mortality rates, and decreased interstitial pneumonia (Schuijt et al. 2016). Taken together,

microbiome diversity appears to play an advantageous role in the host response to pathogens targeting pulmonary tissues. Furthermore, microbiome diversity has proven to be beneficial in the response of pigs to 3 of the most important swine pathogens, PRRSV, PCV2 and *M. hyopneumoniae*.

In addition to microbial diversity, differences in microbiome composition were also detected between high and low growth rate pigs (Fig. 3B). First, high growth rate pigs had increased species present in the *Streptococcaceae* family with a specific increase in the prevalence of the species *Streptococcus equi* (Fig. 3C). Other studies investigating weight gain and the swine microbiome have also found associations with members of the *Streptococcaceae* family. Kim et al. (2016) reported a positive correlation between the abundance of *Streptococcaceae* in feces and weight gain in growing pigs being fed the antibiotic growth promoter tylosin (Kim et al. 2016). In 2013, Pedersen et al. compared microbiome characteristics between obese and lean pigs that were either cloned or not cloned. Although *Streptococcaceae* was 6.3 fold higher in the terminal ileum of obese cloned pigs when compared to their lean counterparts, this finding was not consistent in samples collected from the colon. In obese cloned and non-cloned pigs, the abundance of *Streptococcaceae* in the colon was decreased when compared to lean pigs (Pedersen, Andersen, Hermann-Bank, et al. 2013). Collectively, these results suggest that *Streptococcus* species may play a role in weight gain and the availability of nutrients in swine; however, this role may be dependent upon location in the gastrointestinal tract.

A second microbiome composition shift occurred within the Archaea family of *Methanobacteriaceae*. These archaebacteria were the only microbes detected at an overall

higher prevalence rate in low growth rate pigs. Species detected within the *Methanobacteriaceae* family included *Methanobacterium* sp., *Methanobrevibacter ruminantium*, and *Methanobrevibacter smithii*. Similar to our findings, Luo et al. (2012) reported that lean pigs had a greater abundance and diversity of methanogens in feces when compared to obese pigs (Luo et al. 2012). Interestingly, methanogens have also been associated with host metabolism and several weight phenotypes in humans (Barlow, Yu, and Mathur 2015); however, studies have been inconsistent in their findings (Zhang et al. 2009, Armougom et al. 2009, Million, Maraninchi, et al. 2012, Lee et al. 2011, Million et al. 2013, Schwiertz et al. 2010). For example, Million et al. (2013) found that the prevalence of *Methanobrevibacter smithii* was negatively correlated with body mass index when comparing the feces of obese, overweight, lean, and anorexic individuals in France (Million et al. 2013). In contrast, Lee et al. (2011) reported that the presence and abundance of methanogens was associated with higher body mass index and waist circumference in Korean women (Lee et al. 2011). For cattle, sheep and goats, it is well known that methane production by methanogens in the rumen results in lost available energy from the diet (Hook, Wright, and McBride 2010, Johnson and Ward 1996). In monogastrics, however, additional research into how methanobacteria impact nutrient availability and weight gain is needed to better define their role in host metabolism.

The final microbiome composition feature associated with improved growth was the presence of increased species in the *Ruminococcaceae* family. *Ruminococcaceae* species are common gut inhabitants of both ruminants and monogastrics, considered beneficial due to a major role in cellulose digestion and the production of short-chain fatty acids. The positive correlation between *Ruminococcaceae* species abundance and level of dietary fiber in swine has

long been established (Varel, Robinson, and Jung 1987, Varel 1987, Varel and Yen 1997). Recently, high relative abundance of the *Ruminococcaceae* family was associated with reduced fecal *Salmonella* shedding in pigs after infection with *Salmonella enterica* serovar Typhimurium (Bearson et al. 2013). Other beneficial effects of *Ruminococcaceae* have been shown in chickens where improved feed conversion ratios were associated with increased caecal *Ruminococcaceae* abundance (Stanley et al. 2016) and in mice, where *Ruminococcus gnavus* was shown to reverse impaired growth phenotypes after transplanting the microbiota from malnourished children into germ-free mice (Blanton et al. 2016). Due to their role in metabolism and overall gut health, increasing *Ruminococcaceae* species through inoculation or through fiber source may be a potential target for improving the growth of health-challenged pigs.

One finding from our initial study that was inconsistent with the current study was the increase in non-pathogenic *Escherichia coli* in the feces of pigs with improved clinical outcomes (Niederwerder et al. 2016a). Several study design differences may account for this discrepancy. Perhaps the most likely is timing of collection (i.e., PRRSV/PCV2 co-infection presumably shifts the microbial populations and collecting feces prior to or after viral infection would impact the microbiome). Second, pigs in the worst clinical outcome group had been treated with antibiotics. It is possible that antibiotic therapy targeted and reduced *E. coli* prevalence in pigs with poor outcomes. Nonetheless, due to the potential role of *Escherichia coli* on weight (Looft et al. 2012, Schieber et al. 2015), further studies into the effects of *E. coli* on outcome after PRRSV/PCV2 co-infection are warranted.

3. Microbiome trends after co-infection indicate that improved growth rates of pigs infected with porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) are determined by microbiome composition prior to challenge

Introduction

In December 2016, the US swine herd showed the highest hog inventory since 1943, and the highest recorded breeding herd productivity in our history. The November 2016 USA swine exports were a record high at 510 million pounds and US pork exports are expected to increase to 5.44 billion pounds in 2017. In the last 30 years, porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) have cost billions of dollars in production loss on a global scale. PRRSV is considered the most economically detrimental swine pathogen and has the most potential to impact the global swine industry (Chand, Trible, and Rowland 2012b). As a single-stranded RNA virus in the family *Arteriviridae*, PRRSV causes respiratory disease and negatively impacts weight gain in developing pigs (Benfield, Nelson, Collins, Harris, Goyal, Robison, Christianson, et al. 1992). PCV2 is a single stranded DNA virus in the family *Circoviridae* that has the capacity to cause a variety of disease syndromes known as porcine circovirus associated diseases (PCVAD). PCVAD includes respiratory disease, enteric disease, skin and renal disease, as well as muscle wasting (Segalés 2012). Both PRRSV and PCV2 can present subclinically or result in systemic infections that can cause primary lung pathology, and modulate the immune response. Type I interferon production is antagonized by PRRSV which suppresses innate immunity (Chen et al. 2010, Han and Yoo 2014) while PCV2 causes lymphoid depletion in lymph tissues (Opriessnig and Langohr 2013). Although these viral infections can have a profound impact alone, co-infection with both viruses enhance disease and can result in a wide range of overt clinical signs. Despite this, the majority of pigs co-infected with PCV2 and PRRSV result in subclinical infections with overall morbidity being less than 30% (Niederwerder, Bawa, Serão, et al. 2015).

The collection of microorganisms located within the gastrointestinal tract can be defined as the microbiome. These microorganisms play a critical role both in nutrient availability and immunity (Honda and Littman 2016, Turnbaugh et al. 2006), having been associated with outcome during systemic viral infections in both humans and mice models. Microbiome associations have been found with viruses primarily affecting the respiratory tract, such as respiratory syncytial virus and influenza virus (Fujimura et al. 2014, Ichinohe et al. 2011), as well as viruses considered immunomodulatory, such as human immunodeficiency virus (Mudd and Brenchley 2016). Disease progression, airway inflammation, immune response, and morbidity can all have associations with microbiome composition and diversity during these viral infections.

The focal point of our study is to understand the role of the microbiome and its impact on the response to systemic infection in swine. To follow up on our previous study regarding the initial porcine microbiome and its impact on the outcome of PRRSV and PCV2 co-infection, we followed the same protocols to observe changes in microbiome during peak viremia (21 days post-infection) and viral clearance (42 days post-infection). Furthermore we compared changes in specific microbiome species between the initial microbiome we previously studied (0 days post-infection) with 21 dpi and 42 dpi to identify important trends. We continued to implement use of the Lawrence Livermore Microbial Detection Array (LLMDA), which was developed to detect all known microbes for which whole genome sequences are available. Current as of June 2013, this technology allows for the detection of 8,101 microbes and has been used in the detection of known and unknown microbes in porcine feces, serum, lung, oral fluids, lymph node suspension and tonsil (Niederwerder et al. 2016a, Jaing et al. 2015a).

In a recent study, we investigated the associations between the microbiome and clinical outcome in pigs following PRRSV/PCV2 co-infection (Niederwerder et al. 2016a). Best and worst clinical outcome pigs were selected based on the presence and severity of clinical disease as well as weight gain after co-infection. The fecal microbiomes of best clinical outcome pigs were characterized by increased diversity and increased prevalence of *Escherichia coli* 70 days after co-infection. We followed this study up by investigating the microbiome characteristics that predispose outcome and the role of the microbiome in subclinical infections (Ober et al. 2017). Though these studies confirm a potential role for the microbiome in outcome post PRRSV/PCV2 co-infection, it still remains unknown how the microbiome affects viremia, viral clearance, and differentiated growth rates.

In the current study, we followed up on our previous data by investigating the microbiome changes during peak viremia and after viral clearance that may impact high and low growth rates after co-infection with in subclinically affected pigs. We hypothesized that microbiome composition differences prior to co-infection with PCV2 and PRRSV would be maintained during the co-infection period; however these results did not remain significant. Our results demonstrate that microbiome composition on 0 dpi may play a larger role in the outcome of subclinical disease in pigs than microbiome composition during viremia or after viral clearance.

Materials and Methods

Animals and Housing: At weaning, three week old barrows (n=50; average age 23.5 ± 2.6 days) were obtained from a PRRSV negative high health commercial herd. These piglets were not vaccinated for PCV2 and although these piglets were derived from a sow herd that was previously vaccinated with a PCV2 capsid subunit vaccine they were used without regard to maternal antibody. All pigs kept under BSL-2 condition at the Kansas State University Large Animal Research Center and were housed in a single environmentally controlled room. Groups of 8-10 piglets were randomly allocated into 13.4 m^2 pens with ad libitum access to food and water. A 4-week acclimation period was allotted to all pigs prior to co-infection. This current study was conducted as a subcomponent of a larger project focused on assessing the role of host genetics in determining the outcome following co-infection with PRRSV and PCV2. All studies utilizing animals and viruses were done in accordance with the Federation of Animal Science Societies (FASS) Guide for the Care and Use of Agricultural Animals in Research and Teaching, the USDA Animal Welfare Act and Animal Welfare Regulations, and approved by the Institutional Animal Care and Use Committee as well as the Institutional Biosafety Committee at Kansas State University.

Viruses: Both viral isolates of PRRSV and PCV2b used in this study were originally derived from the lymph node of a pig with severe postweaning multisystemic wasting syndrome (PMWS) as previously described (Trible and R.R. Rowland 2012)(16). The PCV2b isolate (GenBank accession no. JQ692110) was attained by preparing a lymph node suspension enriched from PCV2 as previously described (Niederwerder, Bawa, Serão, et al. 2015, Niederwerder et al.

2016b)(8,14). PRRSV (isolate KS62; GenBank accession no KM035803) was isolated by propagation on MARC-145 cells. It was then recombined with the PCV2 homogenate to infect cesarean-derived, colostrum deprived (CD/CD) pigs. A lymph node/lung combined homogenate was further prepared from the CD/CD pigs, and PRRSC and PCV2 were further isolated by the same methods described. The inoculum was analyzed yielding two positive viral results of Torque teno sus virus (TTSuV) and porcine endogenous retrovirus (PERVs) but was negative for other heat-stable agents(Jaing et al. 2015b). Both viruses were titrated as previously described (Ober et al. 2017).

Experimental design and sample collection: All 50 pigs were infected with PRRSV and PCV2b at roughly 8 weeks of age (average age 54.5 ± 2.6 days). A 2mL dose was created by recombining both viruses and consisted of $10^{3.6}$ $TCID_{50}$ PCV2b and 10^{5} $TCID_{50}$ PRRSV in MEM. The pigs were inoculated by delivering 1mL intramuscularly and 1mL intranasally. Fecal samples were collected from all 50 pigs prior to co-infection. Body weights and blood samples of each pig were collected on 0, 7, 14, 21, 28, 35, and 42 days post infection (dpi). An additional blood sample was taken from each pig on 4 dpi. 20 pigs were selected on day 35 to represent high growth rate pigs (n=10) and low growth rate pigs (n=10) by calculating the average daily gain (ADG) between 0 and 35 dpi as the change in weight (kg) over the change in time. Further information regarding calculations and figures illustrating growth rate group selection can be found in our previous paper(Ober et al. 2017). All pigs were assessed daily by a veterinarian or a veterinary assistant for the presence of clinical signs that are associated with PRRSV/PCV2 co-infection (dyspnea, coughing, nasal discharge, aural cyanosis, open mouth breathing, decreased

body condition, muscle wasting, lethargy, depression, joint effusion, lameness, and pallor or jaundice). Any pig that exhibited overt clinical disease requiring veterinary medical treatment was excluded from selection. This would include pigs needing treatment for dyspnea, mucoid rhinorrhea, lameness with joint effusions, pallor or jaundice with muscle wasting, or lethargy or depression with pyrexia. At 42 dpi the 20 selected pigs were humanely euthanized in accordance with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals and complete necropsies were performed. For more information regarding the outcome of the necropsies, refer to our earlier publication (Ober et al. 2017).

Microarray Analysis of Fecal Samples: The Lawrence Livermore Microbial Detection Array (LLMDA) was designed to detect all sequenced microbes and was developed at Lawrence Livermore National Laboratory. The LLMDA oligonucleotide probes are 60 nucleotides in length and designed to detect all sequenced microbial families with a large number of probes per sequence and to improve sensitivity in the evaluation of microbial nucleic acids in a variety of samples. For this study, the microbiome of the fecal samples were analyzed using version 7 of the LLMDA in the 4plex 180K probe format. This version of the array targets all vertebrate infecting microbes including 3,856 viruses, 3,855 bacteria, 254 archaebacteria, 100 fungi, and 36 protozoa. This high-density array and statistical analysis method has previously been tested extensively in a multitude of studies used for microbial detection in clinical samples and environmental studies (Ober et al. 2017, Niederwerder et al. 2016b, Jaing et al. 2015b, Rosenstierne et al. 2014b, Gardner et al. 2010b)

DNA and RNA from fecal samples were extracted using the PowerViral™ Environmental RNA/DNA Isolation Kit (MO BIO, San Diego, CA) in accordance to the manufacturer's instructions. Each sample initially consisted 600 µl of PV1 added to 0.25 g of feces in a bead beating tube included in the PowerViral™ kit. Samples were homogenized and lysed using the vortex adaptor for the MO BIO Vortex-Genie 2 for 10 minutes at maximum speed. The manufacturer's standard protocols were used to further purify the samples. 100 µl of RNase-Free water was used to elute all samples. The purified nucleic acids were quantified using the Life Technologies Qubit fluorimeter. For each sample, 10 µl of the extracted DNA and RNA was amplified using the random amplification procedure as previously described (18). The amplified cDNA and DNA was purified with the Qiaquick PCR purification columns (Qiagen) and quantified using the Qubit fluorometer. Between 400-500 ng of amplified cDNA and DNA were fluorescently labeled using a one-coloring labeling kit (Roche NimbleGen, Madison, WI) following the manufacturer's instructions.

The Agilent Technologies Oligo aCGH/ChIP-on-Chip Hybridization kit (Santa Clara, CA) was used to prepare the comparative genomic hybridization (CGH) mix. For each sample, 10 µg of fluorescently labeled DNA was mixed with CGH mastermix, denatured at 95˚C for 3 min, and incubated at 65˚C until the arrays were ready to load. The LLMDA version 7 4x180K format microarray (Agilent Technologies Inc., Santa Clara, CA) was utilized for this experiment. After being loaded onto the array, each sample hybridized for 40 hr at 65˚C in a microarray rotator oven (Agilent Technologies Inc., Santa Clara, CA) set to a speed of 20. The standard manufacturer's protocol was then used to wash the microarrays with aCGH/ChIP-on-chip Wash Buffer 1 and Oligo aCGH/ChIP-on-chip Wash Buffer 2 (Agilent Technologies Inc., Santa Clara,

CA). The arrays were each washed in CGH Wash 1 for 5 min at room temperature, followed by CGH Wash 2 for 1 min at 37˚C. Nitrogen gas was used to remove particulate matter from the surface of each array. Using the MS200 microarray scanner (Roche NimbleGen, Madison, WI), all arrays were scanned at a resolution of 2 μM.

Microarray data was generated from the microbe sequences at ≥99% threshold using the CLiMax method developed at Lawrence Livermore National Laboratory (Gardner et al. 2010b) (17). The log likelihood for each of the positive targets is estimated from the BLAST similarity scores of the array feature and target sequences, together with the feature sequence complexity and other covariates derived from BLAST results.

Statistical Analysis: The statistical analyses were performed using GraphPad Prism 7.02 software (La Jolla, CA). Individual species and family comparisons between each growth rate group was assessed using Fischer's exact test. The Mann-Whitney U test was utilized to compare Microbiome diversity and number of species within family and phylum between groups. Fisher's exact test was used to compare proportions of each growth rate group with individual species at the three different time points.

Results

All pigs were selected, PRRSV and PCV2 viremia was measured, and pathology assessed in accordance with our previous paper (Ober et al. 2017). Similar to our original paper, the presence of microbial families and species were detected by using the Lawrence Livermore Microbial Detection Array. Throughout the entire study a total of 34 microbial families and 140 individual microbial species were detected. The results from day 0 were previously reported (Ober et al. 2017). On 21 dpi, there were 31 microbial families that were detected (Figure 11) and 113 individual microbial species detected. On 42 dpi there were 25 microbial families (Figure 11) that were detected and 86 individual microbial species detected. On days 21 and 42 the families *Mycoplasmataceae*, *Prevotellaceae*, and *Streptococcaceae* were detected at 100% prevalence in both high growth and low growth rate pigs. The majority of pigs on 21 and 42 dpi, had the bacterial families *Lachnospiraceae*, *Lactobacillaceae*, *Methanobacteriaceae*, and *Vibrionaceae* detected in their feces. The families *Planctomycetaceae*, *Polyangiaceae*, *Erysipelotrichaceae*, and *Coriobacteriaceae* were only detected in pigs at 21 dpi while the family *Bacillaceae* was only detected on 42 dpi. There was not a significant difference detected in the proportion of high growth and low growth groups within each family detected both on 21 dpi and 42 dpi.

We wanted to investigate the microbiome differences between the 2 growth groups on 21 dpi and 42 dpi in the same manner that we investigated 0 dpi microbiome differences. The number of species detected within each family was compared on 21 dpi and on 42 dpi. Previously on 0 dpi, we had found that there was a trend towards a greater species diversity within the *Streptococcaceae* family in high growth rate pigs, a significant decrease in

the species diversity of the *Methanobacteriaceae* family in high growth rate pigs, and an increased number of *Ruminococcaceae* species in high growth rate pigs. Despite this previous data, there was no statistical significance when comparing these two growth groups (Figure 12) on either day in terms of species diversity differences. This new information, taken with our original findings suggests that the microbiome composition at the time of viral infection may play a larger role in determining the outcome during co-infection than microbiome composition during peak viremia or after viral clearance. There was no statistically significant individual species variation on either 21 or 42 dpi. Furthermore, we previously determined that the difference in family diversity between the two growth rates was statistically significant on 0 dpi and the species diversity between the two groups followed a similar trend. On 21 and 42 dpi there was no longer a statistically significant difference in family diversity or species diversity between the two groups (Figure 12), further supporting that 0 dpi microbiome composition may play a larger role in disease outcome than days after disease progression.

The trends within individual species of bacteria and viruses were compared between the two growth groups. We looked at the presence of specific species on 0 dpi and compared the initial number of pigs that were positive for each species with the number of pigs that were positive for each species at the end of the study on 42 dpi. We then compared the initial number of pigs with a species on 0 dpi with pigs on 21 dpi and then with 21 dpi against 42 dpi. The seven bacterial families with specific species that had statistically significant increases or decreases in number over the experiment included *Lactobacillaceae, Streptococcaceae, Lachnospiraceae, Mycoplasmataceae, Clostricaceae, Methanobacteriaceae,* and

Spirochaetaceae. The viral family, *Astroviridae*, was the only viral family that had specific viral species experience a statistically significant decrease over the course of the experiment.

The family *Lactobacillaceae* had the most individual species that showed statistically significant trends during our experiment. The four species were *Lactobacillus crispatus, Lactobacillus acidophilus, Lactobacillus sakei,* and *Lactobacillus amylovorous*. For all 4 species, in both high growth and low growth groups there was a decrease in the number of positive pigs for each species on 0 dpi when compared with 42 dpi. From 0 to 21 dpi, the high growth rate group experienced a statistically significant decrease from 8 to 2 in pigs positive for *Lactobacillus crispatus* (p=0.0230, Fisher's exact test). This number dropped again from 2 to 1 when comparing 21 dpi with 42 dpi in the high growth rate group. The difference in the number of high growth rate pigs positive for *Lactobacillus crispatus* on 0 dpi compared with the number positive on 42 dpi was also significant, exhibiting an overall decrease from 8 to 1 (p=0.0198, Fisher's exact test).

There were 9 pigs in both the high growth rate and the low growth rate groups that were positive for *Lactobacillus acidophilus* on 0 dpi. For both high growth and low growth rate groups 9 pigs were positive for *Lactobacillus acidophilus* on 0 dpi and for both groups 3 were positive on 42 dpi, each group experienced a significant decrease over the experiment (p=0.0198, Fisher's exact test). Between 0 and 21 dpi each group experienced a statistically significant decrease with high growth rate pigs experiencing a decrease from 9 to 2 (p=0.0055, Fisher's exact test) and low growth rate pigs decreasing from 9 to 3 (p=0.0198, Fisher's exact test). Between 21 dpi and 42 dpi the high growth rate group increased from 2 to 3 while the low growth rate group remained the same.

All 10 pigs in the high growth rate group were positive for *Lactobacillus sakei* on 0 dpi. This number decreased to 6 on 21 dpi (p=0.0867 decrease from day 0 to day 21, Fisher's exact test) and to 5 on 42 dpi (p=>0.9999 decrease day 21 to day 42, Fisher's exact test). The decrease that occurred between 0 and 42 dpi for the high growth rate group was significant (p=0.0325, Fisher's exact test). The low growth rate groups initially had 7 pigs positive for *Lactobacillus sakei* which decreased to 3 on 21 dpi and then increased to 5 on 42 dpi, there were no significant changes for the low growth rate group.

Both high growth and low growth groups each had 9 pigs positive for *Lactobacillus amylovorous* on 0 dpi. Both groups experienced a statistically significant drop to 3 pigs on 21 dpi (p=0.0198, Fisher's exact test). Low growth rate pigs increased to 6 positive for *Lactobacillus amylovorous* on 42 dpi, which high growth rate pigs increased to 5 (p=0.3698 and p=0.6499 respectively, Fisher's exact test). Interestingly, in 3 out of 4 of the species *in Lactobacillaceae* that experienced a decrease in the number of pigs positive for that species, the statistically significant decrease occurred between initial infection (0 dpi) and peak viremia (21 dpi).

Streptococcus equinus Streptococcus bovis and *Streptococcus thermophilus* were the two *Streptococcaceae* species that showed an important difference between the three different time points. *Streptococcus equinus Streptococcus bovis* was initially found in 8 pigs in the high growth rate group and 6 pigs in the low growth rate group, by 42 dpi, both growth rate groups were reduced to 0. The decrease from 0 to 42 dpi in both groups was significant with a p-value of 0.0007 for the high growth rate group, and a p-value of 0.0108 for the low growth rate group. On day 21 the high growth rate group decreased from 8 to 5 (p=0.3034, Fisher's exact test), while the low growth rate group decreased from 6 to 3 (p=0.3698, Fisher's exact test). From 21 dpi to

42 dpi, high growth pigs positive for *Streptococcus equinus Streptococcus bovis* significantly decreased from 5 to 0 (p=0.0325, Fisher's exact test) and low growth rate pigs decreased from 3 to 0 (p=0.2105, Fisher's exact test). *Streptococcus thermophilus* also showed significant decrease in both growth groups between 0 dpi and 42 dpi. There were initially 9 high growth pigs and 7 low growth pigs with *Streptococcus thermophilus* in their microbiome which were both reduced to 1(p=0.0030 high growth; p=0.0198 low growth, Fisher's exact test). The high growth decreased in numbers positive for *Streptococcus thermophilus* to 6 on 21 dpi and to 1 on 42 dpi. The low growth rate pigs decreased to 3 positive pigs on 21 dpi and to 1 on 42 dpi. There were no further significant findings.

There was a single bacterial species, *Dorea longicatena*, in the family *Lachnospiraceae* that had significant data changes during our experiment. The high growth rate group initially had 7 positive pigs on 0 dpi which decreased to 1 by 42 dpi (p=0.0198, Fisher's exact test). Between 0 dpi and 21 dpi the number of high growth rate pigs with *Dorea longicatena*, remained the same at 7. For the high growth rate pigs the significant decrease from 7 to 1 occurred between 21 dpi and 42 dpi (p=0.0198, Fisher's exact test). When observing changes for the low growth rate group, there were no significant changes in the number of pigs positive *for Dorea longicatena* during our experiment (4 on 0 dpi, 3 on 21 dpi, and 1 on 42 dpi; 0 to 21 dpi, p=>0.9999; 21 to 42 dpi, p=0.5820; 0 to 42 dpi, p=0.3034, Fisher's exact test).

Of the *Mycoplasmatacea* family, *Mycoplasma arthritidis and Mycoplasma hyopneumonia* both showed significant changes between time points during this study. There was 1 pig in the high growth rate group that had *Mycoplasma arthritidis* on 0 dpi, this increased to 5 pigs on 21 dpi, and then to 7 pigs on 42 dpi. Although the increase between 0 dpi and 21 dpi and

21 and 42 dpi were not significant (p=0.1409 and p=0.6499 respectively, Fisher's exact test), the overall increase between 0 and 42 dpi was significant (p=0.0198, Fisher's exact test). The low growth rate group also have 1 pig with *Mycoplasma arthriditis* on 0 dpi which increased to 7 pigs on 21 dpi, and remained at 7 pigs on 42 dpi. The increase in the number of low growth pigs with *Mycoplasma arthriditis* between 0 dpi and 21 dpi as well as the overall increase when comparing 0 dpi with 42 dpi were both significant (p=0.0198 for both, Fisher's exact test). There was no significant data regarding the changes in *Mycoplasma hyopneumoniae* for the high growth group (8 pigs on 0 dpi, 4 pigs on 21 dpi, and 7 pigs on 42 dpi). The low growth rate group initially had 10 pigs with *Mycoplasma hyopneumoniae* on 0 dpi which significantly decreased to 5 pigs on 21 dpi, followed by a slight increase to 6 pigs on 42 dpi. Between 0 dpi and 21 dpi this decrease was significant (p=0.0325, Fisher's exact test).

Clostridium sartagoforme and *Clostridium botulinum* both increased in the number of pigs with each species during our experiment for both growth groups. *Clostridium sartagoforme* increased from 0 pigs positive on 0 dpi in the high growth rate group to 4 pigs on 21 dpi and then 5 pigs on 42 dpi. The incremental increases from 0 dpi to 21 dpi and then from 21 to 42 dpi were not significant however, the overall increase from 0 to 42 dpi was significant (p=0.0325, Fisher's exact test). Although there was a steady increase in *Clostridium sartagoforme* in the low growth rate group there were not statistically significant changes. Conversely, in regards to *Clostridium botulinum*, there were no significant increases in the high growth rate pigs, yet in the low growth rate group there was a significant increase between 0 and 21 dpi. In the low growth rate group there were initially 2 pigs positive for *Clostridium botulinum* on 0 dpi which significantly increased to 8 positive pigs on 21 dpi (p=0.023, Fisher's exact test). Between 21 dpi and 42 dpi

this number decreased to 7 (p=>0.9999, Fisher's exact test), the overall change from 2 pigs on 0 dpi to 7 pigs on 42 dpi was also not statistically significant.

Of the *Methanobacteriaceae* family, *Methanobrevibacter ruminantium* showed an overall significant increase in the high growth rate group from 2 pigs on day 0 to 9 pigs on 42 dpi (p=0.0055). Although this species of bacteria steadily increased in the number of pigs to 4 pigs at 21 dpi and to 9 pigs at 42 dpi, these incremental increases were not significant (p=0.06285 and p=0.0573 respectively, Fisher's exact test). There were no statistically significant changes noted for *Methanobrevibacter ruminantium* in the low growth rate group. The other species was *Methanobacterium sp SWAN* which showed a statistically significant decrease between 21 dpi and 42 dpi in the high growth rate group. On 0 dpi the high growth rate group initially had 5 pigs positive for *Methanobacterium sp SWAN*, this number of pigs was increased to 8 on 21 dpi (p=0.3498 from 0 to 21 dpi, Fisher's exact test), and then significantly decreased from 8 pigs on day 21 to 1 pig on day 42 (p=0.0050, Fisher's exact test). The overall decrease from 5 to 1 was not significant (p=0.1409, Fisher's exact test). The low growth rate group of pigs did not yield any statistically significant differences in pigs positive for *Methanobacterium sp SWAN*.

There were 2 bacterial species that showed significance in the family *Spirochaetaceae*. The first was *Treponema succinifaciens*, which in the low growth rate group significantly increased in the number of pigs with this species from 0 to 8 between 0 and 21 dpi (p=0.0007, Fisher's exact test). The number of low growth rate pigs then decreased to 7 on 42 dpi. The decrease between 21 dpi and 42 dpi was insignificant but the overall increase in the number of low growth pigs with *Treponema succinifaciens* on 0 dpi when compared to 42 dpi was significant (p=0.0031, Fisher's exact test). There were no significant findings in the changes to

the number of pigs with *Treponema succinifaciens* in the high growth rate group. The other species, *Treponema brennaborense*, showed a similar statistically significant increase in the number of pigs with this species between 0 and 21 dpi in the low growth rate group. On 0 dpi, 1 pig in the low growth rate group was positive for *Treponema brennaborense* which increased to 7 pigs on 21 dpi (p=0.0198, Fisher's exact test). This number then decreased to 5 pigs on 42 dpi. Neither the overall increase from 1 on 0 dpi to 5 on 42 dpi, nor the decrease from 7 on 21 dpi to 5 on 42 dpi were significant (p=0.1409 and p=0.6499 respectively, Fisher's exact test). There were no significant findings for *Treponema brennaborense* in the high growth rate pigs. Interestingly, there were significant increases within the number of pigs in the low growth groups for both species that occurred between 0 dpi and 21 dpi which the high growth rate groups did not yield significant data for the *Spirochaetaceae* family.

Overall, there were five viral families detected in all pigs over the course of the experiment (*Astroviridae, Circoviridae, Reoviridae, Pircornaviridae*, and *Parvoviridae*). Of these, two species from the family *Astroviridae*, were the only viral species that experienced a statistically significant change during the experiment. There was an overall decrease in the proportion of pigs positive for *Astrovirus wild boar* in both the high growth rate pigs and the low growth rate pigs. 9 pigs in both groups tested positive for *Astrovirus wild boar* on 0 dpi. On 42 dpi, 4 pigs in the high growth rate group and 5 pigs in the low growth rate group tested positive for *Astrovirus wild boar* (p=0.0573 and 0.1409 respectively; Fisher's exact test). Between 0 dpi and 21 dpi the high growth rate group experienced statistically significant decrease in the number of pigs positive for *Astrovirus wild boar*, decreasing from 9 to 3 (p=0.0198, Fisher's exact test). Between 21 and 42 dpi there was an increase from 3 to 4 in pigs testing positive for *Astrovirus*

wild boar (p=>0.9999, Fisher's exact test). The low growth experienced insignificant decreases from 9 to 8 and from 8 to 5 between 0 and 21 dpi and 21 and 42 dpi respectively (p=>0.9999 and p=0.3498, Fisher's exact test). The other viral species, *Porcine Astrovirus*, also experienced an overall decrease in positive pigs in both the high growth group and the low growth group. There were 9 high growth rate pigs that initially were positive for *Porcine Astrovirus* which decreased to 5 on 21 dpi and then to 3 on 42 dpi. The overall decrease between 0 and 42 dpi was statistically significant (p=0.0198, Fisher's exact test). The low growth rate group also had 9 pigs initially positive for *Porcine Astrovirus* on 0 dpi which decreased to 7 on 21 dpi, and then to 5 on 42 dpi (p=0.582 from 0 to 21 dpi, p=0.6499 from 21 to 42 dpi, p=0.1409 from 0 to 42 dpi).

Discussion

This current study is an extension of our previous study focusing on determining if the fecal microbiome characteristics present at the time of viral exposure were associated with outcome in subclincally affected pigs after co-infection with PRRSV and PCV2. We had previously determined that initial microbiome characteristics of high growth rate pigs included decreased *Methanobacteriaceae*, increased *Streptococcaceae*, and increased *Ruminococcaceae* as well as overall increased diversity (Ober et al. 2017). For this study we sought to observe the microbiome diversity between the high growth rate and low growth rate groups during peak viremia on 21 dpi and at viral clearance 42 dpi. Additionally, we pursued identification of significant trends of individual microbial species, comparing 0, 21, and 42 dpi, within both growth groups.

We previously reported that high growth rate pigs had an increased species diversity at 0 dpi (Ober et al. 2017). There were not statistically significant difference in microbial diversity between high growth rate and low growth rate groups on 21 dpi and 42 dpi. Our findings suggest that initial microbiome colonization may be an important factor in determining the outcome of disease. Decreased microbial species diversity has been associated with inflammatory bowel disease, asthma, and *Clostridial difficile* colitis in humans (Ott et al. 2004, Thavagnanam et al. 2008, Chang et al. 2008). Furthermore it has been found in humans that the initial diverse microbiome composition can improve overall immune health such as increases secretory immunoglobulins such as IgA (Sjögren et al. 2009).

Lactobacillaceae are a non-pathogenic group of bacteria found in the intestines of humans and animals that produce lactic acid as a result of carbohydrate fermentation and have

also been found to present colonization of detrimental enteric pathogens through ability to adhere to the intestinal lining (Huang et al. 2004, Lee et al. 2012) as well as stimulating promotion of a healthy gastrointestinal microbiome (Walter et al. 2008). Although four species of *Lactobacillaceae* showed a significant decrease over our study, *Lactobacillus amylovorus* and *Lactobacillus acidophilus* may have the most impact. When compared with untreated piglets challenged with enterotoxegenic *Escherichia coli* (ETEC), piglets fed *Lactobacillus sobrius*—a phenotypically similar bacteria to *Lactobacillus amylovorous*—exhibited reduced levels of ETEC in their ileum and additionally had an improved daily weight gain (Konstantinov et al. 2008). Million et al performed a meta-analysis on the effect of Lactobacillus species on weight gain in humans and animals and found that *Lactobacillus acidophilus* administration results in significant weight gain (Million, Angelakis, et al. 2012). In pigs, weaning piglets supplemented with *Lactobacillus acidophilus* in combination with other lactic acid producing bacteria showed improved feed conversion, feed intake, and weight gain (Giang et al. 2010). The significant decrease in the presence of this bacteria during viral co-infection with PCV2/PRRSV may have negatively impacted both groups. Possible supplementation with selected *Lactobacillus* species during infection may improve outcome in pigs affected with PCV2/PRRSV co-infections.

We had previously determined that high growth rate pigs had an increased number of species present in *Streptococcaceae* family and also an increased prevalence in the specific species *Streptococcus equi* on 0 dpi. We did not find significant data when comparing the number of individual species in the *Streptococcaceae* family between high growth or low growth groups on 21 or 42 dpi, nor when comparing overall number of species. However, when we addressed the trends in the numbers of individual species, both high and low growth rate groups

showed a significant decrease in numbers for *Streptococcus equinus Streptococcus bovis* and *Streptococcus thermophilus* from 0 dpi to 42 dpi. The *Streptococcus equinus/Streptococcus bovis* complex are commensal and opportunistic pathogens of the GI tract previously found in pigs, ruminants, humans, and horses (Jans et al. 2015). In humans, *Streptococcus bovis* bacteremia is associated both with endocarditis as well as colon cancer (Ferrari et al. 2008). In 2005, Bin-Nun et al found that prebiotic supplementation, including *Streptococcus thermophilus*, in low-birth weight human neonates decreased both the incidence and severity of necrotizing enterocolitis (Bin-Nun et al. 2005). Pre-treatment with *Streptococcus thermophilus* in rats subjected to 5-Fluorouracil, significantly reduced histopathogy associated with chemotherapy induced intestinal mucositis (Whitford et al. 2009). Additionally, in a study comparing the growth of children supplemented with Bifidobacterium Bb12 with or without *Streptococcus thermophilus* against unsupplemented children, those that received probiotic supplementation with *Streptococcus thermophilus* showed improved growth over the other groups (Nopchinda et al. 2002). Our study shows that co-infection with PRRSV/PCV2 may be detrimental to the growth of both beneficial and harmful Streptococcal species. Further research into supplementation of *Streptococcus thermophilus* during disease in pigs are warranted to view potential benefits of weight gain.

Of the *Lachnospiraceae* family, *Dorea longicatena* was the only species that showed a significant change during the experiment. High growth rate pigs experienced a significant decrease between 21 dpi and 42 dpi. In a 2015 study in post-menopausal women it was found that *Dorea longicatena* was negatively correlated with insulin resistance (Brahe et al. 2015) but this was the only relevant paper that we found during our literature search regarding *Dorea longicatena* and weight gain.

Mycoplasma arthritidis showed significant increases in both low and high growth rate groups over the course of the study, and specifically increased between 0 dpi and 21 dpi in the low growth rate group. *Mycoplasma arthritidis* is not known to cause disease in pigs, rather it causes rheumatoid polyarthritis in mice and rats (Kirchhoff et al. 1989). Interestingly our study does not reflect the results of there have been previous studies reporting that *Mycoplasma hyopneumoniae* potentiating the outcome of PRRSV. Thacker et al reported that pigs exposed to *Mycoplasma hyopneumoniae* 21 days prior to inoculation with PRRSV exhibited far more severe outcomes of PRRSV infection (Thacker et al. 2000). We saw a significant decrease in the presence of *Mycoplasma hyopneumoniae* in the low growth rate group between 0 dpi and 21 dpi.

Of the *Clostridiaceae* family, we saw a significant increase in *Clostridium sartagoforme* from 0 dpi to 42 dpi in the high growth rate group while we saw a significant increase in *Clostridium botulinum* between 0 dpi and 21 dpi in low growth rate pigs. *Clostridium sartagoforme* is an anaerobic bacteria that possesses genomes that aid in the digestions of cellulose and chitin and produce volatile fatty acids (Nathani et al. 2016). Although there are no specific literature references to the impact of *Clostridium sartagoforme* on growth rate, it may be hypothesized that an increase in the presence of this bacteria may allow for more efficient digestion of plant materials and an increase in abundance of nutrients that lead to higher growth rates. *Clostridium botulinum* can be detected at a high prevalence in many animal species feces and spores are ubiquitous in the environment. *Clostridium botulinum* has previously been isolated and identified from the swine intestine (Myllykoski et al. 2006) but has been described as a weak competitor against other bacterial species in the microbiome (Kautter et al. 1966). The

decrease in other bacterial species is likely the cause of *Clostridium botulinum's* increase in the low growth pigs.

We previously reported that on 0 dpi the low growth rate group of pigs had a higher prevalence rate of *Methanobacteriaceae* species than the high growth pigs. Yet during our follow-up study, *Methanobrevibacter ruminatium* had a significant increase in prevalence of this species in high growth rate pigs between 0 and 42 dpi while *Methanbacterium sp SWAN* had a significant decrease in the prevalence of this species in high growth rate pigs between day 21 and 42 dpi. In ruminants, contrary to our findings, it has been suggested that *Methanobrevibacter ruminantium* can cause a 6% loss of ingested energy (Leahy et al. 2010). *Methanobacterium sp. SWAN* is equivalent to *Methanobacterium paludis* (Cadillo-Quiroz et al. 2014). A literature search of both these species names yielded no results regarding the impact of these specific species on animal weight loss or gain. Further research into these species is indicated to provide a better understanding of relationship between these bacteria and outcome.

The low growth rate group experienced a significant increase in two spirochetes during our study. Both *Treponema succinifaciens* and *Treponema brennaborense* significantly increased between 0 dpi and 21 dpi, with *Treponema succinifaciens* experiencing an overall significant increase from 0 dpi to 42 dpi. *Treponema succinifaciens* is a non-pathogenic spirochete that is often isolated from the swine colon (Cwyk and Canale-Parola 1979). Although *Treponema brennaborense* was once thought to be the causative agent of bovine digital dermatitis (Schrank et al. 1999), further studies have found *Treponema brennaborense* to be minimally pathogenic (Brandt et al. 2011). Although neither *Spirochateaceae* species are pathogenic, there may be a

negative correlation between the prevalence of these species in pigs and the outcome of subclinical PRRSV/PCV2 infections.

Species in the family of virus known as *Astroviridiae* are non-enveloped RNA viruses that generally cause gastrointestinal disease in many species of mammals. In our study the high growth rate group experienced a significant decrease in the number of pigs with *Astrovirus wild boar* between 0 and 21 dpi and a significant decrease in *Porcine astrovirus* between 0 and 42 dpi while there was no significant change for either virus in the low growth rate group. A study in broiler chickens with Runting-stunting syndrome, a multifactorial syndrome that inhibits the growth of young birds, found that *Astroviridae* was one of the most abundant viral families found in the virome of affected birds (Devaney et al. 2016). In turkey poults, co-infection with turkey *astrovirus type-2*, turkey rotavirus, and *Turkey reovirus* has been shown to cause a decrease in weight gain (Spackman, Day, and Pantin-Jackwood 2010). There is a fair amount of literature characterizing astroviruses in pigs but not describing any correlations between astroviruses and weight loss or gain, further research is needed to explore the impact astroviruses have on the outcome of disease in swine.

4. Conclusion

The first objective of these experiments was to first determine what microbiome characteristics predispose outcome in pigs with subclinical infections involving PRRSV and PCV2 and then to determine what role the microbiome plays in these subclinical infections. The conclusion drawn from the information collected from exploring the first objective was as follows: Infection with PRRSV and/or PCV2 results in significant production losses to the swine industry. In this study, both microbiome diversity and composition were associated with response to co-infection with PRRSV and PCV2, including lung pathology, virus replication, and growth rate. Microbiome characteristics of high growth rate pigs included increased diversity, decreased *Methanobacteriaceae*, increased *Streptococcaceae*, and increased *Ruminococcaceae*. Modulating the piglet microbiome to have one or more of these characteristics may be an alternative tool for control of disease associated with PRRSV and PCV2

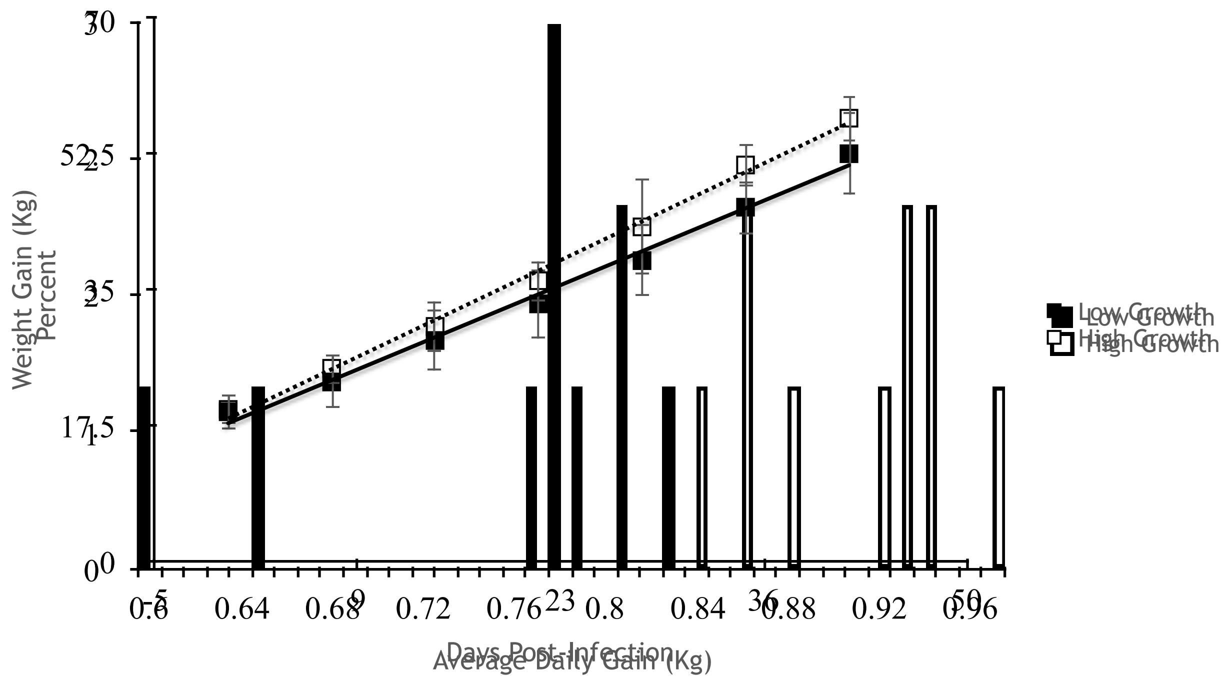
The second experimental objective was to determine if these changes in the microbiome were consistent during the post-infection period. From the results gathered while exploring the second objective, we concluded that individual or co-infections with PRRSV and PCV2 results detrimental production losses in the pork industry on a worldwide level. In this study no significance in microbiome diversity during peak viremia or after viral clearance were noted. Furthermore, these results support our hypothesis that modulating the piglet microbiome at weaning as opposed to during disease or after disease may have a positive impact on outcome. Supplementing pigs with select bacterial species that are reduced in the microbiome during infection during swine post-weaning periods may improve outcome during or after disease associated with PRRSV and PCV2.

5. Figures and Tables

Table 1. Total PRRSV and PCV2 virus replication between 0-42 dpi reported as area under the viremia curve (AUC)

PRRSV		PCV2	
Low Growth Pigs	High Growth Pigs	Low Growth Pigs	High Growth Pigs
83.3	56.0	0.0	0.0
92.3	69.9	28.4	12.2
93.9	73.1	44.5	17.6
95.3	76.5	50.2	33.0
99.1	79.6	56.8	35.7
99.3	83.3	63.3	37.9
100.7	84.2	74.4	39.4
103.2	85.9	77.7	42.4
103.9	87.1	86.0	60.5
115.6	87.7	94.9	77.5
*Mean = 98.6	*Mean = 78.3	‡Mean = 57.6	‡Mean = 35.6
SD = 8.5	SD = 9.9	SD = 28.5	SD = 22.6

*Significant difference between PRRSV means, p = 0.0001, unpaired t-test. ‡Trend towards greater total PCV2 replication in low growth pigs, p = 0.07, unpaired t-test.


Weight Gain (Kg)
Percent
0
17.5
35
52.5
70
0.6
0.64
0.68
0.72
0.76
0.8
0.84
0.88
0.92
0.96
-5
9
23
36
50
Days Post-Infection
Average Daily Gain (Kg)
Low Growth
High Growth

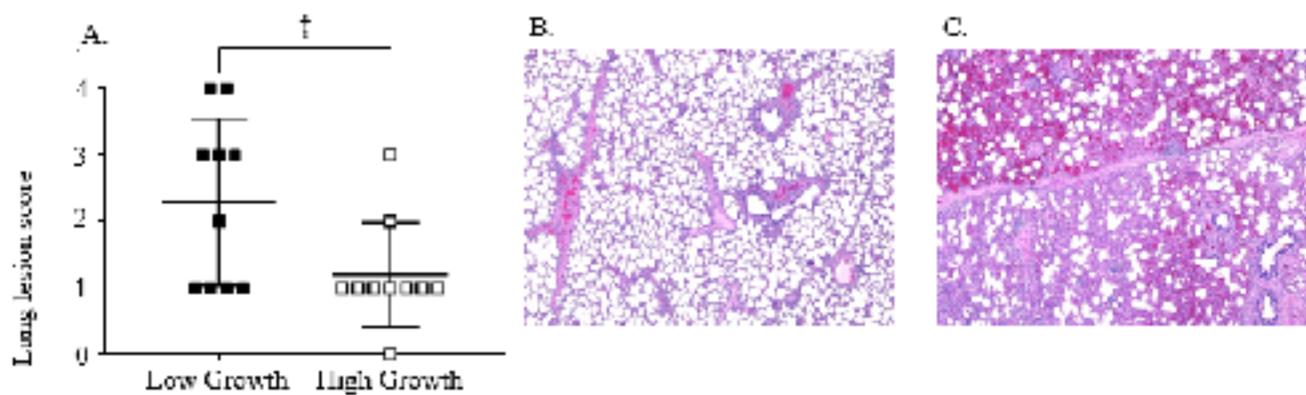


Figure 3. Microscopic lung lesions in pigs with high and low growth rates after co-infection with PRRSV and PCV2. A. Data is shown as the microscopic lung lesion scores with group means and standard deviations represented by horizontal lines. Scores were assigned as follows: 0, no significant lesions; 1, mild multifocal interstitial pneumonia with <50% lung lobe involvement; 2, mild to moderate multifocal interstitial pneumonia with 50–75% lung lobe involvement; 3, moderate to severe multifocal interstitial pneumonia with 50–75% lung lobe involvement; 4, severe diffuse interstitial pneumonia with >75% lung lobe involvement. High growth rate pigs had lower lung lesion scores (†p = 0.06, Mann-Whitney U test). B. H&E stained lung from a high growth rate pig with mild interstitial pneumonia (4X). C. H&E stained lung from a low growth rate pig showing severe diffuse interstitial pneumonia (4X).

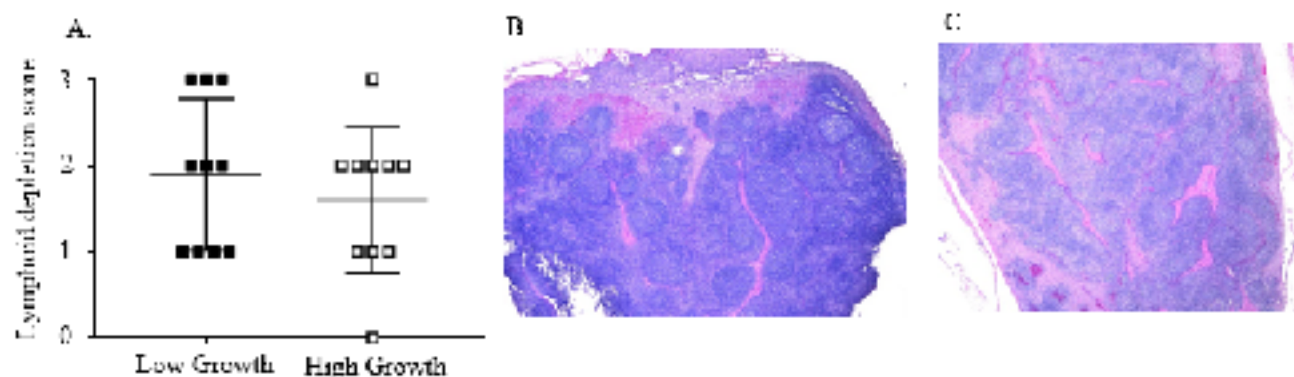
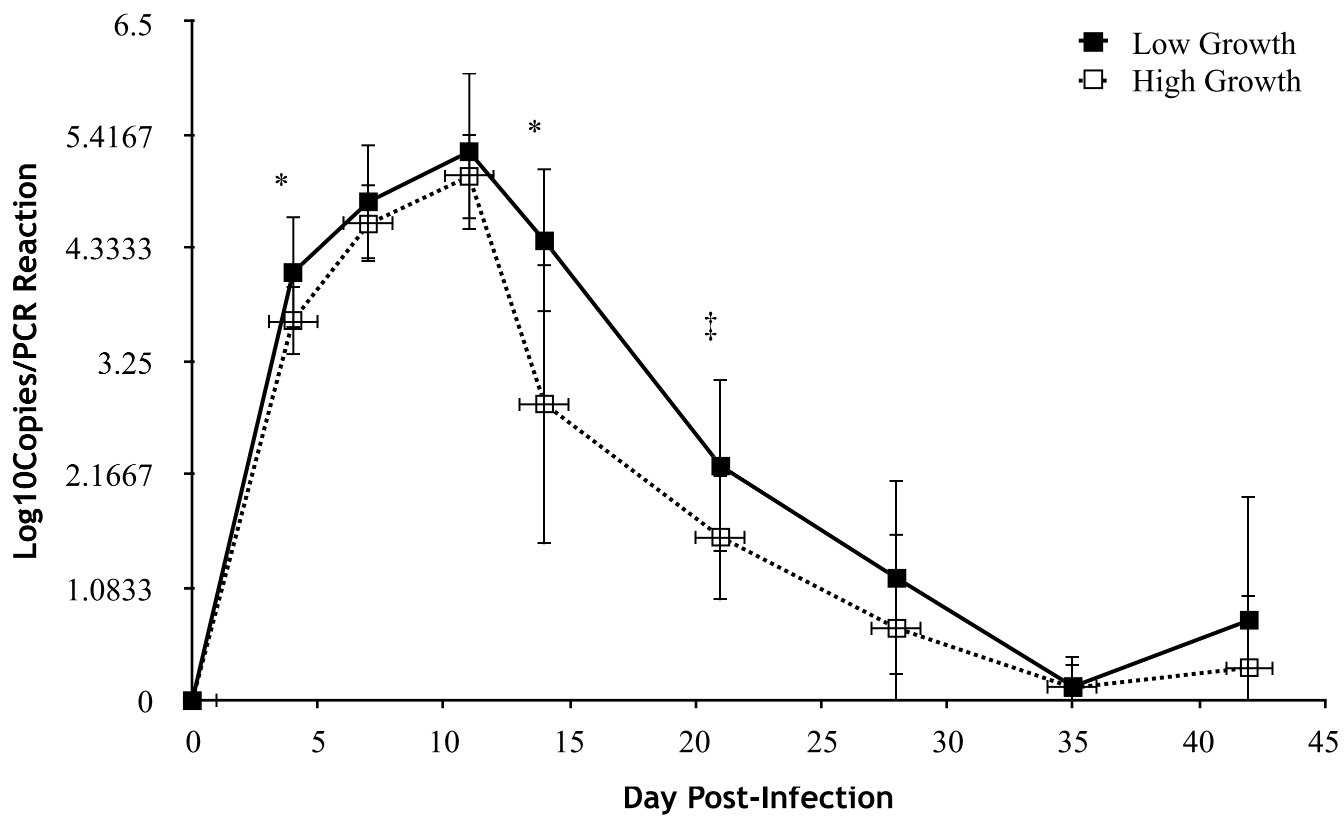
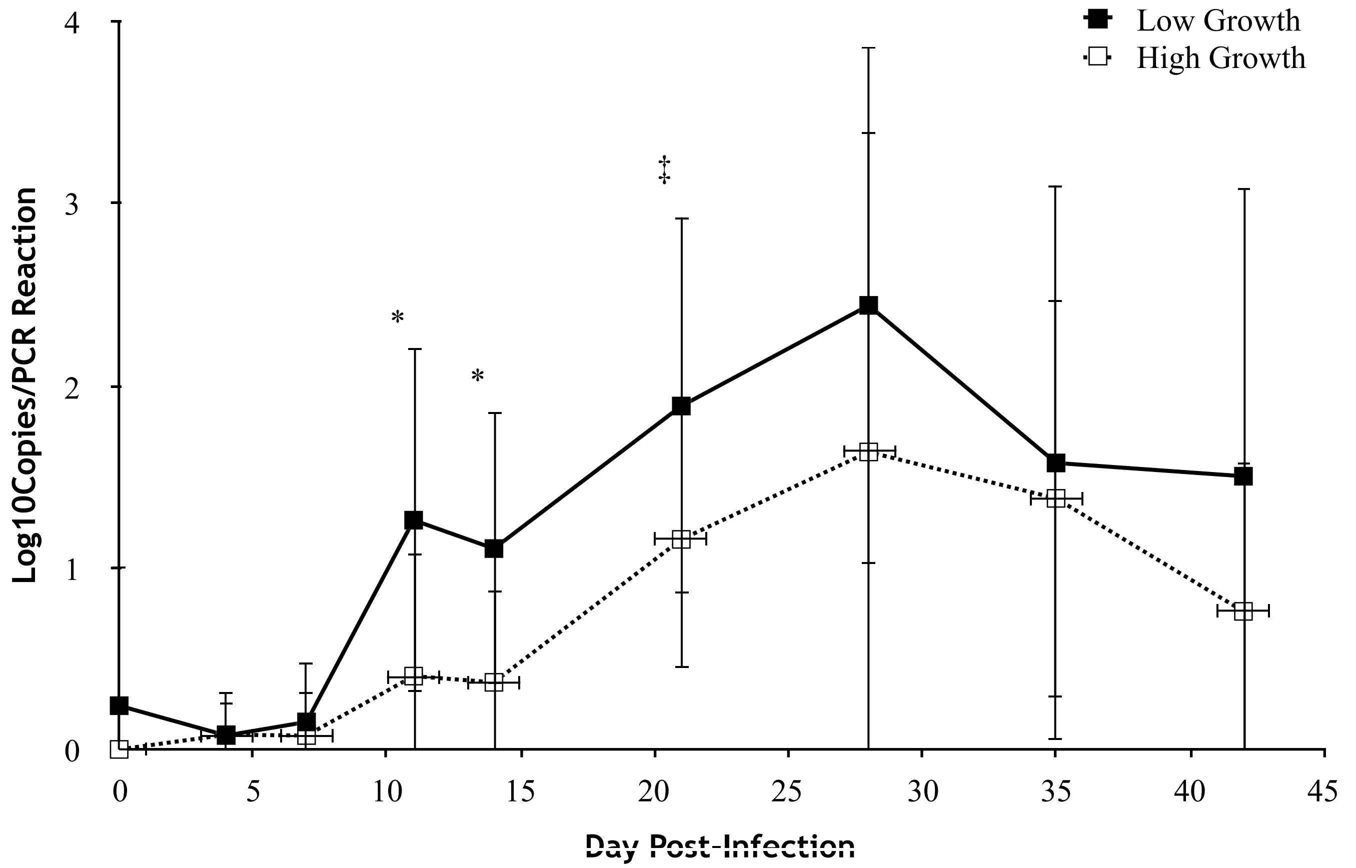
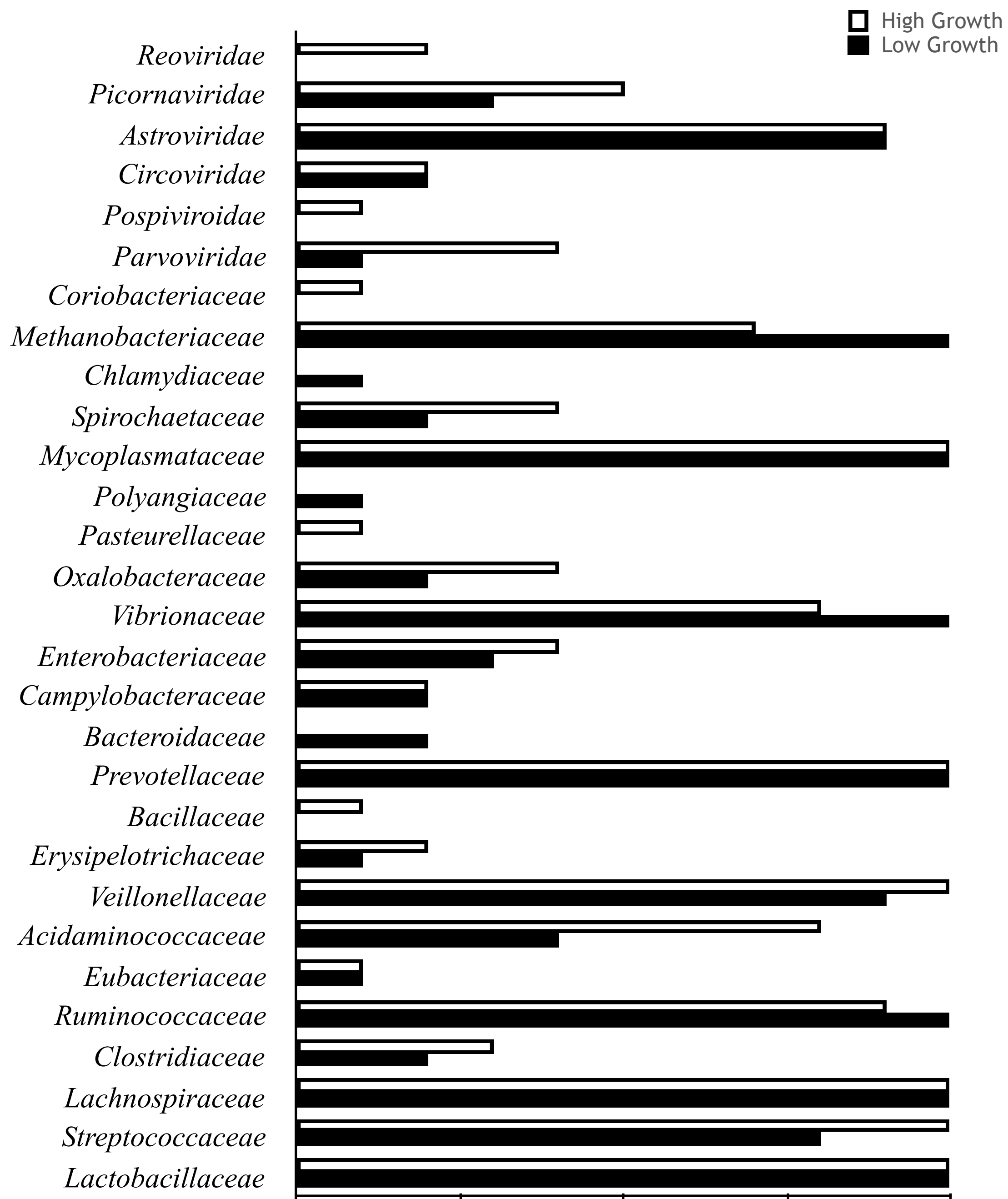


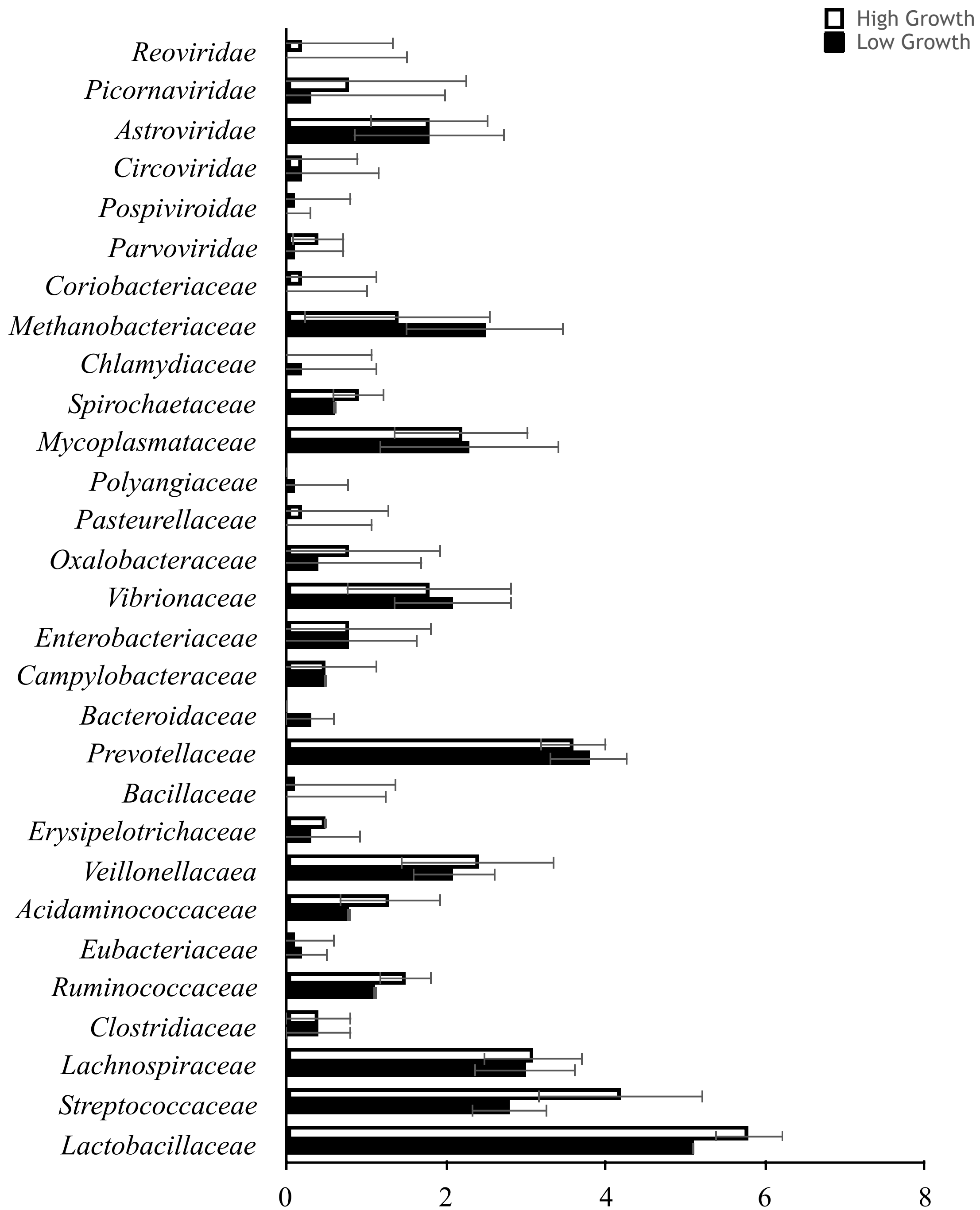
Figure 4. Microscopic lymphoid depletion in pigs with high and low growth rates after co-infection with PRRSV and PCV2. A. Data is shown as lymphoid depletion scores with group means and standard deviations represented by horizontal lines. Scores were assigned as follows: 0, no lymphoid depletion, 1, mild lymphoid depletion, 2, moderate lymphoid depletion, 3, severe lymphoid depletion. No significant difference was detected between the two groups (p = 0.604, Mann-Whitney U test). B. H&E stained lymph node from a high growth rate pig with no lymphoid depletion (2X). C. H&E stained lymph node from a low growth rate pig with severe lymphoid depletion (2X).



Low Growth
High Growth
Log10Copies/PCR Reaction
6.5
5.4167
4.3333
3.25
2.1667
1.0833
0
*
*
‡
0
5
10
15
20
25
30
35
40
45
Day Post-Infection


Low Growth
High Growth
Log10Copies/PCR Reaction
Day Post-Infection
*
*
‡
0
1
2
3
4
0
5
10
15
20
25
30
35
40
45


High Growth
Low Growth
Reoviridae
Picornaviridae
Astroviridae
Circoviridae
Pospiviroidae
Parvoviridae
Coriobacteriaceae
Methanobacteriaceae
Chlamydiaceae
Spirochaetaceae
Mycoplasmataceae
Polyangiaceae
Pasteurellaceae
Oxalobacteraceae
Vibrionaceae
Enterobacteriaceae
Campylobacteraceae
Bacteroidaceae
Prevotellaceae
Bacillaceae
Erysipelotrichaceae
Veillonellaceae
Acidaminococcaceae
Eubacteriaceae
Ruminococcaceae
Clostridiaceae
Lachnospiraceae
Streptococcaceae
Lactobacillaceae


High Growth
Low Growth
Reoviridae
Picornaviridae
Astroviridae
Circoviridae
Pospiviroidae
Parvoviridae
Coriobacteriaceae
Methanobacteriaceae
Chlamydiaceae
Spirochaetaceae
Mycoplasmataceae
Polyangiaceae
Pasteurellaceae
Oxalobacteraceae
Vibrionaceae
Enterobacteriaceae
Campylobacteraceae
Bacteroidaceae
Prevotellaceae
Bacillaceae
Erysipelotrichaceae
Veillonellacaea
Acidaminococcaceae
Eubacteriaceae
Ruminococcaceae
Clostridiaceae
Lachnospiraceae
Streptococcaceae
Lactobacillaceae
0
2
4
6
8

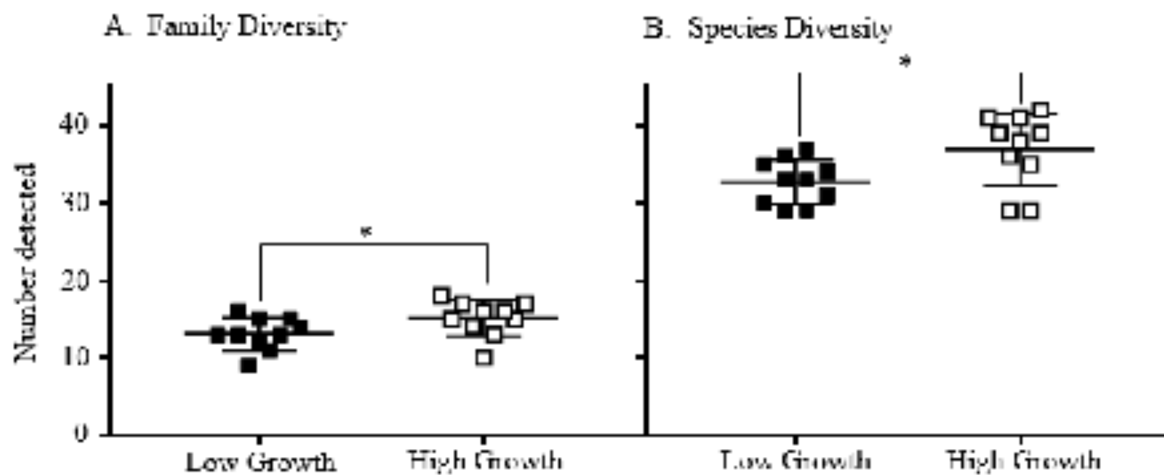
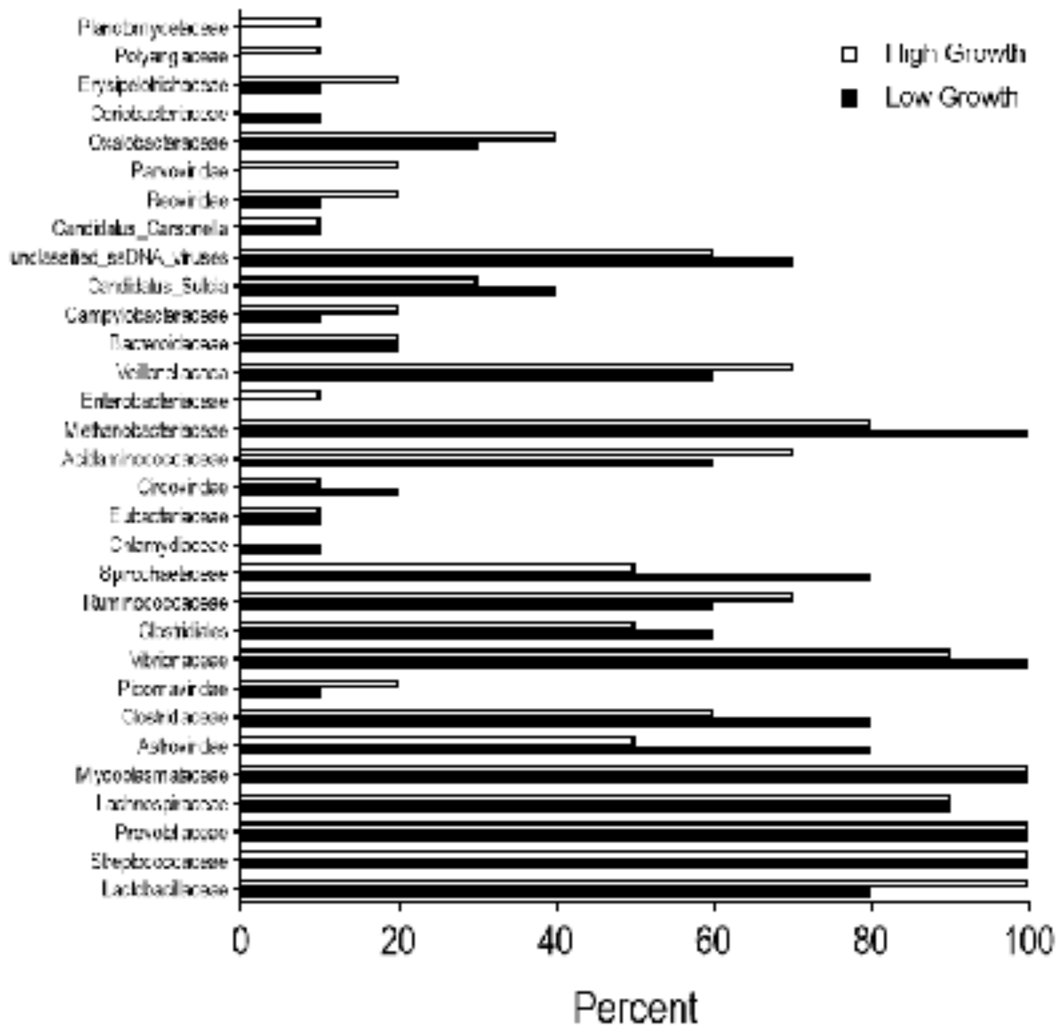


Figure 10. Fecal microbiome diversity in pigs with high and low growth rates after co-infection with PCV2 and PRRSV. Data is shown as the total number of microbial families (A) and microbial species (B) detected by DNA microarray on 0 days post-infection (dpi) for individual pigs. Group means and standard deviations are represented by horizontal lines. Significantly greater microbiome diversity was detected on both a family and species level in high growth rate pigs (*p = 0.0385 and p = 0.0328, respectively; Mann-Whitney U test).



Day 21
High Growth
Low Growth
Planctomycetaceae
Polyangiaceae
Erysipelotrichaceae
Coriobacteriaceae
Oxalobacteraceae
Parvoviridae
Reoviridae
Candidatus_Carsonella
unclassified_ssDNA_viruses
Candidatus_Sulcia
Campylobacteraceae
Bacteroidaceae
Veillonellaceae
Enterobacteriaceae
Methanobacteriaceae
Acidaminococcaceae
Circoviridae
Eubacteriaceae
Chlamydiaceae
Spirochaetaceae
Ruminococcaceae
Clostridiales
Vibrionaceae
Picornaviridae
Clostridiaceae
Astroviridae
Mycoplasmataceae
Lachnospiraceae
Prevotellaceae
Streptococcaceae
Lactobacillaceae
0
20
40
60
80
100
Percent

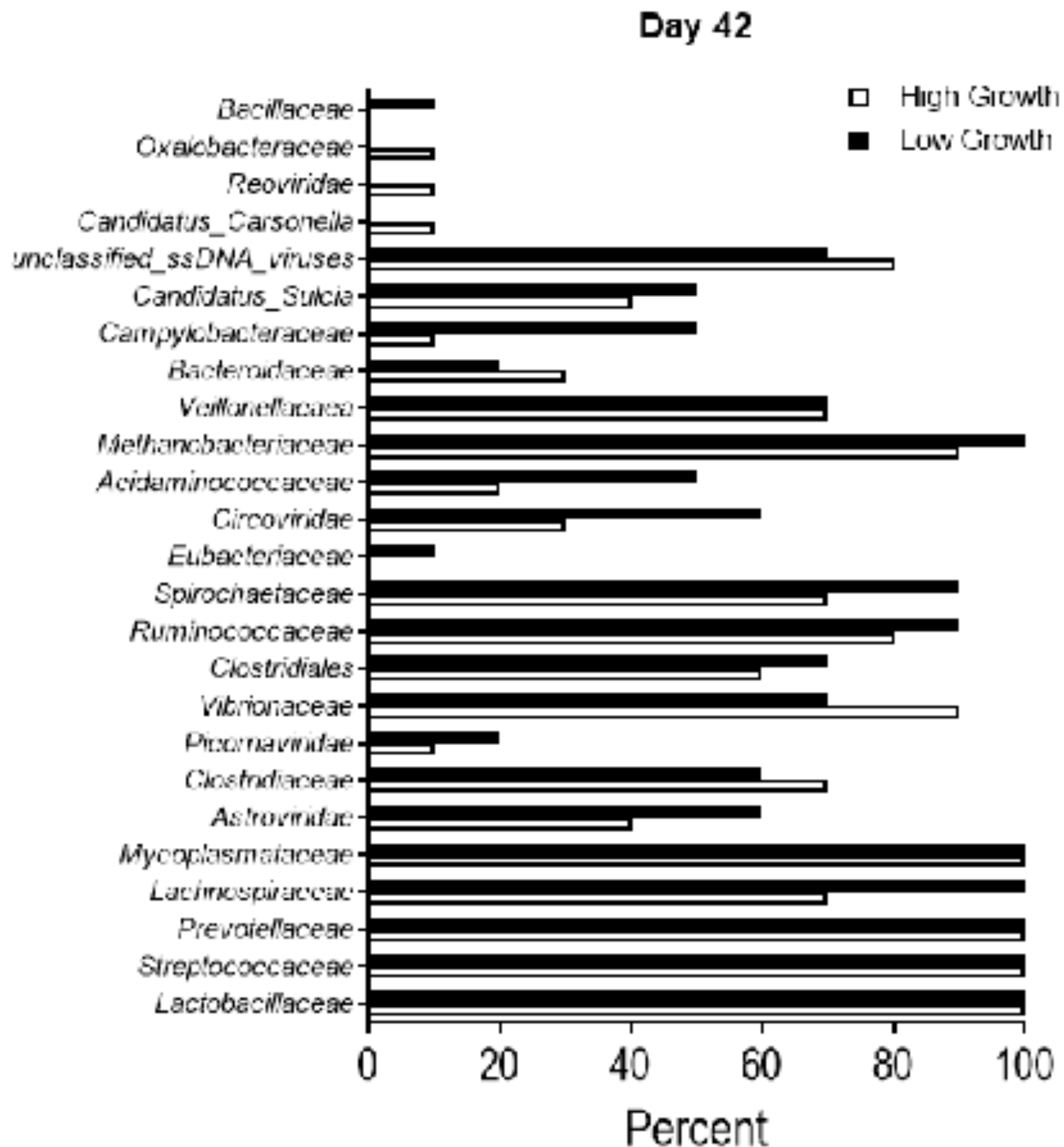


Figure 11. 21 and 41 dpi microbiome composition of pigs with high and low growth rates after co-infection with PRRSV and PCV2. A. Microbiome family composition on day 21 is shown as the percent of low growth pigs (n=10) and high growth pigs (n=10) with each detected family on the array. **B.** Microbiome composition on 42 dpi is shown in the same manner as 21 dpi. There was not a significant difference detected in the proportion of high growth and low growth groups within each family detected both on day 21 and day 42 (Fisher's exact test, P>0.05).

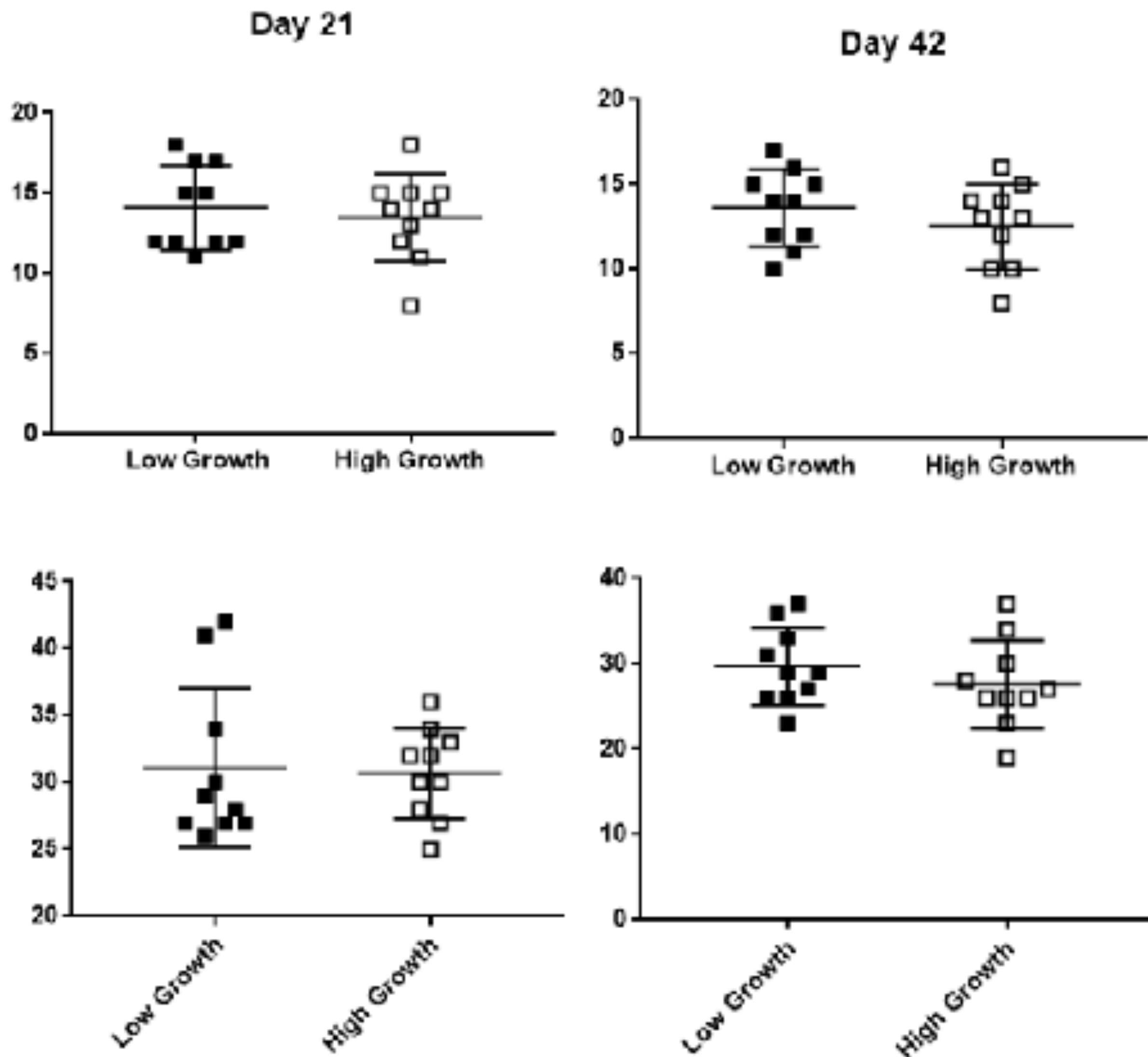


Figure 12. Microbiome diversity in pigs with high and low growth rates after co-infection with PRRSV and PCV2 21 dpi and 42 dpi. The total number of microbial families if seen in A and microbial species is seen in B. Group means and standard deviations are shown via horizontal lines. On day 21 and day 42 there was not a statistically significant difference in family diversity or species diversity between the two groups.

Table 2. Trends in specific bacterial species from 0 dpi to 42 dpi.

Species	Growth Rate	0-42 DPI		
		0 dpi	42 dpi	p-value
Lactobacillus crispatus	High	8	1	0.0055*
	Low	5	1	0.1409
Lactobacillus acidophilus	High	9	3	0.0198*
	Low	9	3	0.0198*
Lactobacillus sakei	High	10	5	0.0325*
	Low	7	5	0.6499
Streptococcus equinus Streptococcus bovis	High	8	0	0.0007*
	Low	6	0	0.0198*
Streptococcus thermophilus	High	9	1	0.003*
	Low	7	1	0.0198*
Dorea longicatena	High	7	1	0.0198*
	Low	4	1	0.3034
Mycoplasma arthritidis	High	1	7	0.0198*
	Low	1	7	0.0198*
Clostridium sartagoforme	High	0	5	0.0325*
	Low	1	5	0.1409
Methanobrevibacter ruminatium	High	2	9	0.0055*
	Low	6	9	0.3034
Treponema succinifaciens	High	2	6	0.1698
	Low	0	7	0.0031*

* Statistically significant date obtained through Fisher's Exact test.

Table 3. Trends in specific bacterial species from 0 dpi to 21dpi

Species	Growth Rate	0-21 DPI		
		0 dpi	**21 dpi**	**p-value**
Lactobacillus crispatus	High	8	2	0.023*
	Low	5	2	0.3498
Lactobacillus acidophilus	High	9	2	0.0055*
	Low	9	3	0.0198*
Lactobacillus amylovorus	High	9	3	0.0198*
	Low	9	3	0.0198*
Mycoplasma arthriditis	High	1	5	0.1409
	Low	1	7	0.0198*
Mycoplasma hyopneumoniae	High	8	4	0.1698
	Low	10	5	0.0325*
Clostridium botulinum	High	3	4	>0.9999
	Low	2	8	0.023*
Treponema succinifaciens	High	2	2	>0.9999
	Low	0	8	0.0007*
Treponema brennaboense	High	0	5	0.6285
	Low	1	5	0.0198*

* Statistically significant date obtained through Fisher's Exact test.

Table 4. Trends in specific bacterial species from 21 dpi to 42dpi

Species	Growth Rate	21-42 DPI		
		21 dpi	42 dpi	p-value
Streptococcus equinus Streptococcus bovis	High	5	0	0.0325*
	Low	3	0	0.2105
Dorea longicatena	High	7	1	0.0198*
	Low	3	1	0.582
Methanobacterium sp SWAN	High	8	1	0.0055*
	Low	5	5	>0.9999

* Statistically significant date obtained through Fisher's Exact test.

Table 5. Trends in specific viral species from 0 dpi to 42dpi

Species	Growth Rate	0-42 DPI		
		0 dpi	42 dpi	p-value
Porcine Astrovirus	High	9	3	0.0198*
	Low	9	5	0.01409

* Statistically significant date obtained through Fisher's Exact test.

Table 6. Trends in specific viral species from 0 dpi to 21 dpi

Species	Growth Rate	0-42 DPI		
		0 dpi	21 dpi	p-value
Astrovirus wild boar	High	9	3	0.0198*
	Low	9	8	>0.9999

* Statistically significant date obtained through Fisher's Exact test.

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