Challenges of porcine reproductive and respiratory syndrome virus (PRRSV) and the use of the fecal microbiome as an alternative control method

by

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B.S., North Carolina State University, 2010
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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine and Pathobiology
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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is the most economically devastating swine disease in the United States (U.S.). In the most recent analyses, PRRS was estimated to cost U.S. swine producers approximately $664 million per year with over $360 million alone due to loss in growing pigs. In a population of growing pigs, PRRS results in reduced weight gain, respiratory disease and immunosuppression, increasing infections by primary and secondary pathogens. Significant genetic variation exists among PRRS virus (PRRSV) isolates, which correlates to differences in clinical disease presentation, as well as difficulty in the production of broadly protective vaccines.

The objective of the first study was to characterize the clinical outcome of large population of nursery pigs infected with two heterologous PRRSV isolates, NVSL 97-7895 (NVSL; n =189) and KS 2006-72109 (KS06, n=200) and followed for 42 days post-infection. NVSL infection led to delayed, chronic disease resulting in significantly higher morbidity characterized predominately by respiratory disease with a high frequency of altered ambulation, decreased body condition, altered mentation and lower average daily gain (ADG), as well as greater virus replication and increased parenteral antibiotic usage. In contrast, KS06 infection was characterized predominately by fever and acute mortalities early in the infection period. Overall, these results provide evidence that genetically diverse PRRSV isolates manifest differently in both phenotypic presentation and duration.

The objective of the second study was to investigate fecal microbiota transplantation (FMT) as a means to prevent porcine circovirus associated disease (PCVAD) in pigs co-infected with PRRSV and PCV-2d. One group of pigs (n = 10) was administered the FMT while a control group (n = 10) was administered a sterile mock-transplant. Over the 42-day post-infection

period, the FMT group showed fewer PCVAD-affected pigs, as evidenced by a significant reduction in morbidity and mortality in transplanted pigs, along with increased antibody levels. Overall, this study provides evidence that FMT decreases the severity of clinical signs following co-infection with PRRSV and PCV-2 by reducing the prevalence of PCVAD.

The objective of the third was to identify gut microbiome characteristics associated with improved outcome in pigs immunized with a PRRS MLV and co-challenged with PRRSV and PCV2b. Twenty-eight days after vaccination and prior to co-challenge, fecal samples were collected from an experimental population of 50 nursery pigs. At 42 days post-challenge, 20 pigs were retrospectively identified as having high or low growth outcomes during the post-challenge period. Gut microbiomes of the two outcome groups were compared using the Lawrence Livermore Microbial Detection Array (LLMDA) and 16S rDNA sequencing. High growth outcomes were associated with several gut microbiome characteristics. Overall, this study identifies gut microbiomes associated with improved outcomes in PRRS vaccinated pigs following a polymicrobial respiratory challenge and provides evidence towards the gut microbiome playing a role in PRRS vaccine efficacy.

The objective of fourth study was to determine the effects of FMT on PRRSV modified live virus (MLV) vaccination. Pigs were split into four groups; two groups of pigs (FMT; $n = 20$) were administered a fecal microbiota transplant while two control groups ($n = 20$) were administered a sterile mock-transplant for 7 days prior to vaccination. One FMT and one control group were then vaccinated with the PRRSV MLV vaccine, allowed to mount an immune response (28 days), then were infected with PRRSV and followed for 42 days. During the 28 day vaccination period transplanted pigs had lower, however not significant, viremia levels. Over the 42-day post-infection period, while PRRS MLV vaccination decreased viremia and increased

antibody load, there was no effect seen due to transplantation. FMT resulted in overall decreases in microbial diversity; however, shifts in microbial composition were consistent to previous studies. Overall, this study supports the idea that FMT improves PRRSV MLV vaccination by reducing vaccine-associated viremia.

The data presented in this dissertation provided evidence PRRSV genetic differences resulting in diverse phenotypic outcomes, and how the gut microbiome can be used to improve or aid in current therapies.

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Dedication

To my wife, Katherine. If it weren't for you, I wouldn't be here today. I love you the mostest.

Chapter 1 - Challenges Associated with PRRSV Control and Alternative Control Methods

Porcine reproductive and respiratory syndrome

Porcine reproductive and respiratory syndrome (PRRS) is a devastating disease, leading to substantial economic losses to swine operations around the world. In the most recent analyses, PRRS was estimated to cost U.S. swine producers approximately $664 million per year.[1] Infections lead to increased morbidity and mortality due to respiratory disease, decreased reproductive performance, and weight gain reduction in growing pigs. PRRS leads to infections by both primary and secondary pathogens. The syndrome was first described in the U.S. in the late 1980s and in Europe in the early 1990s.[2,3] 'Abortus blauw' (Dutch for "blue abortion") was one of the first names given to PRRS due to disease characterized by abortions and notable aural cyanosis in some sows.[2,4] Blue ear disease, Mystery swine disease (MSD), Swine infertility and respiratory syndrome (SIRS), as well as Porcine epidemic abortion and respiratory syndrome (PEARS) were a few of the names given to this disease until the term PRRS was finally established in the early 2000s.[5] Since that time, viral genetic diversity has led to clinical disease variation, with some isolates producing severe disease, while others produce minimal to no disease.[6-13] Genetic diversity also results in difficulty controlling disease; specifically in production of broadly protective vaccines.[14,15]

1.1.1 The PRRS virus

The causative agent of PRRS is the PRRS virus (PRRSV). PRRSV is a single stranded, positive sense virus in the family Arteriviridae.[2,16] Originally this family included four viruses; PRRSV, equine arteritis virus, simian hemorrhagic fever and lactate dehydrogenase-elevating

virus (LDV); however, since that time the family has been expanded to 23 species, additionally including multiple simian arteriviruses, an arterivirus in common brushtails of New Zealand and one in an African forest giant pouched rat.[17,18] PRRSV preferentially infects the cells of the monocyte/macrophage lineage, especially porcine alveolar macrophages (PAM), in the natural pig host.[19] The virus was isolated by Wensvoort's group in The Netherlands in 1991,[2,4] and termed the Lelystad virus. In the early 1990's, Collins' and Benfield's teams isolated the same virus in North America, naming it VR-2332.[20,21] In 2017, PRRSV was re-classified and placed in the subfamily *Variarterivirinae* with LDV.[22]

1.1.2 PRRSV 1 and 2

PRRS virus was recently separated into two species; PRRSV-1 or European PRRSV (prototype Lelystad virus) and PRRSV-2 or North American PRRSV (prototype VR-2332) within the genus *Betaarterivirus*.[23] PRRSV-1 is now classified as *Betaarterivirus suid 1,* while PRRSV-2 is classified as *Betaarterivrius suid 2.* These two species share ~70% identity at the nucleotide level.[24-27] PRRSV has an inherently high mutation rate due to the considerable error frequency during RNA replication;[28,29] as such, many isolates of PRRSV exist within the two species, constituting a diverse genetic population.

1.1.3 Transmission

The most common mode of transmission is pig-to-pig, however, virus persistence is due to transmission between herds. In addition to movement of pigs, transmission between herds can occur through semen transfer and iatrogenically through needles as well as mechanical vectors such as houseflies and moquitos.[30-32] PRRSV is shed in all body secretions, including respiratory fluids, blood, colostrum, milk, semen, urine, and feces, as well as being transmitted

transplacentally and transvaginally.[33-50] PRRSV can be spread by the aerosol route,[51-53] as well as through cuts and scrapes where oropharyngeal fluids come in contact with blood.[36]

In comparison to other animals, pigs are often kept in large groups within relatively small places, allowing for rapid disease spread and increased disease persistence. Pathogens can enter farms through fomites such as clothes, boots or trucks.[30,32] Recently, our group established that PRRSV can also be transmitted through feed ingredients,[54] making feed movement a risk factor for disease spread. Management strategies can also affect susceptibility to respiratory disease. If dust from the ground or feed is stirred in the air and not filtered out, this can irritate the respiratory tract, increasing access of pathogens to respiratory epithelial cells. Additionally, if pens are not cleaned regularly, ammonia can build up in the air, also becoming a respiratory irritant. Therefore, cleaning of pens is critical for maintaining a healthy respiratory epithelium to minimize the risks associated with respiratory pathogens.

1.1.4. Clinical signs

Clinical signs vary with age, with older animals displaying more reproductive disease and younger animals exhibiting more respiratory signs. Other systemic signs, such as fever, arthritis and nervous abnormalities occur variably in both young and old animals. The following sections give a brief summary of reproductive, respiratory as well as systemic disease in both young and mature animals.

1.1.4a Reproductive and systemic signs in sows and boars

Reproductive disease due to PRRS are a major cause of economic loss, and were some of the earliest signs recognized by producers on their farms.[3,55] Initially encephalomyocarditis virus, *Chlamydia psittaci*, porcine parvovirus and mycotoxins, which can also cause reproductive

failure were implicated in disease, until the PRRS virus was isolated and demonstrated to be the caus. Boars have respiratory signs associated with PRRS infection as well as anorexia and lethargy. Virus is shed in semen which is a significant transmission route for both natural and artificial insemination.[39,56,57] PRRS infection has been shown to decrease semen quality, however, it is unclear if and how this affects fertility.[58-62] In sows, PRRS can lead to late term abortions, mummified and stillborn piglets, and delayed return to estrus.[3,55,63,64] Aural and vulvar cyanosis have also been reported.[64,65] Sows with severe disease may experience agalactia,[64] as well as nervous signs such as ataxia, circling and paresis.[58,66,67] Mortality rates in sows when infected with most isolates is less than 5%.[63,64] In 2006, highly pathogenic strains of PRRS (HP-PRRS) led to increased disease severity with abortion rates of up to 100% and sow mortality approaching or greater than 10%.[68-70] In late 2020, a new PRRSV variant resulted in Iowa and Minnesota outbreaks leading to increased sows off feed, increased sow mortality, as well as increased abortions and mummies.[71]

1.1.4b Respiratory and systemic signs in suckling, weanling and growing pigs

Clinical features of PRRS in nursery and growing pigs when initially described three decades ago are still consistent with clinical characteristics of current-day outbreaks in the field, most commonly associated with respiratory disease and secondary bacterial infections. Clinical signs of respiratory disease are often characterized by tachypnea, dyspnea, open-mouth breathing, and forced abdominal respiration.[2-4,63,72,73] Additional respiratory signs include coughing and serous to mucopurulent rhinitis. Systemic signs associated with PRRS include polyarthritis, anorexia, decreased growth rates, pyrexia, lethargy, depression, diarrhea, and rough hair coat.[3,63-65,69,72] Neurological signs, such as ataxia, circling and paresis, can also be seen in more severe cases.[74] The emergence of HP-PRRS in 2006 lead to increased and sustained

pyrexia as well as increased morbidity and mortality rate.[68-70] The 2020 PRRSV variant discovered in the U.S. lead to increased piglet and post-weaning mortality, as well as slower growth in finishing pigs.[71]

1.1.5 Porcine respiratory disease complex (PRDC)

Porcine respiratory disease complex (PRDC) is a term used to describe multifactorial porcine respiratory infections involving one or more virus or bacteria.[75-77] PRDC leads to increased morbidity and mortality, increased antibiotic usage, and therefore increased economic losses. Common characteristics include decreased feed intake, dyspnea, coughing, rhinitis, and fever. Major pathogens associated with PRDC can be both viral and bacterial pathogens. Viral pathogens include porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), pseudorabies virus (PRV), and porcine circovirus type 2 (PCV-2). Bacterial pathogens include *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Streptococccus suis*, *Pasteurella multocida*, *Actinobacillus pleuropneuomoniae*, *Haemophilius parasuis*, *Streptococcus suis*, and *Bordetella bronchiseptica.*[75,76] Polymicrobial infections are common and can lead to enhanced disease.[78]

Factors affecting disease risk include age, immune status, genetics, environmental conditions and management practices.[79] Immunocompromised animals, including young and/or stressed animals, are more susceptible to PRDC. Early in life, stressful events include teeth clipping, tail docking, or antibiotic and vaccine administration. Removal from the sow in young piglets is also stressful and can increase disease susceptibility. Excessive heat and cold, crowding or mixing of pigs, and shipping can also increase stress and impact piglet health.[80-84] Different pig breeds have variability in respiratory disease susceptibility.[85,86] Additionally, our lab has demonstrated

genetic variants that are associated with PRRSV resistance (See Section *1.3.1 Genomics and Gene Editing*).[87-89]

1.1.6 PRRSV/PCV2 co-infection model

1.1.6a Porcine circovirus 2 (PCV2)

PCV2, a single-stranded DNA virus in the family *Circoviridae,*[90] is estimated to cause economic losses up to $20/pig in unvaccinated herds[91] due to a group of syndromes termed porcine circovirus associated disease (PCVAD), which includes muscle wasting, weight loss and respiratory disease.[92] Within the fetus, the main target cells of PCV2 are cardiomyocytes, hepatocytes and cells of the monocytic lineage, while postnatally, macrophages are the target cell.[93]

PCVAD is associated with two main syndromes; postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS). PCV2 infection causes lymphoid depletion[94,95] resulting in lesions within the lymphoid, renal, pulmonary and hepatic lesions.[96] PMWS was originally described in Canada in 1997[97,98] with notable respiratory disease (tachypnea, dyspnea, and interstitial pneumonia), muscle wasting and jaundice occurring in nursery-aged pigs.[98,99] PDNS was originally described in 1993[100] and has not been reproduced by PCV2 infection alone; however, the virus is thought to contribute to this disease syndrome.[101] PDNS occurs in older pigs, growers and finishers and is associated with vascular disease leading to cyanotic and hyperemic skin as well as anorexia, depression, weight loss and respiratory disease.[102] Vascular disease also causes glomerulonephritis, with increasing severity often worsening prognosis of an animal.[102]

1.1.6b PRRSV/PCV co-infection model

The PRRSV/PCV-2 co-infection is typical of PRDC cases seen in the field and using this model simulates PRDC in a laboratory setting with not only respiratory but also systemic disease. PRRSV infection is immunomodulatory leading to downregulation of key immune pathways. It is hypothesized that PRRS attenuates the immune response through upregulation of regulatory T (T_{reg}) cells,[103-105] Interleukin (IL)-10,[103,106-110] and tumor growth factor (TGF)-beta,[104,109] which suppresses activation of T_H2 lymphocytes and macrophages, as well as downregulation of Type I interferon (IFN) responses,[111-114] which further prevents activation of macrophages and T lymphocytes. Most PRRS-related deaths are the result of co-infection with other pathogens.[115] Models for PCVAD include co-infection with PRRSV and PCV2, both of which cause systemic infections primarily targeting pulmonary tissue. [116-118] PRRSV and PCV2 result in systemic infections and modulation of host immunity, [119-122] reducing the rate of weight gain and increasing the likelihood of primary and secondary polymicrobial disease syndromes in swine. Therefore, this model system could also provide data applicable to other types of infections. Co-infections with these two pathogens also lead to increased antimicrobial usage, which is an ever growing concern in the face of rising antimicrobial resistance. This co-infection model is also advantageous to look at the selection pressures in the host and the viruses that result from co-infections. Specifically, PCV2 replication has been demonstrated to be enhanced by PRRSV infection or vaccination with a modified live virus (MLV).[116,117,123]

1.2 Current control strategies and associated challenges

Since the introduction of PRRS, multiple control methods have been attempted with variable success. Substantial genetic variation creates challenges in stimulating long-term and broadly protective immunity, either through natural exposure or through MLV vaccination.[14,15]

In 2012, Holtkamp et al. developed a risk assessment for producers to help identify and mitigate risks for disease introduction, specifically focusing on PRRSV.[124,125] These types of assessments are critical to provide scientists and producers knowledge to further decrease risks and improve control of PRRS disease.[126,127] Biosecurity measures, herd management and eradication programs have been employed; however, these have not been able to eliminate disease. No matter what control method is used, surveillance is critical to determine prevalence of subclinical disease, movement of infections, and success or failure of control methods.[128-131]

1.2.1 Biosecurity

Currently, the most effective PRRSV control method is to prevent entry onto farms and productions.[127,132,133] Any movement onto or off a site is a risk for disease transmission, whether through animals, humans, vehicles, or materials. Strict management of what and who comes onto the production site is essential. Pork producers have drastically changed how they manage herds to decrease disease introduction. Quarantine of new animals being brought to a site not only allows acclimation but can also identify infected animals before they are introduced into the herd.[134] Management of human and supply movement is also critical. Many farms institute shower-in and shower-out protocols, benches to distinguish between clean and dirty sides, changing into site-specific clothing, as well as the irradiation of items brought into animal areas. Trucks moving supplies, such as feed and animals, often travel from farm to farm increasing the risk of disease spread.[135,136] This risk can be mitigated by truck washes in between sites and having specific areas where trucks are allowed to offload, minimizing interaction with animals or goods that will come in contact with the animals.[136] PRRSV has been demonstrated to travel through the aerosol route;[51-53] therefore, air filtration within barns has been shown to decrease the risk of PRRSV introduction[137-146] as well as improve air conditions in the barn, bettering

health and further decreasing respiratory disease.[147] Management of animals during an outbreak can also be critical. Sick animals must be quickly identified and removed from the herd to prevent further spread.

1.2.2 Early segregated weaning

Another mitigation strategy is to use segregated early weaning. Although colostrum and milk can provide beneficial immunoglobulins, the sow can also transmit diseases to the piglets. Therefore, early weaning can be used as a way to minimize the risk of transmission. Segregated early weaning, by removing the piglet from the sow, and to an off-site nursery for weaning is used.[148] Ideally, pigs are then moved to a finisher site for the remainder of their lives. Consequently, pigs of similar age and disease susceptibility are kept together. All-in/all-out methods, where disinfection can occur between each group of pigs, can also minimize disease spread. However, early weaning can lead to decreased immunity,[149] due to the lack of secretory immunoglobulins obtained from the sow's milk, as well as increased gut permeability and risk to enteric infections.[150-153]

1.2.3. Eradication

Elimination of PRRSV leads to improved health within herds. Several methods are used, including total or partial depopulation/repopulation, test/removal and herd closure; these are reviewed extensively elsewhere.[154] Briefly, herd closure is the most common technique employed and involves a cease in animal introduction while current animals build immunity to disease, whether by natural infection or vaccination.[155] Depopulation, whether total or partial, is costly to the producer and is only used when economically justified. Partial depopulation may be useful in situations where mass vaccination and unidirectional pig flow or herd closure

occurs.[156,157] Testing and removing positive animals is also costly and labor intensive but can eliminate persistently infected animals in a herd. [158-161]

1.2.3 PRRSV modified live vaccine (MLV)

PRRS modified live virus (MLV) vaccines are widely used in PRRS-endemic herds to reduce losses associated with PRRSV infection. In experimental and field settings, PRRS MLV immunization has the potential to improve weight gain, reduce viral replication and pulmonary pathology, as well as decrease clinical disease after wild-type PRRSV exposure.[162,163] However, several challenges remain for PRRS MLV vaccine safety and efficacy, including the potential for reversion to virulence, recombination with wild-type strains,[164] potentiation of primary and secondary pathogens,[116] and incomplete protection against emerging wild-type strains.[165] Additionally, the vaccine does not prevent weight gain variation associated with PRRSV infection.[166] PRRSV vaccines are most effective against homologous strains and may provide little to no protection again various heterologous strains.[167-169] As such, the currently available commercial vaccines are generally considered inadequate for disease control.[170,171]

1.2.4 Supportive Care

Once there is an outbreak within a farm, supportive therapy can be used to decrease clinical signs and improve weight gain. Antimicrobials, including oxytetracycline (Liquamycin®; LA-200®) or ceftiofur hydrochloride/sodium (Excenel® or Excede®), are used for treating lameness or respiratory disease associated with PRRSV infection. With growing concerns of antibiotic stewardship, careful consideration for their usage is critical. The non-steroidal anti-inflammatory medication flunixin meglumine (Banamine®) is administered for lameness and/or pyrexia. While providing analgesia, these medications do nothing for the underlying viral cause of disease.

1.3 Alternative Control Methods

Since current PRRSV control methods are inadequate, there has been a significant research shift towards alternative approaches. Additionally, growing concern for antimicrobial resistance necessitate these alternative treatments. While many other approaches have been considered, gene editing, antivirals and the fecal microbiome are some of the most researched to help in the disease treatment as well as control and will be discussed in the remainder of this chapter.

1.3.1 Genomics and Gene Editing

Genomics has been used to not only be used to identify pigs that are more PRRSV resistant but also aid in the development of knockout pigs that are completely resistant to PRRSV infection. Initially, Ait-ali et al. found that porcine alveolar macrophages in Landrace pigs showed lower levels of PRRSV replication compared to other breeds,[172] suggesting a genetic component to resistance. In 2011, the PRRS Host Genetics Consortium (PHGC) was formed to further investigate these differences.[173] The PHGC is composed of groups from the USDA, academia and industry with an interest in identifying host genetics associated with PRRS resistance. Genome-wide association studies (GWAS) were used to identify single nucleotide polymorphisms (SNPs) that were associated with PRRS resistance. Approximately 200 commercial pigs were infected for each study; clinical data, weight and serum samples for viremia were collected over time to identify variances in disease susceptibility. Within chromosome 4, the SNP WUR10000125 (WUR) was associated with variation in weight gain and PRRS serum viremia.[87,89,174] Specifically, the "B" allele was found to be advantageous over the "A" allele at this SNP. The "B" allele for the WUR SNP was also associated with decreased virus load and increased average daily gain after vaccination and decreased virus load after challenge.[175] Additionally, the PHGC looked at differences associated with PRRSV/PCV2 co-

infections,[176] finding SNPs associated with both PRRSV and PCV2 viral load as well as average daily gain.

In addition to GWAS, significant research has been done to identify and alter receptors associated with PRRSV infection. Many receptors have been hypothesized and are reviewed comprehensively;[177] however, a few notable cases will be highlighted. Initially, heparan sulfate and CD169 (sialoadhesin; siglec-1) were thought to be the main receptors involved in PRRSV internalization and infection.[178] Heparan sulfate is a polysaccharide expressed on the cell membrane and in the extracellular matrix of most mammalian cells and is involved in leukocyte development and migration, immune activation, and inflammation.[179] It has been demonstrated as the receptor for multiple viruses.[180-184] CD169 is a transmembrane glycoprotein found only in macrophages and binds sialic acid located on the surface of pathogens,[185-190] aiding in internalization.[191] Subsequent studies disproved these receptors being critical for infection, showing that blocking heparan sulfate did not prevent infection [192] and CD169 transfection into non-permissive cells did not result in PRRSV infection.[193] Another study also found that knocking out the CD169 gene did not prevent PRRSV infection.[194] While heparan sulfate and CD169 are still considered important for binding of the PRRS virus to macrophages,[195,196] further research identified CD163 as the critical receptor for PRRSV infection.[196,197]

Porcine CD163 is a transmembrane protein within the scavenger receptor cysteine-rich superfamily (SRCR-SF) and is involved in cellular immune response regulation. Of the nine SRCR domains, only SRCR2, SRCR3 and SRCR5 have known biological functions. SRCR2 is involved in the binding of erythrocytes and bacteria.[198,199] SRCR3 binds and causes internalization of hemoglobin-haptoglobin complexes (cell-free hemoglobin),[200] which are formed after hemolysis, and is involved in anti-inflammatory processes.[201] SRCR5 plays a role in

PRRSV infection;[202] since its identification, CRISPR technology has been used to delete this domain, verifying that without CD163 pigs are resistant to PRRSV infection.[203,204] Additional studies have demonstrated that domain five of the scavenger receptor is most involved in infection[205] and that replacing domain five with a human CD163 homolog[202] also leads to decreased PRRSV infection. While these breakthroughs brought great promise to the swine industry, there have been setbacks to implication of genetically modified animals within production settings. There is still skepticism in the general public about genetically modified organisms and implications for human health. Without approval from consumers it is unlikely that we will see these PRRSV-resistant pigs on the market.

1.3.2 Antivirals

Another alternative control is antivirals, which can directly inhibit PRRSV binding, uptake and replication. Compounds have been studied for their antiviral activity against PRRSV. Du et al. found that tilmicosin had antiviral activity against both PRRSV-1 and PRRSV-2 in porcine alveolar macrophages.[206] A monoclonal anti-idiotypic antibody against glycoprotein (GP)-5 of PRRSV has also been shown to decrease PRRSV infection.[207] Blebbistatin, a myosin II ATPase inhibitor, prevented PRRSV replication *in vitro* as well as *in* vivo.[208] More recently, since the CD163 receptor was demonstrated critical in PRRSV infection, monoclonal antibodies have been produced to bind to the SRCR5 domain and decrease PRRSV infection.[209] MicroRNAs (miRNAs) have also been investigated for their anti-PRRSV effects. Reviewed extensively elsewhere,[210] miRNAs and other antisense RNAs (small interfering RNAs, short-hairpin RNAs, artificial miRNAs, and morpholino oligomers) can target not only the PRRSV genome and proteins directly[211-221] but also pathways involved in PRRSV infection.[212,222-225] MicroRNA 181 was specifically found to target the CD163 receptor.[226] Zhang et al. also found heme oxygenase

produces carbon monoxide and biliverdin to block PRRSV replication.[227] In 2015, single-chain antibody fragments, known as nanobodies were investigated due to ease of manipulation over full-length antibodies.[228] It was found that these fragments could target nonstructural PRRSV proteins, inhibiting viral replication.[228,229]

Chinese herbal medications have also been more recently investigated for their anti-viral effects. Matrine, derived from plants in the genus *Sophora*, is an alkaloid that has been demonstrated to have antiviral effects. Specifically, Sun et al. found PRRSV antiviral activity within porcine alveolar macrophages.[230] They then developed a mouse model, in which matrine not only had antiviral activity against PRRSV and PCV2[231] separately, but it also had antiviral activity against PRRSV/PCV2 co-infection.[231] The same group also found that a derivative of tanshinone IIA (sodium tanshinone IIA sulfonate) also had anti-PRRSV effects *in vitro*.[232] Other groups have shown that Chinese herbal medications, specifically xanthohumol,[233] epigallocatechin-3-gallate in green tea (*Camellia sinensis*),[234] glycyrrhizin in licorice roots (*Glycyrrhiza glabra*),[235] *Thymus vulgaris* and *Nepeta cataria* hydrosol,[236] curcumin in turmeric rhizomes (*Curcuma longa*),[237] tea seed saponins,[238] *Cryptoporus volvatus* extract,[239] tetrahydroaltersolanol C[240] and ginsenoside Rg1[241] had anti-PRRSV effects *in vitro*. These and others are extensively reviewed in Du et al. and Bell-Onaghise et al..[210,242]

While antivirals can help ease clinical signs due to disease, they may not prevent infection. Currently, administration in sick pigs must be done individually, similar to many antimicrobials, which is labor-intensive. Additionally, many studies mentioned above have been done *in* vitro and have yet to be validated *in vivo* and more specifically pigs. Therefore, antiviral use still have limited application in the field until administration can be validated and be applied at the herd level.

1.3.3 The Pig Gut Microbiome

The gastrointestinal tract is a dynamic place of exchange between the outside world and the host. Nutrients, host epithelial and immune cells, secretory immunoglobulins, metabolites and microorganisms converge and interact within the gastrointestinal tract. The host epithelium must obtain nutrients for metabolism while keeping out luminal pathogens and parasites. Within this microenvironment, the immune system is held in a delicate balance between defense of invading pathogens and tolerance to self and food antigens.

The gut microbiome is the collection of microorganisms, composed of bacteria, viruses, fungi, archaea and protozoa living in the gastrointestinal tract. The composition of the gut microbiome varies by location with increasing numbers as well as diversity of microbes from the stomach to the colon. Of the gut microorganisms, bacteria has been the most studied in the gut microbiome. In 1903 the first study describing bacterial species in the gut was done by Heinick et al., isolating bacteria such as *Bacterium coli*, *Bacterium lactis-aerogenes*, as well as staphylococci species within the intestine, cecum and rectum of 23 healthy pigs.[243] It was not until the mid-1900s that further experiments were done looking at normal gastrointestinal bacteria. In 1934, Kraneveld and Djaenoedin found *Clostridium welchii* in rectal samples from 50 healthy pigs.[244] In 1936, *Lactobacillus acidophilus* was found to be more prevalent in healthy pigs than diarrheic pigs.[245] In 1940, another study found similar bacteria as Heinick; in addition, they isolated *Lactobacillus acidophilus* and anaerobic spore-formers in healthy pigs.[246] In 1945, Levine et al. isolated colonic bacterial species and administered them to healthy pigs;[247] this was effectively the first study in pigs that determined certain bacterial species could be present within the gastrointestinal tract without causing disease, including some *Salmonella* species, *Proteus* species, as well as Eberthella- and Shigella-like species. While these bacteria as well as other

specific pathogens had been studied prior, the first documented studies of the pig gut microbiome as a whole, then called the intestinal 'flora' or 'microflora', occurred in the early 1950s.[248-252] These studies focused on the relationship between antibiotics and the gut microbiome. Up to the late 1990s, most gut microbiome studies were cultured based, limiting the number of detectable bacteria. Within these studies, lactobacilli, streptococci, bacilli and Bacteroides species were often isolated,[249,253-257] both in unweaned and weaned pigs. Results from these early studies should be approached with caution as bacterial culture can lead to bias not only by what species were culturable but also based on which media and techniques were used within the study.

In the late 1990s, 16S ribosomal DNA (rDNA) sequencing started to become more widely used, further increasing our knowledge of the gut microbiome as well as decreasing variability between and within studies.[258,259] A relatively new sequencing method at the time, 16S rDNA sequencing takes advantage of the unique bacterial small-subunit ribosomal DNA to aid in species identification, both described and unknown. One disadvantage, in comparison to bacterial culture is that while nucleic acid may be present, bacteria may not be viable. The two major bacterial phyla found in the gut microbiome were Firmicutes and Bacteroidetes;[260-266] however, Proteobacteria can be found at a higher proportion within the ileum.[264,267] Notable bacteria within the Firmicutes phylum include *Bacillus*, *Staphylococcus*, *Streptococcus*, *Lactobacillus, Ruminococcus* and *Clostridium* species. Notable bacteria within the Bacteroidetes phylum include *Bacteroides, Prevotella,* and *Flavobacterium* species. Notable bacteria within the Proteobacteria phylum include *Salmonella, Helicobacter, Klebsiella,* and *Pseudomonas* species. At the genus level, *Prevotella* species have been found to represent the largest group.[261]

Weaning has been shown to result in a transient decrease in overall diversity and number of bacteria then eventually rebounds.[268,269] During this time aerobes, which were prevalent immediately after birth, are replaced with a large anaerobic population.[270] Prior to weaning, some studies was found that a higher level of *Lactobacillus* species are found within the gastrointestinal tract, which keep pH low by producing lactic acid from fermentation of milk-based oligosaccharides,[271] thereby reducing pathogen invasion; however, at weaning *Lactobacillus* numbers decrease, resulting in increased pH, and growth of pathogenic species.[272,273] However, a newer study demonstrated increased *Lactobacillus* prevalence after weaning.[274] Further studies should be done to elucidate the changes in this bacterial genus during the time of weaning. After weaning, species such as *Prevotella*[265,266,274] and *Bacteroides*[270] increase in abundance and are associated with increased growth rates. *Prevotella* and *Bacteroides* species have been shown to breakdown polysaccharides found in plants.[275,276]

Many factors can affect the diversity and composition of the gut microbiome. Diet, genetics and age have been shown to affect the gut microbiome [277-279] In swine, the first exposure to microbes is from the sow's vaginal, fecal and skin microbiomes. Mode of delivery (vaginal vs caesarean), sow diet, piglet diet, antibiotics, handling, and environment can all affect a growing pig's microbiome. These factors have been extensively reviewed by Niederwerder 2016,[280] as well as Guevarra et al. 2019.[281]

The gut microbiome plays many roles within the host, and is not merely a passerby in the gastrointestinal tract. It forms a mutualistic relationship with its host, facilitating feed digestion and nutrient absorption, promoting the development and regulation of the immune system and providing a protective barrier against pathogenic organisms.[282-287] The following sections characterize the role of the gut microbiome.

1.3.3a Weight gain

PRRSV infection reduces nutrient digestibility and feed efficiency of growing pigs.[288] In some of the earliest gut microbiome work evaluating lean versus obese humans, gut microbiomes with an increased Firmicutes abundance and decreased Bacteroidetes abundance were classified as having increased nutrient extraction capabilities.[289,290] The trend of Firmicutes: Bacteroidetes ratios being increased in high growth pigs after co-infection with PRRSV and PCV2 has also been reported by our group.[291] Moreover, other groups have demonstrated a positive correlative relationship between Firmicutes bacterial abundance and weight gain[292,293] and a negative correlative relationship between Bacteroidetes bacterial abundance and weight gain[294] in swine. In a contrasting study, Oh et al. 2020 recently reported no significant differences in the relative abundance of these two phyla between high and low growing pigs.[295] Further work should be done to verify or disprove this, as gut microbiome therapy could change due to the findings.

1.3.3b Intestinal Barrier

Even before the innate or adaptive immune system, the gut microbiome can provide a first line of defense against invading pathogens. Some gram negative bacteria, such as *Proteobacteria* and *Verrucomicrobia*, use mucin as an energy source and, therefore adhere to the mucous layer of the gastrointestinal tract.[296] Bacteria within the mucous layer, with mucins, secretory immunoglobulin A (IgA) and other antimicrobial peptides can serve as a primary barrier against invading pathogens.[297] Additionally, a normal gut microbiome is integral to maintaining the integrity of tight junctions between gastrointestinal epithelial cells; disruption of the gut microbiome can lead to leaky tight junctions and result in disease and pathology.[298,299]

1.3.3c Immune modulation

Gut-associated lymphoid tissue (GALT) is the largest aggregation of immune cells and lymphoid tissue in the whole body. GALT is a part of the larger mucosal-associated lymphoid tissue (MALT) system, which is the first line of defense against pathogens that enter the body. GALT consists of not only isolated lymphoid follicles and immune cells but also structured aggregates of lymphoid cells known as Peyer's patches (PPs) within the gastrointestinal tract. There are two types of PPs in the pig; discrete patches in the jejunum and upper ileum and a long continuous patch in the terminal ileum. The continuous ileal PP is not considered a primary lymphoid organ as in other species such as the sheep,[300,301] and therefore may be more involved in tolerance to commensal organisms and pathogen invasion monitoring. It has been suggested that even though the ileal lymphoid node is not a primary lymphoid organ, that it could increase the efficiency of mucosal immunity [302] by sampling of luminal antigen.

The gut microbiome interacts with GALT to develop and regulate the immune system. The gut microbiome interacts with GALT through microfold cells, where they are taken up by the gastrointestinal tract and processed.[303,304] Weak stimulation of naïve T cells from commensal antigens on antigen presenting cells leads to expression of anti-inflammatory cytokines interleukin 10 (IL-10) and tumor growth factor beta (TGFβ), signaling the maturation of regulatory T cells (T_{regs}).[305-307] The major role of T_{regs} in the gastrointestinal tract is to balance pro-inflammatory with anti-inflammatory functions, creating an environment of tolerance to self as well as food and commensal organisms. The function of T_{regs} was theorized as early as 1969, when thymectomized mice lead to spontaneous murine autoimmune ovarian disease.[308] Studies later confirmed the presence of an "anti-inflammatory" T lymphocyte, called a suppressor T lymphocytes in the early 1970s.[309,310] Uncertainty of presence of these cells in the 1980s lead to a

decrease in research, but advances in identification markers lead to the resurgence of the cell now known as the $T_{reg.}$[311] Commensal bacteria also lead to IgA production, the most secreted immunoglobulin in the gut lumen; IgA is important for opsonization and neutralization of pathogens,[312] thereby preventing bacterial overgrowth.[305-307] The gut metatranscriptome, the gut microbiome's gene expression, has been preliminary explored. [313] These studies are critical for identifying transcriptionally active microbes that modulate host metabolism and immunity. Only one such study was specific to the swine metatranscriptome,[314] therefore, there is potential for expansion in this research area.

The pig gut microbiome is essential for normal immune development. Rothkötter et al;[315] discovered that without a gut microbiome, T-lymphocytes within the lamina propria are significantly decreased. Another study found that conventional pigs have longer and more developed Peyer's patches in contrast to their gnotobiotic counterparts.[316] Additionally, conventional pigs were found to have significantly more CD4+ and CD8+ cells, and therefore helper T (T_H) cells and cytotoxic T lymphocytes (CTLs), respectively to aid in pathogen defense, compared to gnotobiotic pigs. Gnotobiotic pigs have decreased quantities of fecal secretory IgA[317] and by adding specific bacterial monocultures, secretory IgA levels can be restored.[318]

1.3.3d Gut-Lung Axis

The gut-lung axis, or the interaction between the gastrointestinal tract and lungs, has gained more attention in recent studies.[319-327] The lung was first thought to be a relatively sterile environment; however, recent studies have suggested that the lungs themselves are inhabited by their own specific microbiome, which can be affected by disease.[328-333] One recent study found that healthy porcine lungs contained *Methylotenera*, *Prevotella*, *Sphingobium* and *Lactobacillus* species.[334] Communication exists between the mucosal tissues of the gut and lung, not only due

to the physical closeness of these systems but also due to systemic movement and the stimulation of the immune system from gut microbes and their metabolites. Metabolic products of gut microbiota mediate communication between the gastrointestinal tract and extra-gastrointestinal tissues. Metabolic products absorbed into the gastrointestinal epithelium go into the lymphatics and the blood stream and can then communicate with other body systems, including the respiratory tract.

In humans, respiratory disease such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, pneumonia, and influenza have been characterized as having a gastrointestinal component.[321,325,326,335] Interestingly, within the last year research has increased on the gut-lung axis as it relates to severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Clinical trials suggest adjunct probiotics in treatment of SARS-CoV-2 to improve outcome.[336] A study in mice analyzing the pulmonary and intestinal flora after FMT found that the operational taxonomic unit (OTU) values significantly decreased in the phyla Proteobacteria, Firmicutes and Bacteroidetes in feces and in the genera *Pseudomonas*, *Sphingobium*, *Lactobacillus*, *Rhizobium*, and *Acinetobacter* in pulmonary tissue.[337] However, the implications for these changes in disease are unknown and should be further explored.

1.3.3e Gut microbiome and swine respiratory disease

Research exploring the gut-lung axis in pigs is limited. The mechanism by which the gut microbiome impacts outcome in pigs exposed to respiratory pathogens is largely unknown, but is believed to be associated with immune modulation and microbial metabolic products.[280] Previous work by our group has demonstrated several gut microbiome characteristics that are associated with improved outcome parameters in pigs co-infected with PRRSV and PCV2. Specifically, increased gut microbiome diversity, increased *Ruminococcaceae* species, and increased

Streptococcaceae species were associated with reduced virus replication, improved weight gain, and decreased morbidity.[118,291,338] Associations between increased gut microbiome diversity and beneficial outcome characteristics following *Mycoplasma hyopneumoniae* induced respiratory disease have also been published. In one 2013 study, an oral microbial inoculum prior to *M. hyopneumoniae* challenge resulted in decreased coughing and lung pathology.[287] A more recent study demonstrated decreased *M. hyopneumoniae*-associated lung pathology was associated with increased *Ruminococcaceae* abundance,[339] substantiating our findings that this bacterial group within the gut microbiome is correlated to improved respiratory disease outcomes.

1.3.3f Gut microbiome and vaccines

Despite PRRS MLV vaccines being widely used to reduce PRRS-associated losses in endemic herds, the currently available commercial vaccines are inadequate for disease control.[171] There is a growing body of evidence for the role of the gut microbiome in response to vaccination for infectious diseases of humans and livestock.[340,341] Research focused on gut microbiome associations with PRRS vaccine efficacy limited. While one study showed no effect of an oral single-strain probiotic on PRRS vaccine response,[342] another demonstrated improved immune response. The second study showed increased antibody titer, milder pathogenic damage and shorter viral clearance time, following PRRSV vaccination were associated with increased relative abundance of *Lactobacillus* species.[343] The relationship between the immune response to other infectious disease vaccines and gut microbiome characteristics has been described.[340,341,344] IgA seroconversion after oral immunization with a live human rotavirus vaccine has been correlated with fecal microbiome characteristics such as increased *Streptococcus bovis* and reduced species in the Bacteroidetes phylum.[345] Pigs with high IgG production after

immunization with a killed *Mycoplasma hyopneumoniae* vaccine had increased *Lachnospiraceae*, *Prevotella* and *Fibrobacter* bacteria.[346]

1.3.3g Gut microbiome therapeutics

Modulation of the gut microbiome also presents an opportunity to affect multiple body systems, compared to many other therapy types only affecting one system, or even possibly one metabolic pathway. The presence of beneficial bacteria and their metabolites can prevent pathogen invasion by preventing colonization, secreting antimicrobial substances or priming the immune system to defend the body.

The gut microbiome can lead to changes not only locally, but also systemically, similar to how mucosal vaccination can result in systemic effects. The effects of gut microbiome therapeutics were first predicted when germ free mice were found to be more susceptible to respiratory infection.[347] The gut microbiome leads to migration of immune cells to the intestine as well as homing to distant locations. This is in part due the same receptors being localized to many part of the body. Any cell that migrates to survey the environment or to be activated will not only travel through lymph but also through the bloodstream. B lymphocytes that secrete IgA have been shown to be capable of binding to integrins at distant sites to cause effects in other organs such as the lung. Segmented filamentous bacteria in the gastrointestinal tract were found to decrease susceptibility to *S. aureus* mediated through T_H17 pathways.[348] T_H17 cells are responsible for neutrophil recruitment, which can then phagocytose pathogens. In addition to lymphocytes, cytokines can travel to distant sites to affect the immune system. In one study, they found that the normal gut microbiome lead to T_H17 production of IL-17 in the lungs which decreased susceptibility to *S. pneumoniae* and *K. pneumoniae*.[349] Another study found that *B. fragilis* produces polysaccharide A (PSA) which has been shown to induce IL-10 systemically

within mice;[305] implications for disease susceptibility have yet to be determined. Therefore, innate and adaptive components of the immune system can contribute to the systemic effects seen in gut microbiome therapeutics.

1.3.3.1 Probiotics

The purpose of probiotics is to increase beneficial microbes within the gut microbiome. These consist of live bacterial cultures, or occasionally yeast and fungal organisms. Probiotics can help increase nutrient digestion by fermentation of feed material and nutrients within the gastrointestinal tract.[350] They can also provide competitive exclusion of pathogens and enhance the intestinal epithelial barrier[297-299,351] by producing antimicrobials (see Section 1.3.3.3. Postbiotics). Specifically in pigs, it has been found that probiotics can lead to decreased shedding of *Salmonella enterica* as well as *Escherichia coli*.[352,353] Finally they have been shown to increase T_{reg} cells and IgA production leading to increased self-tolerance and decreased autoimmune disease within the gastrointestinal tract.[305,354-357]

1.3.3.2 Prebiotics

Prebiotics aid in the growth of beneficial microbes, and therefore, the chance of survival and colonization. They function as nutrients for probiotics and increase activity of probiotics, further leading to the production of postbiotics.[350,358] They have also been shown on their own to competitively exclude pathogens and increase T_H1 response, [359-362] therefore leading to macrophage recruitment and pathogen phagocytosis. Prebiotics vary in composition but are often comprised of complex carbohydrates and sugars. Some examples of prebiotics include whole grain wheat and corn that contain oligosaccharides, fructans, dextrins and lactulose.[359,361-363]

1.3.3.3. Postbiotics

Bacterial metabolites and cell components, known as postbiotics, can also benefit the host by increasing immunity and decreasing disease susceptibility. The hypothesized mechanism of benefit includes increased expression of the anti-inflammatory cytokine IL-10 to promote self, commensal and food tolerance, antimicrobial activity through increased cytokines and competitive exclusion of pathogens through antimicrobial products such as bacteriocidin.[364-367] Further work should be done to elucidate postbiotics and their mechanism of action.

1.3.3.4. Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is the process by which fecal microbes are transplanted from a healthy donor into a diseased or young individual. FMT has demonstrated promising results for use as an alternative tool for antimicrobial agents in the face of increasing antimicrobial resistance. The process of fecal microbiota transplantation, at the time called transfaunation, was described in 17th century ruminants.[368] Where pre-, pro-, and post-biotics are relatively well-defined, FMT may not be. FMT may contain some combination of pre-, pro-, and post-biotics, as well as other, possibly unknown elements. These additional elements include secretory IgA, undigested food particles (e.g. cellulose), inorganic substances such as iron phosphate and calcium phosphate, dead donor cells and their metabolites, as well as proteins, fats and carbohydrates. In terms of microorganisms, FMTs may be comprised of a considerable amount of dead microorganisms in combination with live microorganisms. The implications of these dead microorganisms is largely unknown and should be further considered.

FMT has been shown to transmit phenotypes, such as immune function and obesity.[289,369,370] Other phenotypes, such as early weaning stress, could also be transmitted through the gut microbiome but have yet to be determined. It is difficult to define how certain microbes affect phenotypic differences due to the complex relationship that the gut microbiome has with the

hosts. While some microbes may have isolated effects on the host, it is likely that microbes work synergistically. A recent study[371] attempted to parse out these phenotypes by colonization of gnotobiotic mice with well-defined fecal samples. Many of these inoculations resulted in upregulation of T_{regs} and metabolic pathways. Obesity phenotypes have also been shown to be transmissible through fecal transplantation.[289,369] When the gut microbiome from an obese mouse is transplanted into a thin mouse, the thin mouse becomes obese. In contrast, a recent study in pigs given a FMT from high feed efficiency pigs led to decreased feed efficiency and weight.[372] Interestingly, when characterized, transplanted pigs had higher levels of *Bacteroidetes,* which is consist with mouse obesity experiments. These studies highlight the complexity of the gut microbiome and significant effects when transplanted into other animals.

For several diverse human disease states, FMT has been shown to improve treatment outcome or resolve complex disease conditions. Although recurrent *Clostridium difficile* infections are by far where FMT is most commonly used, other digestive diseases have also been linked with improvement due to FMT treatment, including inflammatory bowel disease, ulcerative colitis, and metabolic syndrome.[373-375] The mechanism by which FMT effectively improves outcome in these disease conditions is thought to be associated with the restoration of normal flora and improvement of nutrient digestion.

FMT and microbiome modulation has also been associated with the improvement of non-digestive diseases, such as neurologic and respiratory diseases. For these diseases, the mechanism is likely more complex, although the benefits appear to be, at least in part, immunological. For example, FMT restored the production of the inflammatory cytokine tumor necrosis factor alpha (TNF-α) produced by macrophages and monocytes leading to apoptosis of

infected cells,[376] in microbiota depleted (by antibiotics) mice after respiratory infection with *Streptococcus pneumoniae*.[377]

Recent studies have analyzed the effects of the gut microbiome and FMT in swine respiratory disease. The mechanisms by which FMT improves outcome, both in health and disease, are generally unknown. One study in pigs found that FMT increased microbial diversity and FMT in led to decreased coughing, decreased lung lesions and improved immune responses after *Mycoplasma hyopneumoniae* infection.[287] Martin et al. 2015 established that FMT could be used to decrease necrotizing enterocolitis; however, this study also found there was an increase in the amount of mortalities in these piglets.[378] The focus of our research group is to understand how the gut microbiome can be modulated to increase weight gain and decrease clinical signs associated with PRRSV/PCV-2 co-infections. Increased microbial species prior to[291] and after PRRSV/PCV-2 co-infection[328] were associated with increased weight gain. FMT could be used to negate the effects of early weaning by improving weight gain and immune response to respiratory diseases. The limited research demonstrates the need for additional studies to characterize the effects of FMT in pigs.

Chapter 2 - Phenotypic differences in clinical disease response associated with two North American porcine reproductive and respiratory syndrome virus (PRRSV) isolates in nursery pigs

Abstract

Porcine reproductive and respiratory syndrome (PRRS) is the most economically devastating swine disease in the United States (US). In a population of growing pigs, PRRS results in reduced weight gain, respiratory disease and immunosuppression, increasing infections by primary and secondary pathogens. Significant genetic variation exists among PRRS virus (PRRSV) isolates, which presumably correlates to differences in morbidity, mortality, and clinical presentation. The objective of this study was to compare morbidity and mortality of two heterologous PRRSV-2 (North American) isolates after experimental infection in large nursery pig populations. Two experimental populations of approximately 200 commercial crossbred pigs were infected with either NVSL or KS06 and followed for 42 days post-infection (dpi). Overall morbidity rates after infection with NVSL or KS06 were 39.2% and 23.5%, respectively. However, the mortality rate after infection with NVSL was approximately half that of KS06; 4.8% and 9.0%, respectively. The time course of clinical disease post-infection with NVSL was chronic, with clinical signs occurring at a similar rate throughout the 6-week trial. In contrast, clinical disease post-infection with KS06 was acute, with peak clinical signs primarily occurring between 4 and 14 dpi. Respiratory disease, altered ambulation, decreased body condition, altered mentation, and fever occurred in a significantly higher proportion of pigs infected with NVSL. Increased viral load and reduced weight gain corresponded to the prolonged clinical course of

disease in NVSL-infected pigs. Overall, these results provide evidence of diverse clinical phenotypes that may occur during infection with heterologous PRRSV isolates.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) causes substantial economic losses to swine operations around the world. In the most recent analyses, PRRS was estimated to cost US swine producers approximately $664 million per year,[1] with over $360 million alone due to losses in growing pigs. The causative agent of PRRS is PRRS virus (PRRSV), a single-stranded RNA enveloped virus in the *Arteriviridae* family,[379] which leads to increased morbidity and mortality due to respiratory disease, decreased reproductive performance, and reduced weight gain in growing pigs. Additionally, PRRS leads to immunosuppression, secondary infections and increased usage of parenteral antibiotics. PRRS was first described in the US in the late 1980s[3] and Europe in the early 1990s[380] with isolation of the virus shortly thereafter.[2] 'Abortus blauw' (Dutch for "blue abortion") was one of the first names given to PRRS due to abortion and notable aural cyanosis in some sows.[4] Subsequently, PRRSV has been characterized by a vast range of clinical signs, including respiratory disease such as tachypnea, dyspnea, open-mouth breathing, forced abdominal breathing, conjunctivitis, sneezing, coughing, serous to mucopurulent rhinitis and ocular discharge.[3,63,72,73] Additional PRRS-associated clinical signs include polyarthritis, altered ambulation, reduced appetite, decreased growth rate, pyrexia, lethargy, depression, diarrhea, and rough hair coat.

PRRSV is separated into two lineages comprised of PRRSV-1 or European PRRSV and PRRSV-2 or North American PRRSV.[23,381] PRRSV has an inherently high mutation rate due to the error frequency during virus replication;[29] as such, many PRRSV isolates exist within the two lineages constituting a diverse genetic population. Genetic variation creates challenges in

stimulating long-term and broadly protective immunity, either through natural exposure or through modified live virus vaccination.[15,382,383] Furthermore, infection with heterologous isolates can lead to severe outbreaks, even in endemic herds with underlying immunity.[384,385] For example, a new PRRSV-2 variant recently resulted in outbreaks affecting previously exposed and vaccinated populations, including increased sows off feed, increased post-weaning mortality, increased abortions, and reduced finisher weight gain.[386] To understand how genetic virus variation relates to clinical disease variation, phenotypic data must be standardized; however, there is limited quantitative data in large controlled studies to describe clinical progression associated with different PRRSV isolates.

The PRRS Host Genetics Consortium (PHGC) was developed to understand how host genetics affects PRRSV infection response.[173] The objective of this consortium was to identify genotypes (i.e., candidate genes, single nucleotide polymorphisms, and genomic regions) that confer phenotypic resistance or susceptibility to PRRS. Previous publications have described the advantage of the B allele in the single nucleotide polymorphism WUR10000125 by reducing viral load and increasing weight gain after PRRSV infection.[87] Furthermore, Hess et al. (2016) described the role of host genetics in the response of pigs to two heterologous PRRSV-2 isolates, NVSL and KS06, including differences in heritability, viremia and weight gain.[174] However, a standardized comparison of clinical outcomes across the two PRRSV isolates in large populations of nursery pigs had yet to be reported.

In the study described herein, we characterized the clinical outcome of pigs infected with either NVSL or KS06 in a subset of two PHGC trials, including the quantification of morbidity and mortality, as well as defining the time course and frequency of clinical signs. This report provides evidence for acute and chronic clinical disease associated with PRRSV infection across

well-controlled experimental studies, where differences in viral isolate correlated to a significant shift in clinical presentation.

Materials and Methods

Selection of Trials. The populations of pigs involved in the current study were part of the PHGC as previously described.[173] Of the 14 PHGC studies included in the host genetics analysis,[387] two studies were selected for inclusion in the clinical disease analysis described herein. The rationale for selecting these two studies was to minimize variation associated with factors other than viral isolate. Specifically, 1) both studies were performed in the same Kansas State University facility, 2) health evaluations had been standardized between the two studies, 3) complete medical records were available for all pigs across the two studies and 4) pigs in the two studies were obtained from the same commercial source and had similar genetic backgrounds.

Experimental Design. Experiments involving animals and viruses were performed 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. The study involving NVSL occurred between the dates of April 1 and May 13, 2013, while the study involving KS06 occurred between the dates of October 24 and December 5, 2011. Commercial crossbred barrows (Pietrain x Large White) were transported to the Kansas State University Large Animal Research Center at weaning. For both trials, nursery pigs were obtained from a single high-health commercial source (Topigs; Burnsville, MN) that was negative for PRRSV and *Mycoplasma hyopneumoniae*. Both studies were conducted under biosafety level 2 (BSL-2) conditions with the same environmental (temperature and humidity) conditions.

Pigs were prophylactically administered a single dose of the broad spectrum antibiotic tulathromycin (Draxxin®) prior to shipment. Upon arrival at Kansas State University, piglets were randomly allocated into 9 identical pens (144 ft^2) with 22-23 pigs/pen. Piglets were then allowed 5 or 6 days to acclimate to their new environment prior to infection with PRRSV. After the acclimation period, all pigs (average age 26.0 ± 1.5 days for NVSL; average age 25.5 ± 1.5 days for KS06) were experimentally infected with 10^5 50% tissue culture infectious dose ($TCID_{50}$) NVSL-97 or KS06. A 2 mL dose was administered, with 1 mL delivered intranasally and 1 mL delivered intramuscularly. Pigs were given access to food and water *ad libitum* throughout both studies. At 42-43 dpi, all remaining pigs were humanely euthanized in accordance with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals.

Viruses. Two heterologous North American PRRS-2 viruses were used for infection of pigs. The first isolate, NVSL 97-7895 (NVSL; GenBank Accession No. AY545985), is believed to have originated in southeast Iowa in December 1996 from an outbreak characterized by widespread abortion storms, even in herds routinely vaccinated with a modified live PRRS virus vaccine.[67] The virus was isolated, characterized and sequenced by the Diagnostic Virology Unit at the National Veterinary Services Laboratory (NVSL, USDA/APHIS, Ames, IA, USA) in 1997.[388] The second isolate, KS 2006-72109 (KS06; GenBank Accession No. KM252867), originated in north central Kansas in 2006 from a sow herd with clinical signs of respiratory disease in pigs from the farrowing room between 5-14 days of age. The virus was isolated at the Kansas State Veterinary Diagnostic Laboratory for confirmatory diagnosis.[389] Comparing the two isolates, NVSL and KS06 have 89% similarity in glycoprotein 5 (GP5), a major PRRSV

antigen commonly used as a benchmark for genetic variation[390] at both the nucleotide and amino acid levels.[389]

MARC-145 cells were used for PRRSV isolation, propagation and titration. 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). Dilutions were 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.[391]

Clinical evaluation. Pigs were evaluated daily throughout the 42 day trial by a veterinarian or veterinary assistant for the presence of PRRS-associated clinical signs, including tachypnea, dyspnea, coughing, sneezing, mucoid rhinorrhea, open mouth breathing, conjunctivitis, aural cyanosis, altered ambulation (stiffness, lameness, or joint effusion), diarrhea, decreased body condition, and altered mentation (lethargy or depression). Any pig showing clinical signs of PRRS was restrained for a physical examination and rectal temperature was collected. A standardized health evaluation form was developed and utilized during both trials (**Table 2.1**). This form was completed for all pigs showing clinical signs that warranted veterinary evaluation. Included on the health evaluation form were clinical signs characterized by presence or absence in addition to several clinical signs scored based on severity. Clinical signs documented as binomial included diarrhea, pyrexia, tachypnea, mucoid rhinorrhea, coughing, sneezing, dyspnea, open-mouth breathing, ocular discharge, conjunctivitis, altered ambulation, and joint effusion. Clinical signs scored on severity included altered mentation and

response to stimuli as well as body condition. In the calculation of clinical signs for comparing the two viruses in the current analysis, all clinical signs were reduced to binomial variables. For example, attitude was considered normal for any pig characterized as bright, alert, and responsive whereas attitude was considered abnormal for any type of decreased mentation (e.g., quiet, alert and responsive; depressed, alert, responsive; moribund). In addition to the presence of clinical signs, the frequency and duration of each clinical sign were compared across virus isolates.

Pigs with clinical disease were prescribed parenteral veterinary treatment as deemed necessary by the attending veterinarian. Examples of clinical presentations where parenteral treatment was administered included 1) dyspnea or persistent coughing, 2) mucoid rhinorrhea, 3) altered ambulation with or without joint effusion, 4) diarrhea with pyrexia, and 5) lethargy or depression with pyrexia. Antimicrobials, including oxytetracycline (Liquamycin®; LA-200®) or ceftiofur hydrochloride (Excenel®), were typically administered once daily via intramuscular injection for a total of 3 days. The non-steroidal anti-inflammatory medication flunixin meglumine (Banamine®) was typically administered once daily via intramuscular injection for a period of three days. Pigs were monitored for progression or resolution of clinical signs, including daily rectal temperature measurements during treatment and a 3-day post-treatment evaluation period for all pigs recovering from clinical illness. Pigs non-responsive to veterinary treatment or those with progressive clinical disease and compromised welfare were humanely euthanized by the attending veterinarian.

Mortality and morbidity were assessed throughout the 42-day study. Any pig that died or was humanely euthanized due to PRRS was counted as a mortality. In addition to frequency, survival curves were used to assess mortality rates post NVSL and KS06 infection. Any clinical

signs warranting a veterinary evaluation and/or a mortality throughout the 42-day study were counted as a morbidity. Morbidity rates over time were determined by summing the total number of pigs with clinical signs on a given day and dividing by the total number of pigs alive. Mean clinical scores were calculated by summing the total number of clinical signs for all pigs on a given day and dividing by the number of pigs alive. Pigs subclinical for PRRSV infection were assigned zeros, but were included in all analyses of mean clinical scores. Specific clinical signs were also analyzed throughout the 42-day infection period and clinical sign frequency over time was calculated as the number of pigs demonstrating a specific clinical sign divided by the total number of pigs alive. Mean duration of specific clinical signs was calculated by summing the number of days individual pigs had a given clinical sign throughout the study divided by the total number of pigs that had the given clinical sign. For mean duration, zeros were assigned but not included in the calculations.

Individual body weights were measured weekly throughout the 42-day infection period (0, 7, 14, 21, 28, 35, and 42 dpi). Average daily gain (ADG) was calculated as the change in weight (kilograms; kg) over the change in time (days) and was reported in kg. ADG was determined for the entire 42-day study as well as the first and second halves of the study (21 day periods).

Viremia. Blood samples were collected at nine time points (0, 4, 7, 11, 14, 21, 28, 35, and 42 dpi) to determine level and duration of PRRSV viremia as well as total viral load. PRRS viral RNA was extracted using Ambion's MagMAX 96 Viral Isolation Kit (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. 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. Viral load was calculated by Riemann sums; total area of the trapezoids under the line segments connecting weekly viremia measurements. Duration of PRRSV viremia was determined by the total time (in days) a pig had a positive PCR result. Any pig missing initial or terminal data points were excluded from the viral load and duration analysis as endpoints could not be determined.

Statistical Analysis. All data was analyzed using GraphPad Prism® 9.0 software (La Jolla, CA). Comparisons of mortality and morbidity between NVSL and KS06 were performed using Fisher's exact test and corresponding odds ratios. Survival curves were compared using the Mantel-Cox test. Mean weekly weights and all mean PRRSV viremia data were compared between groups using a two-way analysis of variance (ANOVA) with multiple comparisons. ADG and viral load were analyzed using the unpaired *t*-test. Average age at challenge, PRRSV viremia duration, and mean clinical sign duration were analyzed using the Mann-Whitney *U* test.

Results

Overall, 389 commercial crossbred piglets infected with one of two North American heterologous PRRSV isolates were included in this study. Of these, 189 pigs were infected with NVSL and 200 pigs were infected with KS06. The average age at infection was significantly higher in pigs infected with NVSL than KS06 (average age 26.0 ± 1.5 days for NVSL and 25.5 ± 1.5 days for KS06; $p < 0.0001$; Mann-Whitney *U* test; data not shown).

NVSL infection resulted in higher rates of morbidity and antibiotic treatment but lower rates of mortality. Mortality and morbidity rates were used to assess overall differences in clinical disease post-infection. The overall mortality rate combining both studies was 7% or 27 out of 389 pigs. Pigs infected with NVSL were approximately half as likely to die as pigs infected with KS06 which trended towards significance (4.8 vs 9.0% mortality rates, respectively; $p = 0.09$; Mantel Cox Test; **Figure 2.1A, Table 2.2**). Morbidity, in contrast, occurred at a higher rate in NVSL infected pigs; after infection with NVSL, 74 of 189 pigs (39.2%) developed significant clinical disease warranting veterinary evaluation in comparison to 47 of 200 pigs (23.5%) after infection with KS06 ($p = 0.001$; Fisher's exact test; **Table 2.2**). Respiratory disease, associated with mucoid rhinorrhea, dyspnea, conjunctivitis, sneezing, coughing, tachypnea, ocular discharge and open mouth breathing, was approximately six times more likely to occur in NVSL than KS06 infected pigs (33.9 vs 5.5% in NVSL and KS06, respectively; $p < 0.0001$; Fisher's exact test with corresponding odds ratio analysis). Interestingly, while mucoid rhinorrhea occurred in approximately one quarter of NVSL infected pigs (57/189; 30.2%), it was completely absent with KS06 infection ($p < 0.0001$; Fisher's exact test). This may provide evidence for a reduced rate of immunosuppression and secondary bacterial infections with certain isolates. Additionally, compared to their NVSL infected counterparts, no pigs infected with KS06 demonstrated conjunctivitis, sneezing, coughing or ocular discharge. Other signs, such as altered ambulation, decreased body condition, and altered mentation also occurred at a higher rate in NVSL than KS06 infected pigs ($p < 0.01$; Fisher's exact test). However, diarrhea was seen in a similar, low proportion of NVSL and KS06 infected pigs (6.3 and 3.5%, respectively, $p = 0.2$; Fisher's exact test). Morbidity rates were positively correlated to veterinary treatment with parenteral antibiotics. Within the group of clinically

affected pigs, 98 out of 389 pigs (25.2%) received parenteral antibiotic treatment. Parenteral antibiotic treatment was administered to nearly twice the number of pigs infected with NVSL compared to KS06; 34.4% (65/189) of NVSL and 16.5% (33/200) of KS06 infected pigs received treatment ($p < 0.0001$; Fisher's exact test). Overall, NVSL infection resulted in a higher morbidity rate leading to increased usage of parenteral antibiotic treatment, while KS06 infection lead to a higher mortality rate.

NVSL infection resulted in chronic disease while KS06 infection resulted in acute disease. Clinical disease progression was monitored over time to assess disease chronicity in association with infection of the two isolates. Mortalities associated with NVSL were delayed, starting at 10 dpi with less than 1% mortality by 14 dpi. In contrast, mortalities associated with KS06 occurred earlier, beginning at 4 dpi with a 6% mortality rate by 14 dpi. From 14 dpi to the conclusion of the studies, mortalities occurred at a similar rate across both isolates. Comparing the proportion of pigs with clinical signs on each day, morbidity levels first significantly diverged between the two isolates on 7 dpi, when NVSL had significantly less clinically affected pigs (0 out of 189) than KS06 (19 out of 193; $p < 0.0001$; Fisher's exact test; **Figure 2.1B**). This pattern continued until 10 dpi. By 13 dpi, NVSL infected pigs had significantly higher levels of morbidity ($p = 0.03$; Fisher's exact test); NVSL pigs were 3.7 times more likely to have clinical signs than KS06 pigs (95% CI 1.3 - 10.5 %; odds ratio analysis). Between 13 and 41 dpi (28 days total), NVSL infected pigs had significantly higher morbidity rates than KS06 infected pigs ($p < 0.05$; Fisher's exact test). After 41 dpi, clinical signs associated with NVSL infection began to wane until morbidity rates were similar with KS06 infection at the study conclusion. Mean clinical scores were utilized to evaluate overall disease severity (**Figure 2.1C**). While multiple peaks in severity of clinical disease were noted with NVSL infection at 16, 27 and 36 dpi, a

single peak was associated with KS06 infection at 7 dpi. This data suggests that mean clinical score more clearly depicts clinical disease severity than morbidity rates.

Clinical sign frequency and duration was followed over time. Respiratory signs, decreased body condition, altered mentation, diarrhea, and pyrexia followed a time course similar to the overall morbidity time course for both isolates (**Figure 2.2A, C, D, E and F)**. In contrast, altered ambulation primarily occurred in the second half of the study (21-42 dpi) (**Figure 2.2B**). While altered ambulation, diarrhea and fever occurred for similar lengths between the two isolates ($p > 0.1$; Mann-Whitney *U* test; **Figure 2.2B, E and F**), duration of respiratory signs, decreased body condition, lethargy and antibiotic treatment were significantly longer for NVSL versus KS06 ($p < 0.05$; Mann-Whitney *U* test; **Figure 2.2A, C and D**). This data establishes the acute versus chronic clinical disease potential of PRRSV isolates.

NVSL infected pigs had reduced weight gain and increased virus replication compared to KS06 infected pigs. Prior to infection, no significant difference was detected in the weights across the two groups; 6.9 ± 1.3 kg and 6.5 ± 1.3 kg for NVSL and KS06 pigs, respectively (mean ± SD, $p = 0.77$; two-way ANOVA with multiple comparisons; **Figure 2.3A**). Absolute weights between the two groups remained similar until 21 dpi when weights significantly diverged; 12.7 ± 2.4 kg and 13.8 ± 2.8 kg for NVSL and KS06 pigs, respectively ($p = 0.003$; two-way ANOVA with repeated measures). Weights were also significantly lower in NVSL pigs on 35 dpi with NVSL pigs weighing 19.1 ± 3.9 kg and KS06 pigs weighing 20.6 ± 4.2 kg ($p < 0.0001$; two-way ANOVA with repeated measures). Weights were similar between the two groups at the conclusion of the study. ADG during the 42 day trial was significantly lower in pigs infected with NVSL; 0.43 ± 0.10 kg and 0.46 ± 0.10 kg in pigs infected with NVSL and KS06, respectively ($p = 0.03$; unpaired *t*-test; **Figure 2.3B**). Differences in ADG occurred

primarily during the first half of the study where ADG was 0.28 ± 0.08 kg for NVSL and 0.35 ± 0.09 kg for KS06 ($p < 0.0001$; unpaired *t*-test). These data indicate that disease associated with NVSL infection led to reduced weight gain in early infection compared to KS06.

Viremia curves for PRRSV followed a similar time course for both NVSL and KS06 (**Figure 2.4A**). Between 4-14 dpi, NVSL-associated viremia was higher than KS06 ($p <0.0001$; unpaired *t*-test). Viremia levels in both groups peaked at 7 dpi with 6.6 ± 0.7 log_{10} copies/PCR reaction and 5.6 ± 0.4 log_{10} copies/PCR reaction in NVSL and KS06 infected pigs, respectively ($p < 0.0001$; two-way ANOVA with multiple comparisons). Mean viremia was similar between the two isolates for most of the second half of the study; however, at 42 dpi viremia associated with NVSL infection was higher than KS06 infection (1.0 ± 1.1 log_{10} copies/PCR reaction compared to 0.7 ± 1.0 log_{10} copies/PCR reaction, respectively; $p = 0.01$; two-way ANOVA with multiple comparisons). Total viral load and duration of viremia were calculated as static measurements of overall virus burden (**Figure 2.4B**). Any pig that died or was humanely euthanized was excluded from viral load and viremia duration analyses (12 from NVSL and 18 from KS06). An additional nine pigs in the NVSL and two pigs in the KS06 groups were excluded from viremia duration analysis due to lack of initial or terminal data points. Viral load was higher in NVSL than KS06 infected pigs; 153.4 ± 20.0 and 140.2 ± 17.2, respectively ($p < 0.0001$; unpaired *t*-test). NVSL infection led to a longer viremia duration than KS06 infection; mean duration was 35.1 ± 4.7 days and 33.2 ± 5.0 days, respectively ($p < 0.0001$; Mann-Whitney *U* test; **Figure 2.4C**). These data demonstrate that NVSL infection leads to increased viral levels and viremia duration compared to KS06 infected pigs.

Discussion

The current study quantitatively characterized host phenotypic outcomes in approximately 400 pigs infected with one of two heterologous PRRSV isolates. NVSL infection led to delayed, chronic disease resulting in significantly higher morbidity associated with respiratory disease and increased parenteral antibiotic usage. In comparison, KS06 infection led to acute, severe disease resulting in higher mortalities early in the infection period.

One important consideration revealed herein is how economic and welfare impacts of PRRS can be isolate dependent. Considering NVSL, impacts included increased antibiotic usage, which would result in increased labor and veterinary costs as well as antimicrobial resistance risk. Further, NVSL was associated with reduced weight gain, which would have resulted in longer time to reach market weight and increased feed costs. These costs have previously been estimated by Nauthes et al. (2017), who reported that PRRSV-associated increases in veterinary, labor, and feed costs were approximately $30, $18 and $6 per weaned pig.[392] On the other hand, KS06 infection led to higher death loss, which would have resulted in fewer market pigs and higher post-weaning mortality. PRRS mortalities have previously been estimated to cause a loss of 10 million market pigs/year in the US, which contributes to the $362 million of economic losses in the growing pig population.[166] Further, acute death loss of large numbers of weaned pigs can cause negative impacts to the mental health of animal care personnel and farm staff. Post-traumatic stress disorder was reported after mass culling due to foot-and-mouth disease in the United Kingdom,[393] South Korea[394] and Japan.[395] Taken together, economic and welfare impacts of PRRS can be manifold and vary across isolate.

Interestingly, clinical signs and disease timelines had minimal overlap between the two isolates. Previous studies have also demonstrated a wide variation in the time course of clinical

signs associated with different PRRSV isolates. For example, a 2001 study using the NADC-20 PRRSV isolate found that respiratory signs were elevated between 7 and 15 dpi[396] spanning the period where NVSL signs were rising and KS06 signs were decreasing. Other PRRSV isolates show respiratory disease peaks ranging from 5 – 30 dpi.[6-8,397-400] In contrast to our study that reported altered ambulation in up to 13.2% of infected pigs, altered ambulation was not described as a major clinical component in these studies. Furthermore, all of these previous studies found cyanosis in a proportion of pigs whereas no cyanosis was reported herein. These phenotypic differences may be attributed to differential regulation of virulence factors across the viral genome.

Raising concern, some PRRSV isolates result in clinical signs similar to foreign animal diseases such as African and classical swine fever virus (ASFV and CSFV, respectively). In 2006, a new highly pathogenic PRRSV isolate (HP-PPRSV) emerged in China, resulting in approximately 400,000 pigs euthanized,[69,70] with ASFV- and CSFV-like disease.[401,402] HP-PRRSV resulted in high mortality (approaching 100% compared to 7% in the current study) and morbidity (approaching 100% compared to 31% in the current study), characterized by erythema and central nervous system deficits, while in the current study, these were not observed. HP-PRRSV and ASFV are also known to cause severe pyrexia, approaching 107°F,[69,403,404] while in the current study, a majority of pigs that were pyrectic (50/53 in NVSL and 31/32 in KS06) had temperatures less than 106°F. Therefore, considerable variation in clinical disease is significant, slowing PRRS diagnosis, treatment and control.

Viral replication may be associated with clinical disease and weight gain variation. Previous groups have analyzed viremia and weight gain associated with these two isolates. Ladinig et al. (2015), using a PRRSV reproductive model, found higher fetal viral loads in

NVSL compared to KS06-infected gilts, with peak gilt viremia of both isolates at 7dpi, consistent with the current study.[405] Reduced daily feed intake was also in higher proportion of NVSL than KS06 infected gilts (100 vs 50%, respectively),[405] comparable to our findings that NVSL infected piglets gained less weight than KS06 infected piglets. Interestingly, there were no differences in fetal weights between infections of the two isolates. In Hess et al. 2016,[174] 14 PHGC studies were used to evaluate host genetics and response to PRRSV infection. Peak viremia was sooner for NVSL pigs (7dpi) compared to KS06 pigs (9dpi) and viremic decay was faster after NVSL infection compared to KS06 infection, which differs from the current study. This could be attributed to different PHGC studies analyzed; however, this should be further explored. Similar to the current study, NVSL pigs gained less weight than KS06 pigs within these 14 PHGC studies.[174] While these data demonstrate variation between studies, there are generalized associations that can be made. Both within our studies and others, NVSL clinical signs were delayed compared to peak viremia, while peak viremia was consistent with KS06 clinical sign progression, which could indicate that NVSL disease is due to inflammatory cytokines or other immune responses not measured in this study rather than viral replication. These findings also suggest that supplementing feed may be beneficial to improve weight gain in piglets and gilts, while it may not be helpful with *in-utero* growth during infection. Due to ADG differences between the two isolates, emphasis should be placed on feed supplementation early in infection.

There are several aspects of the current study unrelated to PRRSV infection that may have affected clinical outcome. First, the population of pigs infected with NVSL were older (average of 0.5 days) at the time of infection than pigs infected with KS06. This may be important as age susceptibility of pigs to PRRSV has been well documented.[43,406] In addition,

NVSL infected pigs had one additional day of acclimation prior to infection. Despite the age and acclimation differences, which presumably would give the NVSL pigs an advantage, the NVSL pigs developed higher rates of clinical disease and viral loads with decreased weight gain. The two studies were also performed in different years and although these studies were done in the same facility under the same conditions, we cannot rule out the effect of completion time. Furthermore, analysis of clinical signs is invariably subjective. To minimize subjectivity across observers, body systems were reduced to categories of normal or abnormal, therefore creating a binary, semi-quantitative measurement. This type of analysis was utilized to reduce observation bias and improve the standardized assessment of PRRS clinical disease.

Our findings demonstrate evidence for acute and chronic forms of clinical disease associated with two PRRSV-2 isolates and highlight how different PRRSV isolates can impact pork production. Further investigations to identify genetic markers associated with phenotypic clinical outcomes may increase our understanding of host genetics in disease susceptibility and resistance. This study underscores how clinical disease variation across PRRS isolates contributes to challenges in early and rapid detection of novel PRRSV introductions on farms and in endemic herds.

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Table 2.1. Standardized clinical health evaluation worksheet utilized to characterize clinical signs after infection with porcine reproductive and respiratory syndrome virus

Mentation*:	BAR	QAR	DAR	Moribund
Body Condition:	Normal	Thin	Poor	
Feces:	Normal	Diarrhea		
Temperature:	Normal (<103.6°F)		Febrile (≥103.6°F)	
Respiration:				
Rate	Normal (<30bpm)		Tachypnea (>30bpm)	
Signs	MR	Coughing	Sneezing	Dyspnea
	OMB	OD	Conjunctivitis	
Joints:	Normal	Altered Ambulation†		Joint Effusion

Key: BAR= Bright, Alert, Responsive; QAR= Quiet, Alert, Responsive; DAR= Depressed, Alert, Responsive; bpm= breathes per minute; MR= Mucoid Rhinorrhea; OMB= Open Mouth Breathing; OD= Ocular Discharge

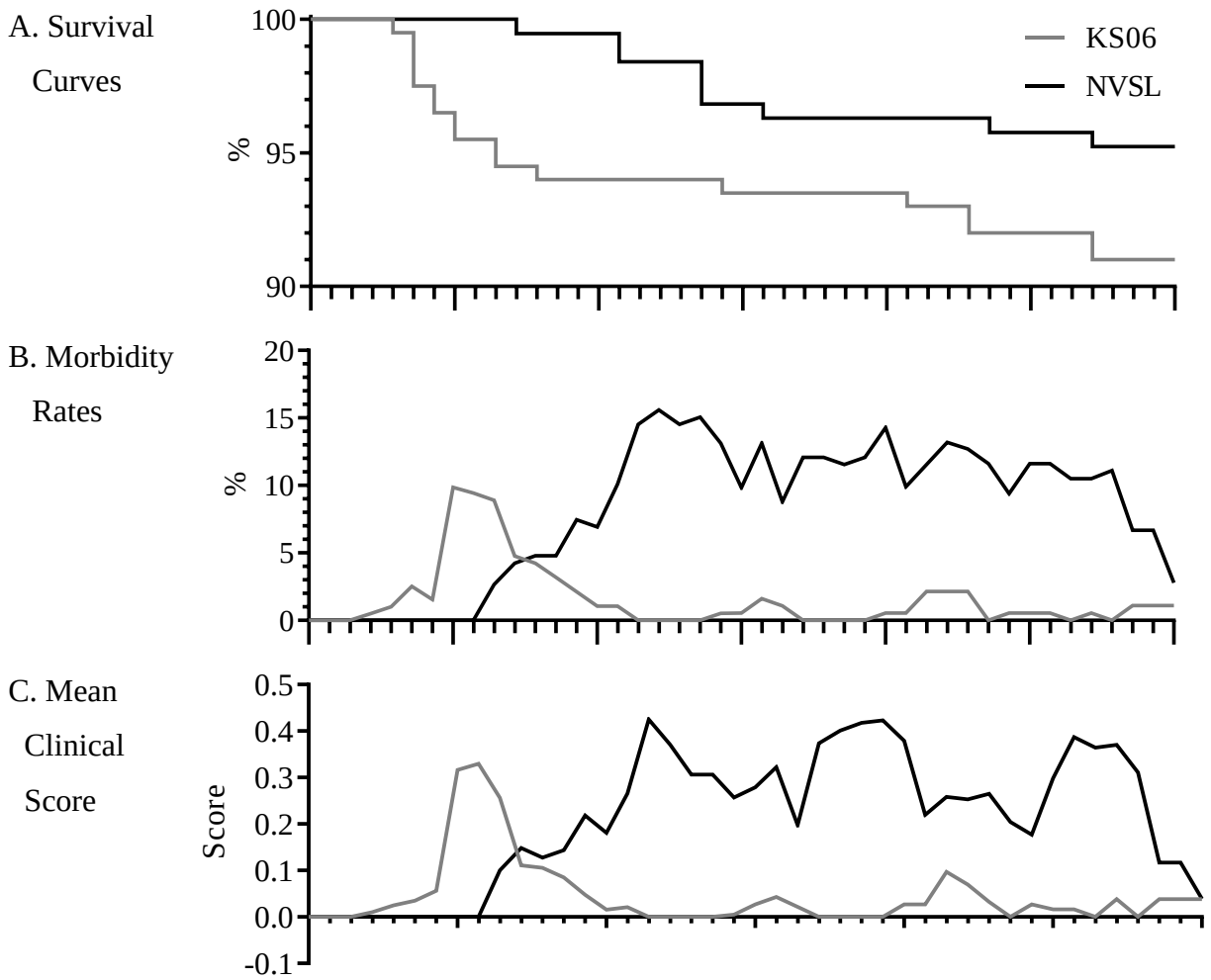
*All pigs described as QAR, DAR, or Moribund were classified as having altered mentation

† Pigs with stiffness, inability to ambulate, reluctance to ambulate, toe-touching lame, or non-weight bearing lame were characterized as having altered ambulation

Table 2.2. Frequency of clinical event documented in nursery pigs infected with one of two isolates of porcine reproductive and respiratory syndrome virus (NVSL or KS06)*

Clinical Event	Total (*n* = 389)	NVSL (*n* = 189)	KS06 (*n* = 200)	p-value
Mortality	27 (7)	9 (4.8)	18 (9.0)	0.09
Morbidity	121 (31.1)	74 (39.2)	47 (23.5)	0.0010
Antibiotic Treatment	98 (25.2)	65 (34.4)	33 (16.5)	<0.0001
Respiratory	75 (19.3)	64 (33.9)	11 (5.5)	<0.0001
Mucoid Rhinorrhea	51 (13.1)	51 (27.0)	0 (0.0)	<0.0001
Dyspnea	23 (5.9)	20 (10.6)	3 (1.5)	0.0005
Conjunctivitis	15 (3.9)	15 (7.9)	0 (0)	<0.0001
Sneezing	16 (4.1)	16 (8.5)	0 (0)	<0.0001
Coughing	11 (2.8)	11 (5.8)	0 (0)	0.0003
Tachypnea	13 (3.3)	3 (1.6)	10 (5.0)	0.0886
Ocular Discharge	13 (3.3)	13 (6.9)	0 (0)	<0.0001
Open Mouth Breathing	4 (1.0)	2 (1.1)	2 (1.0)	>0.9999
Altered Ambulation	32 (8.2)	25 (13.2)	7 (3.5)	0.0007
Stiffness or Lameness	30 (7.7)	23 (12.1)	7 (3.5)	0.0019
Joint Effusion	21 (5.4)	16 (8.5)	5 (2.5)	0.0122
Decreased Body Condition	17 (4.4)	14 (7.4)	3 (1.5)	0.0053
Altered Mentation	63 (16.2)	47 (24.9)	16 (8.0)	<0.0001
Diarrhea	19 (4.9)	12 (6.3)	7 (3.5)	0.2413
Febrile	85 (21.9)	53 (28.0)	32 (16.0)	0.0047

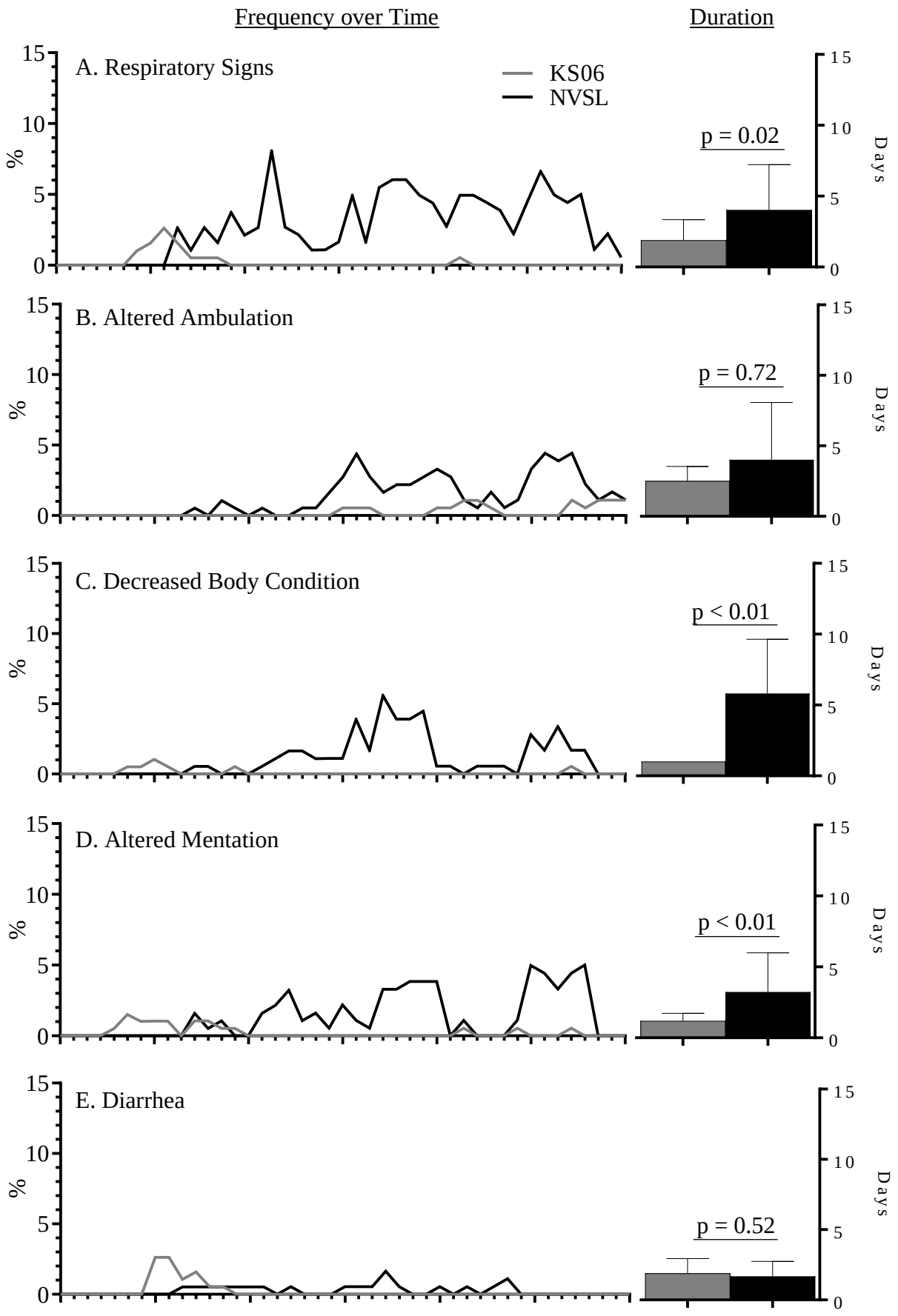
*Data is shown as the number (percentage) of pigs documented with each clinical event. Statistical differences between the two isolates were calculated using the Fisher's exact test.



Day Post Infection	0	7	14	21	28	35	42
No. KS06 Pigs	200	193	188	187	187	184	182
No. NVSL Pigs	189	189	188	183	182	181	180

Figure 2.1. Time course of clinical outcome in nursery pigs infected with KS06 or NVSL isolates of porcine reproductive and respiratory syndrome virus (PRRSV).

Number of pigs used in the analysis are provided below the graphs. A) Data shown as the percentage of pigs that survived during the course of PRRSV infection. Survival curves trended toward being significantly different ($p = 0.09$; Mantel-Cox test). B) Data shown as the percentage of pigs with clinical signs each day. C) Data shown as the mean number of clinical signs based on the number of pigs alive on a given day.


Frequency over Time
Duration
A. Respiratory Signs
KS06
NVSL
p = 0.02
B. Altered Ambulation
p = 0.72
C. Decreased Body Condition
p < 0.01
D. Altered Mentation
p < 0.01
E. Diarrhea
p = 0.52
%
Days
0
5
10
15

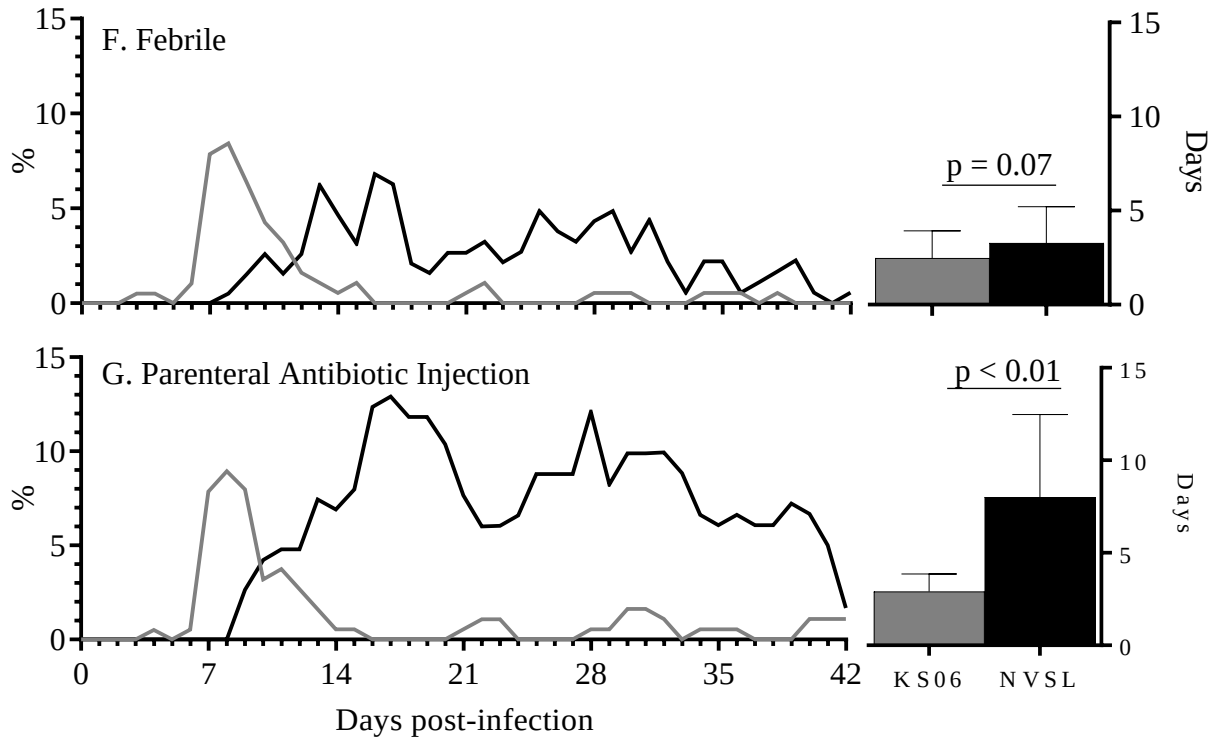
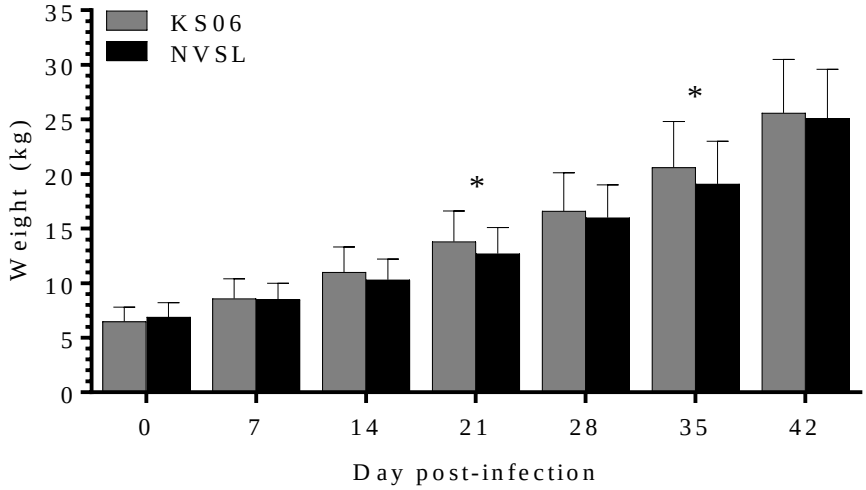


Figure 2.2. Frequency and duration of clinical events over time in nursery pigs infected with KS06 or NVSL isolates of porcine reproductive and respiratory syndrome virus (PRRSV).

Left column: percentage of pigs with clinical signs associated with PRRS. Data is shown as the percentage of pigs with. The right column shows clinical sign duration (data shown in mean days with SD).

A. Weight



B. Average Daily Gain

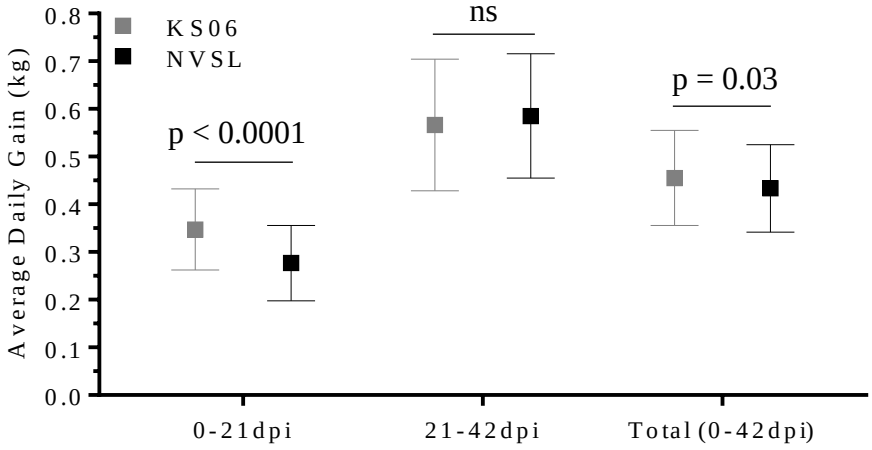
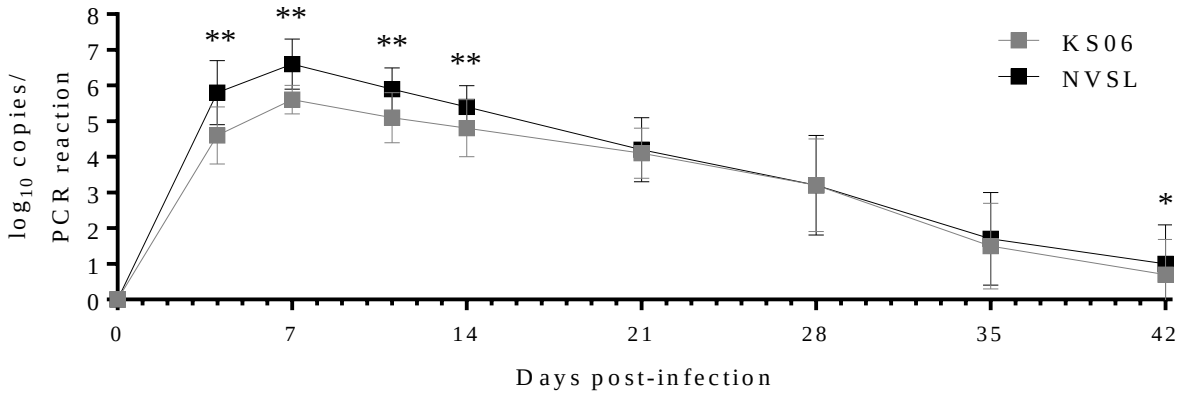


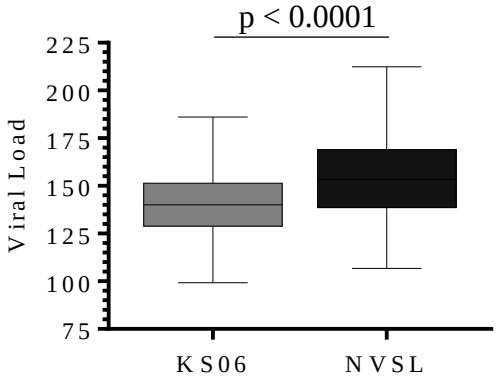
Figure 2.3. Weights over time and average daily gain in nursery pigs infected with KS06 or NVSL isolates of porcine reproductive and respiratory syndrome virus (PRRSV).

A) Data shown as means and standard deviations. Asterisks indicate statistically significant differences ($p < 0.05$ by two-way ANOVA with repeated measures). B) Data shown as means ± 1 standard deviation in kg. Statistically significant differences were determined by unpaired t-test.

A. PRRSV Viremia Time Course



B. PRRSV Viral Load



C. Duration of PRRSV Viremia

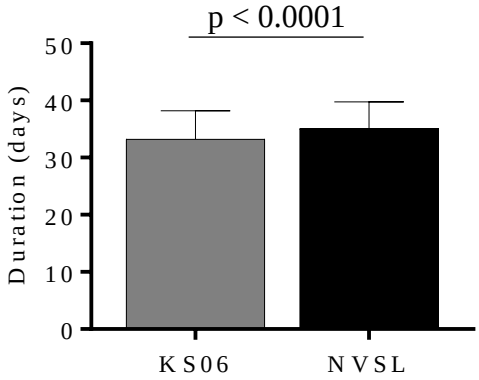


Figure 2.4. Virus detection in serum from nursery pigs infected with KSO6 or NVSL isolates of porcine reproductive and respiratory syndrome virus (PRRSV).

A) Data is shown as mean ± standard deviations (log_{10} copies/PCR reaction). Asterisks indicate statistically significant differences between groups (**$p < 0.0001$, *$p < 0.05$; two-way ANOVA with multiple comparisons). B) Total viral load, estimated as area under the curve through Riemann sums of viremia (log_{10} copies/PCR reaction), is shown as box and whiskers plots with the box indicating the quartiles and the whiskers indicating the range of values. C) PRRSV viremia duration shown as mean plus standard deviation (days).

Chapter 3 - Fecal Microbiota Transplantation Is Associated With Reduced Morbidity and Mortality in Porcine Circovirus Associated Disease

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Abstract

Porcine circovirus associated disease (PCVAD) is a term used to describe the multi-factorial disease syndromes caused by porcine circovirus type 2 (PCV-2), which can be reproduced in an experimental setting through the co-infection of pigs with PCV-2 and porcine reproductive and respiratory syndrome virus (PRRSV). The resulting PCVAD-affected pigs represent a subpopulation within the co-infected group. In co-infection studies, the presence of increased microbiome diversity is linked to a reduction in clinical signs. In this study, fecal microbiota transplantation (FMT) was investigated as a means to prevent PCVAD in pigs co-infected with PRRSV and PCV-2d. The sources of the FMT material were high-parity sows with a documented history of high health status and robust litter characteristics. The analysis of the donated FMT material showed the absence of common pathogens along with the presence of diverse microbial phyla and families. One group of pigs (n = 10) was administered the FMT while a control group (n = 10) was administered a sterile mock-transplant. Over the 42-day post-infection period, the FMT group showed fewer PCVAD-affected pigs, as evidenced by a significant reduction in morbidity and mortality in transplanted pigs, along with increased

antibody levels. Overall, this study provides evidence that FMT decreases the severity of clinical signs following co-infection with PRRSV and PCV-2 by reducing the prevalence of PCVAD.

Introduction

Fecal microbiota transplantation (FMT) is the process by which fecal microorganisms are donated by a healthy individual and subsequently transplanted into a diseased individual. The actual process of fecal transplantation has been described for centuries, being reported as early as the 4th century in China for the treatment of diarrhea in humans[407] and in the 17th century in Italy as transfaunation for the treatment of diseases in ruminants.[368] The recent surge in FMT usage and application is evidenced by a search in PubMed, where publication results for either "fecal microbiota transplantation" or "fecal microbiota transplant" comprise approximately 1,200 publications, with almost all being published within the last decade.[408,409] For several human disease states, FMT has been shown to improve treatment outcome or resolve complex disease conditions. Although recurrent *Clostridium difficile* infections are by far the most frequent use for the application of FMT, other FMT-treatable digestive diseases include inflammatory bowel disease, ulcerative colitis, and metabolic syndrome.[373-375] The mechanism by which FMT is effective for the treatment of digestive diseases is likely associated with the restoration of normal flora. FMT is also associated with the improvement of non-digestive diseases, such as neurologic and respiratory disorders. For these conditions, the mechanism for the beneficial effect is likely more complex. For example, FMT restored the production of cytokines, including TNF-α and IL-10, in antibiotic-treated mice after respiratory infection with *Streptococcus pneumoniae*.[377]

A different and less-explored role for FMT is in the prevention of disease. Traditionally, antibiotics and other growth promoters have been used in food animal production as

prophylactics. Part of the benefit is likely derived from the maintenance of a microbiome that optimizes growth, either through individual species and/or metabolites, along with the prevention of common bacterial infections. As beneficial microbial populations are further characterized for their role in both growth and immunity, FMT or other microbiome therapeutics may provide an alternative to antibiotics and growth promoters in food animal production.

Porcine reproductive and respiratory syndrome virus (PRRSV) is the most costly disease of swine worldwide, with estimated annual losses to the U.S. industry at $664 million, primarily due to respiratory disease and reduced weight gain in growing pigs.[410] Porcine circovirus type 2 (PCV-2) is also a significant and widely distributed pathogen of swine. PCV-2 is a causative factor associated with a group of disease syndromes termed porcine circovirus associated disease or PCVAD, characterized by muscle wasting, respiratory disease, jaundice or pallor, and reduced weight gain in growing pigs.[92] Models for PCVAD include co-infection with PRRSV and PCV-2, both of which cause systemic infections primarily targeting pulmonary and lymphoid tissues.[116-118]

Previous work utilizing a PRRSV/PCV-2 co-infection model has shown a consistent association between increased fecal microbiota diversity and improved outcome in nursery pigs.[118,291] Specifically, both pre and post-infection fecal microbiome diversity was associated with several improved outcomes after co-infection, including reduced clinical disease severity, reduced virus replication, decreased lung lesions, and improved weight gain. Increased microbiome diversity, as measured by a pan-microbial microarray, was correlated with a reduction in PCVAD clinical signs as well as improved growth in subclinically-affected pigs.

The hypothesis tested in the current study was that prophylactic administration of FMT to 3-week old weaned barrows prior to co-infection with PRRSV and PCV-2 would reduce clinical

signs and pathology associated with PCVAD. The sources of the FMT material were high-parity sows with life-long histories of high-health status and efficient production characteristics, such as the absence of frequent antimicrobial treatment, low pre-weaning mortality rates, large healthy litters, and a lack of clinical disease. The results showed that FMT reduces the number of pigs affected by PCVAD, including a reduction in virus load and increased viral antigen-specific antibodies. Therefore, FMT provides a potential therapeutic for the prevention of disease.

Materials and methods

Animals and housing. The use of animals and viruses in research was performed 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. Ten pairs of barrow siblings (n = 20; 24 days of age upon arrival) were obtained at weaning from a single high health commercial source negative for PRRSV, *Mycoplasma hyopneumoniae* and porcine epidemic diarrhea virus (PEDV). Sibling pairs were from 10 different sows and the piglets were not vaccinated for PCV-2. No prophylactic or therapeutic antibiotics were administered at weaning or within 1 week of arriving at Kansas State. All pigs were housed in two identical environmentally controlled rooms at the Kansas State University Large Animal Research Center and maintained under biosafety level 2 (BSL-2) conditions. Each sibling pair was divided into either the control or fecal microbiota transplant (FMT) group; the two groups were balanced according to arrival weight. Pigs were housed in groups of 10 in a 9.1 sq m pen with raised slatted flooring. All pigs were given approximately 24 hours to acclimate to their new environment prior to FMT or mock-transplant treatment. Pigs were given access to food and water *ad libitum*.

Viruses. The PRRS virus (isolate KS62; GenBank accession no. KM035798) used to prepare the inoculum for this study originated from the lymph node of a pig with porcine circovirus associated disease (PCVAD) due to co-infection with PRRSV and PCV-2.[117] PRRSV was isolated by propagation on MARC-145 cells and titrated as previously described.[116]

The PCV-2d virus was a field-derived isolate. Serum containing the field isolate was heat-treated to remove heat-labile pathogens and was subsequently used to infect cesarean-derived, colostrum-deprived (CD/CD) pigs. Lung, spleen and liver samples were collected from the CD/CD pigs at 21 days post-infection (dpi) and tested by qPCR for PCV-2d. Quantification cycle (Cq) values of the tissues used to create the inoculum for the current study were 14.9, 14.2 and 14.2 for liver, lung and spleen, respectively, from a single CD/CD pig. A 10% tissue homogenate was created from the described tissues in Eagle's minimum essential medium (EMEM; Sigma-Aldrich) supplemented with 50 µg/mL gentamicin. Following centrifugation at 100xg for 15 min at 4°C, the supernatant was heat-treated at 55°C for 45 min to inactivate heat-labile pathogens. Analysis of the supernatant using the Lawrence Livermore Microbial Detection Array (LLMDA) confirmed the inoculum was negative for other common pathogens, such as porcine parvovirus, PRRSV, swine influenza virus, and *Mycoplasma hyopneumoniae*, but remained positive for porcine endogenous retroviruses (data not shown), which are ubiquitous in swine. PCV-2d infectivity was titrated on swine testicle (ST) cells. Briefly, serial 10-fold dilutions of PCV-2d challenge stock were added 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 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 a

polyclonal anti-PCV-2b primary antibody and a fluorescein (FITC) AffiniPure Goat Anti-Swine IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). Infected cells were visualized using an inverted fluorescent microscope and the 50% tissue culture infective dose ($TCID_{50}$/mL) was calculated using the method of Spearman and Karber.[411]

To prepare the inocula for pigs, the stock viruses were mixed to yield a 2-mL dose consisting of 10^4 $TCID_{50}$ PCV-2d 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.

Fecal microbiota transplant. Two sows from a commercial farrow-to-wean farm in Kansas were selected as donors for the transplant material. This herd was negative for PRRSV and had recently undergone a *Mycoplasma hyopneumoniae* elimination program. The two sows were selected based on several characteristics, including older age (average age 4.8 years), high parity (9 and 12 litters born prior to donation), large litters with a high percentage of born alive piglets (15.1 ± 2.0 total born; 95% born alive), low pre-weaning mortality, no history of fetal mummification, and no antibiotic treatment received within at least the last 15 months prior to donation. Pre-weaning mortality in these two sows was primarily attributed to crushing injuries. Lifetime number of weaned pigs was 101 and 131 for each sow, respectively. For this study, feces were collected during lactation and sows had not yet weaned their respective litters at the time. Feces were initially screened and confirmed as negative for gastrointestinal parasites using a fecal float qualitative exam by the Kansas State Veterinary Diagnostic Laboratory (KSVDL).

To prepare the FMT, fresh feces was collected naturally during defecation or manually from the rectum of the two sows. Feces were collected on a single time and day, mixed, and processed within approximately 3 hours after collection, during which the fecal microbiota was concentrated and stored using a protocol adapted from the human FMT literature.[412] Specifically,

feces were weighed into 50 gram aliquots and mixed in a standard commercial blender (Oster, Sunbeam Products Inc.) with 250 mL of sterile saline (0.9% sodium chloride irrigation USP, Braun Medical) until homogenized. The fecal slurry was then passed progressively through 2.0, 1.0, 0.5, and 0.25 mm stainless steel sieves into a sieve receiver (Fisherbrand™). The filtered liquid was collected, aliquoted into 50 ml tubes, and centrifuged at 6,000xg for 15 minutes. The supernatant was discarded and each bacterial pellet was resuspended in approximately 20 mL of sterile saline. All resuspensions were gently vortexed prior to mixing the concentrated microbiota in a large beaker. Glycerol (molecular biology reagent grade, MP Biomedicals™) was added to create a 10% glycerol suspension and the transplant material was stored at -80°C until the day of transplantation. On the day of transplantation, the FMT material was thawed for 2 hours on ice and kept cold prior to administration.

The FMT material was submitted to KSVDL for routine bacterial culture, including aerobic culture, anaerobic culture and *Salmonella* enrichment. Species identification was attempted for all bacteria cultured. The FMT material was also fully characterized on the Lawrence Livermore Microbial Detection Array.

Experimental design and sample collection. Approximately 24 hours after arriving at Kansas State University, pigs were administered a fecal microbiota transplant (FMT) or a mock transplant (CONTROL). Mock transplants were made of 10% glycerol in sterile saline. Transplants or mock-transplants were administered as 5 mL doses delivered once daily for seven consecutive days prior to co-infection. To administer the FMT or mock-transplant, 5 mL doses were delivered through flexible dispensing tips (6.4 mm Flexoject™ Dispensing Tips, Innovet). Solutions were delivered slowly on the tongue or in the cheek pouch, allowing the pig to chew on the tip and naturally consume the material over 30 seconds to 1 minute.

At 32 days of age, all 20 pigs were infected with PRRSV and PCV-2d. Body weights of individual pigs were collected upon arrival (-8) and on -7, 0, 7, 14, 21, 24, 28, 32, 35, and 42 dpi. Blood samples were collected from all pigs on -7, 0, 4, 7, 11, 14, 21, 28, 35, and 42 dpi. Fecal samples were collected from all 20 pigs on -7 and 0 dpi. In addition to these planned sample collection times, blood, feces and weights were collected on the day of death or euthanasia. Pigs were humanely euthanized under the direction of the attending laboratory animal veterinarian if 1) pigs had greater than or equal to 20% weight loss, 2) pigs were moribund or nonresponsive to veterinary treatment, or 3) pigs had severe dyspnea or clinical disease that compromised animal welfare. At 42 dpi, all remaining 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/PCV-2 co-infection, such as dyspnea, tachypnea, ocular discharge or conjunctivitis, coughing or sneezing, nasal discharge, aural cyanosis, open mouth breathing, decreased body condition, muscle wasting, rough hair coat, lethargy, depression, joint effusion, lameness, diarrhea, 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 the attending veterinarian, appropriate treatments were administered to pigs with moderate to severe clinical disease. Examples of clinical presentations where treatment was administered included 1) dyspnea and/or tachypnea, 2) mucoid rhinorrhea, 3) conjunctivitis with swelling, 4) pallor or jaundice with muscle wasting, and 5) lethargy or depression with pyrexia. Clinically affected pigs were prescribed parenteral antibiotics, such as ceftiofur hydrochloride or oxytetracycline. Any pig with overt clinical disease and a rectal temperature of ≥104°F was administered

parenteral flunixin meglumine, a nonsteroidal anti-inflammatory drug. Other supportive care, such as oral or subcutaneous fluids, were administered for significantly dehydrated pigs under the direction of the attending veterinarian. Pigs with evidence of conjunctivitis were treated with triple antibiotic ophthalmic ointment (bacitracin-neomycin-polymyxin, Vetropolycin, Dechra). Clinical signs and treatments unrelated to PRRSV or PCVAD (e.g., lacerations, dermatitis, congenital hernias, etc.) were documented and monitored but were not included in the data analysis related to clinical outcome. Treatment days were numerated for individual pigs over time. Treatment days were counted as each day a pig was prescribed a parenteral therapeutic. Mortality rate was calculated from those pigs that died or were euthanized prior to the 42-day termination of the study. Morbidity rate was calculated as the number of pigs demonstrating clinical signs deemed by the attending veterinarian to require veterinary intervention and prescription of parenteral therapy.

At 42 dpi, all surviving pigs (n = 11) were humanely euthanized with intravenous pentobarbital sodium. A board certified veterinary pathologist, blinded to the source of the pigs, performed complete necropsies and histopathology. First, whole body weights were collected post-mortem. Second, lungs and trachea were removed *in toto* immediately after euthanasia and total lung weights were measured. The trachea was excised immediately distal to the larynx. Lung weight to body weight ratio was calculated as a measure of pulmonary pathology. Dorsal and ventral aspects of the whole lung were photographed (Canon EOS Rebel T6 DSLR) and digital images were evaluated after gross necropsy using a photo scoring system. Gross anatomical photo scores were reported as the percentage of whole lung affected by pneumonia (ranging from 0 to 100%). Scores were combined from 5 sections of the lung as previously

described.[413] The photos were evaluated by a board certified veterinary pathologist who was blinded to the source of the lung pictures.

Tissues collected for histopathology included lung (1 section from each lobe) and tracheobronchial lymph node. Additional tissues were collected at the pathologist's discretion by evidence of gross lesions. 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.[116,118] Degree of lymphoid depletion was scored using a 0-3 system as previously described.[118]

PCV-2 immunohistochemical staining. PCV-2 antigen staining in paraffin-embedded tissue thin sections was performed by personnel in the Kansas State Veterinary Diagnostic Laboratory. Briefly, deparaffinized slide-mounted thin sections were first treated with proteinase K (1.2 mg/ml diluted in Bond Enzyme Diluent with 0.35% ProClin 950) for 10 minutes at room temperature (Bond Enzyme Pretreatment Kit, Leica Biosystems). Rabbit anti-PCV-2 antibody (Iowa State University) was diluted at 1:500 in PowerVision IHC/ISH Super Blocking (Leica Biosystems) and applied to the tissue section for 15 minutes at room temperature. Bound antibody was detected by incubation with 25 μg/ml Poly-AP anti-rabbit IgG (Leica Biosystems) in antibody diluent for 25 minutes at room temperature. The complex was visualized using Fast Red chromogen (Bond Polymer Refine Red Detection Kit, Leica Biosystems) and counterstained with hematoxylin.

Measurement of PRRSV and PCV-2 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 25 μL reaction volume.

PCV-2d DNA was quantified using SsoAdvanced Universal SYBR green supermix (Bio-Rad) as previously described.[116,118] The PCR assay results were reported as $\log_{10}$ PCV-2 DNA starting quantity (copy number) per 20 μL reaction volume.

Microsphere immunoassay for detection of PRRSV and PCV-2 antibodies. PRRSV nucleocapsid protein and PCV-2b capsid protein fragments (43-233 and 160-233) were cloned into the pHUE expression vector, as previously described.[414] For protein expression, bacteria were grown in Luria-Bertani (LB) broth plus ampicillin (0.01 mg/ml) and incubated at 37ºC with shaking. Once the OD^{600} reached 0.4-0.6, protein expression was induced by adding 1 ml of 0.1M isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture and bacteria were harvested 4 hours later. Bacteria were pelleted by centrifugation at 4,000xg for 10 min. Soluble proteins were purified using the USB PrepEase Histidine-tagged Protein Purification Kit (Affymetrix) under non-denaturing conditions, according to the manufacturer's directions. Purity was assessed by SDS-PAGE and total protein measured using the Bio-Rad Protein Assay.

Proteins were coupled to carboxylated Luminex MagPlex® polystyrene microspheres according to the manufacturer's directions. For the assay, approximately 2500 antigen-coated beads, suspended in 50 μL PBS with 0.05% Tween-20 and 4% goat serum (PBST-GS), were placed in each well of a 96-well polystyrene round bottom plate (Costar). Sera were diluted

1:400 in PBST-GS and 50 µL was added to each well. The plate was sealed and incubated for 30 min at room temperature with gentle shaking. After the incubation, the plate was placed on a magnet and beads were washed three times with 190 µL of PBST-GS. For the detection of IgG, 50 µL of biotin-SP-conjugated affinity purified goat anti-swine secondary antibody (IgG, Jackson ImmunoResearch) was diluted to 2 µg/mL in PBST-GS and 100 µl was added to each well. The plate was incubated at room temperature for 30 min and washed three times followed by the addition of 50 µL of streptavidin-conjugated phycoerythrin (2 ug/ml in PBST-GS; SAPE). After 30 min, the plate was washed and microspheres resuspended in 100 µL of PBST-GS. Microspheres were analyzed using a MAGPIX instrument (Luminex) and Luminex® xPONENT 4.2 software. A minimum of 50 microspheres was used for the calculation of mean fluorescence intensity (MFI). The sample to positive (S/P) ratio was calculated as the MFI of sample minus MFI of negative control divided by MFI of standard positive control minus MFI of negative control.

Microarray analysis of FMT and fecal samples. The Lawrence Livermore Microbial Detection Array (LLMDA) was used to analyze microbiome composition and diversity of the transplant material and fecal samples. This array detects annotated sequences of microbes associated with infection of vertebrates within GenBank®, the National Institute of Health genetic sequence database. The version 7 of the LLMDA in the 4plex 180K probe format was used in this study. This version of the array targets 4,377 viruses, 5,457 bacteria, 327 archaea, 319 fungi, and 132 protozoa. The LLMDA oligonucleotide probes vary between 50 and 65 nucleotides in length and have roughly equivalent affinities for their complementary target DNA molecules [415]. Probes 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.[118,291,416-418]

The PowerViral™ Environmental RNA/DNA Isolation Kit (MO BIO, San Diego, CA) was used to extract DNA and RNA from the fecal samples. For each sample, approximately 250 mg of feces was added to 600 µl of PV1/β-mercaptoethanol in a glass beat tube included in the kit. Samples were homogenized and lysed by vortexing tubes for 10 minutes at maximum speed. Samples were further processed using the PowerViral™ Kit protocol. All samples were eluted into 100 µl of RNase-Free water. The purified nucleic acids were quantified using the Thermo Scientific™ Nanodrop™ spectrophotometer. For each sample, 10 µl of the extracted DNA and RNA was amplified using the random amplification procedure as previously described [418]. The amplified cDNA and DNA was purified with the Qiaquick PCR purification columns (Qiagen) and quantified using the Nanodrop™ spectrophotometer.

Approximately 1 µg of amplified cDNA and DNA were fluorescently labeled using a one-coloring labeling kit (Roche NimbleGen, Madison, WI). Briefly, the samples were labeled using nick translation with Cy3-labeled random nonamer primers (TriLink Biotechnologies, San Diego, CA) and Klenow DNA polymerase at 37°C for 2 hr. The labeled DNA was precipitated in isopropanol, centrifuged for 10 min, and the pellet was washed and dried. The pellet was then reconstituted in 50 µl of RNase-Free water and quantified using the Nanodrop™ spectrophotometer.

The Agilent Technologies Oligo aCGH/ChIP-on-Chip Hybridization kit (Santa Clara, CA) was used to hybridize samples to the arrays. For each sample, 10 µg of fluorescently labeled DNA was mixed with blocking agent, hybridization buffer and nuclease free water. The samples

were then denatured at 95˚C for 3 min, and incubated at 65˚C for 3 min. Each sample was then immediately loaded onto the array and hybridized for approximately 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 for 5 min at room temperature and Oligo aCGH/ChIP-on-chip Wash Buffer 2 for 1 min at 37°C (Agilent Technologies Inc., Santa Clara, CA). Using the SureScan Microarray Scanner (Agilent Technologies Inc., Santa Clara, CA), all arrays were scanned to a resolution of 3 µm.

Microarray data was generated from the microbe sequences using the CLiMax method developed at Lawrence Livermore National Laboratory,[416] at a detection threshold of ≥ 99%. 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.

Diversity of the fecal samples was measured by calculating the number of families and species detected in each sample. The mean number of families and species in the control and transplant groups as well as the affected and unaffected groups were compared prior to (-7 dpi) and after (0 dpi) FMT. Microbiome composition was compared between these groups at the level of phylum, family and species.

16S rDNA analysis of fecal samples. Pig fecal samples were collected in cryovials and stored at -80°C until shipment to the University of Nebraska-Lincoln for DNA extraction and bacterial community analysis. DNA was extracted using the manufacturer's protocol for Mag-Bind® Soil DNA 96 Kit (Omega Bio-tek, Inc.) with the following modifications: precipitation of nucleic acids was done by using sodium acetate, isopropanol and ethyl alcohol. 0.1X volumes of

10mM sodium acetate was added to each sample tube, which were vortexed and later incubated on ice for 5 min. Subsequently, 1ml of ice-cold isopropanol was added and samples were incubated at -80°C overnight to precipitate the DNA. The following day, samples were centrifuged at 4°C for 15 min at 16,000xg. The supernatants of the resulting samples were discarded and the nucleic acid pellet was washed with 0.5ml of ice-cold 70% ethyl alcohol. The samples were centrifuged for 2 min at 13,000xg, the residual supernatant was discarded, and the nucleic acid pellet was air dried for 3min. The nucleic acid pellet was dissolved in a 0.45ml of Tris (10mM, pH 8) and incubated for 1 hr at 4°C. For further purification of dissolved nucleic acids, the KingFisher (ThermoFisher Scientific) robot was used with reagents from the Mag-Bind® Soil DNA 96 Kit. The resulting DNA was used for the tag-sequencing of the V4 region of 16S rDNA using the universal bacterial primers described previously [419]. A 20 μL PCR reaction contained 1X Terra™ PCR Direct Polymerase Mix, 0.5 μL Terra polymerase, 20 mM of each primer, and 20-50 ng of DNA. The cycling conditions for PCR were the same as previously described [420]. The PCR product size was confirmed by agarose gel electrophoresis. Normalization of the amplified PCR products were performed with Just-a-Plate™ 96 PCR Purification & Normalization kit (Charm Biotech, CA, USA) according to the manufacturer's protocol. Following normalization, 10 μL from each sample were pooled and concentrated using Nucleospin® Gel & PCR Cleanup kit (MACHEREY-NAGEL Gmbh & Co. KG, Duren, Germany) and was eluted using 20 μL of elution buffer. This pooled and purified sample was analyzed in a Agilent 2100 bioanalyzer (Agilent Scientific Instruments, CA, USA) using Agilent High Sensitivity DNA Kit (Agilent Technologies, Inc. Waldbronn, Germany) to ensure the quality and quantity of the targeted V4 region of 16S rDNA. The concentration of the DNA library was determined using the DeNovix QFX Fluorometer (DeNovix Inc. DE, USA) and

using DeNovix dsDNA Fluorescence Quantification Assay (DeNovix Inc. DE, USA). The resulting 16S rDNA libraries were sequenced using the Illumina MiSeq platform utilizing the 2 X 250 paired end sequencing strategy using a MiSeq Reagent Kit V3 (Illumina Inc. CA, USA).

Data processing was performed on a custom pipeline utilizing several publicly available software tools. The paired-end reads were assembled into contigs after quality filtering using MOTHUR v.1.38.1.[421] Operational taxonomic units (OTUs) were generated from the quality filtered sequences using the UPARSE pipeline (USEARCH v7.0.1090) [422] at a threshold of 97% identity. Chimeric sequences were removed using the ChimeraSlayer gold.fa as the reference database using UCHIME.[423] OTUs were aligned against the v128 (SILVA) database and mismatched sequences were discarded. A phylogenetic tree was generated using high quality aligned sequences within MOTHUR v.1.38.1 using the Clearcut algorithm.[424] Taxonomies to the identified OTUs were assigned using QIIME v.1.9.1 pipeline[425] with the Greengenes reference database (gg_13_5_otus). OTUs representing Archaea and Cyanobacteria were removed as Cyanobacterial reads may be a result of contamination of plant chloroplast[426] and the archaea sequences may be biased as the primers used are not designed to universally amplify all archaea. Alpha diversity matrices (Chao1 and Observed OTUs) were calculated using the QIIME v.1.9.1 pipeline. The rarefaction of the OTU table was performed using QIIME v.1.9.1[425] with the lowest number of reads. For the experiment, 27035 was used as the lowest depth. The difference in bacterial communities (beta-diversity) among transplanted and control pigs was determined using the QIIME v.1.9.1 pipeline using distance matrices (weighted UniFrac, unweighted UniFrac and Bray Curtis) from the rarefied-OTU table.

Statistical analysis. For 16s rDNA sequencing, a three-way ANOVA (considering the effect of Treatment, Day and Animal) was performed on the Chao1 and Observed OTUs to

estimate bacterial richness among the transplanted and control pigs with open source statistical software R [427]. The overall bacterial community differences among treatments were determined by applying the permutational multivariate analysis of variance PERMANOVA on the weighted UniFrac distance matrix. Principal coordinate analysis (PCoA) was used on all distance matrices to generate plots which displayed global treatment effects. The PERMANOVA analysis was performed using R[428] (adonis function vegan package)[429] in which treatment was considered as a fixed effect and animal (pig) as a random effect with the pig as the experimental unit. A core microbiome was determined for each treatment group by only selecting the OTUs present in 80% of the animals in each group (8/10). The core OTUs were used to identify differential OTUs between treatments using the linear discriminant analysis (LDA) effect size with LefSe [430]. LefSe analysis was performed using default settings and differential OTUs from all pairwise comparisons to generate heatmaps in R.[428]

All remaining statistical analyses were performed using GraphPad Prism 7.01 software (La Jolla, CA). Mean viremia, antibody levels, and weight measurements were compared between groups using repeated measures analysis with multiple unpaired t-tests. Survival curves were compared using the Mantel-Cox test and daily morbidity rates were compared using the Fisher's exact test. Microscopic lung and lymph node lesion scores were compared between groups using the Mann-Whitney U test. Gross photo scores and lung weight to body weight ratios were compared using the unpaired t-test. Microbiome diversity and number of species within family were compared between groups using the Mann-Whitney U test. Proportions of each group with individual species and families detected were compared using Fisher's exact test.

Results

Characterization of fecal transplant material. Several methods, including aerobic culture, anaerobic culture, microarray, and fecal float, were used to characterize the fecal microbiota transplant material. Fecal floatation for parasites confirmed feces were negative for parasites, including *Ascaris suum,* through standard diagnostic testing at KSVDL. Aerobic and anaerobic culture identified several culturable bacteria known to inhabit the gastrointestinal tract, including non-hemolytic *Escherichia coli*, *Bacillus altitudinis*, *Streptococcus alactolyticus*, *Enterococcus hirae*, non-hemolytic *Staphylococcus* sp., *Bacteroides vulgatus*, and *Clostridium perfringens*. Several additional anaerobic bacteria were cultured but were unable to be identified at the genus or species level; these bacteria included gram negative coccobacilli, gram positive long rods, and large gram positive boxy rods. *Salmonella* enrichment culture was negative. The pan-microbial array detected the most diversity and absolute number of organisms, with 12 phyla, 33 microbial families and 49 microbial species detected (**Table 3.1**). Microbes were very diverse and from the phyla Actinobacteria, Amoebozoa, Bacteroidetes, Basidiomycota, Euryarchaeota, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes, and Tenericutes. Additionally, a single virus was detected in the family *Circoviridae*. The majority of species detected fell within the Proteobacteria phylum (16/49; 32.7%) with the second highest number of species falling with the Firmicutes phylum (9/49; 18.4%) and the third highest number of species falling within the Tenericutes phylum (6/49; 12.2%). Using the above methods, known swine pathogens were not detected.

FMT had no effect on pigs prior to co-infection. Upon arrival to Kansas State University, mean weight of the control group was 7.05 ± 1.46 kg and mean weight of the FMT group was 7.07 ± 1.39 kg ($p = 0.99$, unpaired t-test using repeated measures; **Table 3.2**). No

significant difference in weight gain was noted during the transplantation or mock-transplantation time period, suggesting no detrimental effect of FMT on weight gain in unchallenged conditions; mean weights for control pigs and FMT pigs on 0 dpi were 7.6 ± 1.7 kg and 7.3 ± 1.4 kg, respectively ($p = 0.85$, unpaired t-test using repeated measures). FMT and control pigs appeared clinically within normal limits.

FMT reduced the number of PCVAD-affected pigs. Morbidity and mortality of the FMT and control groups are shown in **Figure 3.**1. Morbidity rates of the control and FMT groups were comparable in the first 22 days after co-infection (**Figure 3.1A**). During this time, 4 control pigs and 3 FMT pigs showed clinical signs, including dyspnea, open-mouth breathing, coughing, tachypnea, mucoid rhinorrhea, conjunctivitis, reduced body condition, lethargy/weakness and pyrexia. Starting on 23 dpi, the morbidity rates of the control and FMT groups diverged, with 5 control pigs (5/8; 62.5%) and only 1 FMT pig (1/8; 12.5%) exhibiting clinical signs sufficient to require veterinary intervention, including depression, dyspnea, tachypnea, coughing, open-mouth breathing, rough hair coat, mucoid oculonasal discharge, pyrexia, emesis and diarrhea, muscle wasting and loss of condition, ataxia, hypoxia and cyanosis. On days 25 and 26 after co-infection, morbidity rates were significantly different ($p = 0.04$; Fisher's exact test), with 75% and 12.5% of control and FMT pigs receiving treatment, respectively. A trend towards significantly higher morbidity was also seen on 27 dpi ($p = 0.09$; Fisher's exact test) and 28 dpi ($p = 0.05$; Fisher's exact test). By 28 dpi, clinical disease had completely resolved in the remaining 8 FMT pigs while 50% of control pigs (3/6) remained on treatment. Clinical disease in affected FMT and control pigs ($n = 9$) was consistent with PCVAD. Unaffected pigs ($n = 11$) had mild to a complete lack of clinical signs. With regards to resulting mortality, initial death rates were similar between the two groups, with 20% mortality

at 21 dpi. However, by the end of the study, the mortality in the control group was 70% compared to 20% for the FMT pigs (**Figure 3.1B**). Mortality between 19 and 30 dpi was due to pigs that died or were euthanized due to severity of clinical disease. Overall, the mortality rate of the control group (7/10, 70%) was significantly higher than that of the FMT group (2/10, 20%; $p = 0.0447$, Mantel-Cox test). When taken together, pigs which received the FMT treatment showed a reduction in PCVAD.

The clinically affected pigs showed a decrease in weight gain beginning at approximately 17 dpi (**Figure 3.1C**). Overall, no significant differences were detected between the two groups in regards to absolute weights during the biweekly or weekly weight measurements throughout the study ($p > 0.05$, repeated measures analysis with multiple t-tests). The one exception was the final weights on 42 dpi, where the 3 remaining control pigs had a mean weight significantly higher than that of the 8 remaining FMT pigs ($p = 0.04$, repeated measures analysis). During peak clinical disease between 17 and 30 dpi, 7 control pigs lost body weight whereas only 2 FMT pigs lost body weight ($p = 0.07$, Fisher's exact test). Control pigs were 9.3 times more likely to lose weight during this period (95% CI: 1 to 56.5). Overall, FMT reduced the number of pigs which lost weight associated with clinical PCVAD.

Representative gross and microscopic lesions seen in pigs with PCVAD are shown in **Figure 3.2**. Images of minimally-affected pigs are included for comparison. Examples of gross lesions included interstitial pneumonia with consolidation and hemorrhage, splenic infarcts, mucohemorrhagic rhinitis, lymphadenopathy with congestion and edema, pericardial effusion, mucohemorrhagic exudate in trachea, serous atrophy of fat, enteritis and intestinal ulceration, infarction of extremities, and tonsillar congestion. On gross examination of lung tissue, pneumonia was overall more severe in the control group, with 70% of the control pigs having

severe interstitial pneumonia with marked edema, coupled with enlarged lymph nodes, characteristic of PCVAD. In addition, a few of the affected pigs in the control group had bronchopneumonia and fibrinous pleuritis. Interestingly, these pigs also had severe suppurative rhinitis. Rhinitis, bronchopneumonia and pleuritis are suggestive of a secondary bacterial infection, likely due to immunosuppression associated with both PRRSV and PCV-2 infections. In contrast, only 30% of the FMT group had severe interstitial pneumonia and enlargement of the lymph nodes on gross examination, characteristic of PCVAD.

Gross lung tissue images were captured during necropsy and subsequently scored for severity of lesions (**Figures 3.2E, 3.2F and 3.3A**). Control pigs had a range of 30.5 to 99.0% of lung affected, with a mean of 82.4 ± 20.3%. FMT pigs had a range of 10.0 to 99.0% of lung affected, with a mean of 53.6 ± 40.6%. These differences had a trend towards significance ($p = 0.06$, unpaired t-test). A lung weight to body weight ratio was calculated for each pig and are depicted in **Figure 3.3B**. Control pigs had significantly higher ratios when compared to FMT pigs ($p = 0.04$, unpaired t-test), indicative of increased cellular infiltrate and edema characteristic of interstitial pneumonia.

Lesions were also assessed through histopathology. Microscopic lesions in the lungs included lymphoplasmacytic and histiocytic interstitial pneumonia, suppurative bronchopneumonia, and interlobular septal edema and hemorrhage. Lymphoid depletion with histiocytic replacement was seen in the tracheobronchial lymph nodes. Lymphoid depletion was scored in all 20 pigs, with 7/10 control and 3/10 FMT pigs having severe lymphoid depletion (**Figures 3.2A and 3.3C**). Although lymphoid depletion scores were generally higher in control pigs, this difference was not statistically significant ($p = 0.155$, Mann-Whitney U test). Severe lymphoid depletion was associated with large amounts of PCV-2 antigen (**Figure 3.2A**). Degree

of interstitial pneumonia was also scored in all 20 pigs, with 6/10 control and 2/10 FMT pigs having severe diffuse interstitial pneumonia with >75% lung lobe involvement (**Figures 3.2C and 3.3D**). Control pigs again tended to have higher overall severity of microscopic lung lesions; however, this difference was not statistically significant ($p = 0.164$, Mann-Whitney U test).

Taken together, the control pigs trended towards having more severe gross and microscopic lesions associated with PCVAD when compared to pigs that received the transplant material, indicating that FMT provided partial protection from both respiratory and lymphoid disease. This difference was seen due to an increased number of PCVAD-affected pigs in the control group.

FMT reduced PRRSV and PCV-2 virus replication and increased antibody production. PRRSV and PCV-2 viremia curves are shown for both individual pigs as well as group means (**Figure 3.4**). PRRSV viremia followed the typical time course, peaking at 7 dpi prior to a gradual decline over the next 5 weeks. Interestingly, most of the PCVAD-affected pigs had PRRS viremia rebound, a phenomenon initially described in 2010 [431] and later by our group [432]. For example, one control pig had peak PRRSV replication at 7 dpi (5.8 $\log_{10}$ copy number/25 µL reaction volume), a gradual decline of PRRSV replication until a low at 21 dpi (2.9 $\log_{10}$ copy number/25 µL reaction volume), and a second peak of PRRSV replication at 30 dpi (5.9 $\log_{10}$ copy number/25 µL reaction volume). When comparing mean PRRSV replication between groups, the only significant difference was seen at 28 dpi where the control group had significantly higher viremia; a mean of 3.9 and 3.0 $\log_{10}$ copy number/25 µL reaction volume was seen for control and FMT groups, respectively ($p = 0.02$, repeated measures analysis).

PCV-2 viremia also followed the typical time course, peaking later in the co-infection period between 14 and 21 dpi, followed by a plateau through the conclusion of the study.

Interestingly, all nine pigs that died had significantly higher levels of PCV-2 replication when compared to pigs that survived the course of the study (**Figures 3.4D, 3.4E and 3.4H**). Specifically, surviving pigs maintained < 7 $\log_{10}$ copy number/20 µL reaction volume in the serum at all measurements whereas pigs that died had > 7 $\log_{10}$ copy number/20 µL reaction volume detected during the study. When comparing the two groups' mean PCV-2 replication, significant differences were seen on 7 and 21 dpi, where the FMT group had a more rapid increase in PCV-2 replication on 7 dpi and a more rapid decline in PCV-2 replication on 21 dpi ($p = 0.02$ and 0.03, respectively; repeated measures analysis). Overall, PCVAD-affected pigs had high levels of PCV-2 and PRRSV in serum at the time of death or euthanasia, confirming the role of viral load in the course of clinical disease (**Figure 3.4G and 3.4**H). In general, virus replication during peak clinical disease was reduced in the FMT group, demonstrating a protective effect of FMT on viral load.

Antibodies were measured against PRRSV N protein, PCV-2 whole capsid protein (CP 43-233), and PCV-2 decoy epitope (CP 160-233). PRRSV antibodies were detected similarly in both groups initially on 7 dpi and peaking between 11 and 14 dpi (**Figure 3.5A**). PRRSV antibodies were detected at a greater level in FMT pigs on 21, 28 and 42 dpi ($p = 0.06$, $p = 0.02$, and $p = 0.05$, respectively; repeated measures analysis). When comparing PCV-2 antibody levels, a similar trend was noted with FMT pigs having higher antibody levels. FMT pigs had higher CP 43-233 antibodies at 21 and 28 dpi whereas the FMT pigs maintained higher CP 160-233 antibodies from 21 dpi until the conclusion of the study. Taken together, FMT promoted the production of higher and more sustained levels of antibodies directed at both PRRSV and PCV-2.

FMT shifted microbiome composition. Fecal microbiomes of the transplanted and control groups were analyzed both before and after FMT or mock-transplantation by a pan-microbial array (LLMDA) and 16S rDNA sequencing. First, microbiome composition and diversity was measured by the LLMDA. Diversity was calculated as the mean number of species and families in each group; after transplantation, the mean number of species was 62.3 ± 2.7 and 59.9 ± 4.2 for the control and FMT groups, respectively. With regards to family diversity, the mean number of families on 0 dpi in the control group was 35.3 ± 2.7 while the transplant group had 33.8 ± 2.6. Interestingly, no significant differences in species or family diversity were detected in the FMT group compared to the controls after transplantation using the pan-microbial array. Microbiome diversity, as measured by the microarray, was also similar in the two groups upon arrival (data not shown).

Microbiome composition was also assessed by the LLMDA through the presence of individual phyla, families and species in the two groups. After 7 days of transplantation, there were 64 total families and 166 total species detected in both the control and transplanted groups. Several differences were detected between the transplant and control groups after transplantation that were not detected upon arrival (**Figure 3.6A**). Specifically, the family *Synergistaceae* was detected at a decreased prevalence rate in the transplanted group after FMT compared to the controls (20 and 70%, respectively; $p = 0.07$; Fisher's exact test) and a bacterium in the *Intrasporangiaceae* family was detected in a higher proportion of the transplant pigs when compared to the control pigs (100 and 50%, respectively; $p = 0.03$; Fisher's exact test). Even though members of the *Intrasporangiaceae* family have been discovered in environmental samples and sequenced [433,434], there is a lack of research exploring the effects of these organisms on the vertebrate gut microbiome.

Finally, species diversity within each family was assessed for differences associated with transplantation (**Figure 3.6B**). Most families had similar species diversity between the control and transplanted groups. However, within the families *Spirochaetaceae* and *Vibrionaceae*, there was greater species diversity in the control group compared to the transplanted group. The mean number of species within the family *Spirochaetaceae* was 2.6 species in the control group, while in the transplant group it was 2.0 species ($p = 0.01$; Mann Whitney U test). The mean number of species within the family *Vibrionaceae* was 1.6 species in controls, while in the transplant group it was 0.8 species ($p = 0.02$; Mann Whitney U test). Overall, the LLMDA failed to detect a global increase in microbiome diversity after 7 days of transplantation; however, several shifts in microbiome composition were detected, primarily based on a reduction in bacteria generally considered pathogenic.

A secondary analysis was performed to assess microbiome diversity between PCVAD-affected and unaffected pigs using the LLMDA. Affected and unaffected pigs had similar mean numbers of families and species when compared on days -7 and 0 post-infection. However, there was a significant increase detected in the number of families in the unaffected pigs between -7 and 0 dpi, with the mean number of families detected increasing from 28.9 to 33.7 during the transplantation period ($p = 0.03$; Mann-Whitney U test). Although an increase in family diversity was also seen in the affected pigs between -7 and 0 dpi, this difference was not statistically significant ($p = 0.20$; Mann-Whitney U test). Species diversity also increased in both affected and unaffected pigs during the transplantation period, albeit at a similar rate ($p = 0.07$ and $p = 0.09$ for unaffected and affected pigs, respectively; Mann-Whitney U test). Taking the results together from the LLMDA, there is some evidence suggesting that pigs unaffected by PCVAD after challenge had a greater increase in microbiome diversity during the transplantation period.

Fecal bacterial communities of the control and transplanted groups were also analyzed using 16S rDNA sequencing both prior to and immediately following transplantation (**Figures 3.7 and 3.8**). A total of 2,446,796 quality-filtered 16S rDNA sequences of the V4 region were generated with an average read depth of 61,169 reads per sample. To determine if sampling depth was adequate for gut microbiota analysis, Good's coverages were calculated, which displayed that 99.4 – 99.8% of the bacterial community in the gut was represented in the dataset.

The Chao1 alpha diversity metric for richness was similar in the transplant and control animals at 7 days post transplantation ($p \geq 0.82$; **Figure 3.7A**). The phylum level distribution of the major taxa in both control and transplanted pigs included Bacteroidetes, Firmicutes, and Proteobacteria. Interestingly, the phylum Actinobacteria was almost half in the transplanted group compared to the control group (1.7% vs 3.3%, respectively; **Figure 3.7B**). The family level analysis of the transplanted and control animals revealed *Prevotellaceae*, *Paraprevotellaceae*, Bacteroidales S24-7, *Lactobacillaceae*, *Christensenellaceae*, *Lachnospiraceae*, *Ruminococcaceae*, and *Veillonellaceae* to be the predominant families (**Figure 3.7C**). Phyla composition was not significantly different across the transplant and control animals. Bacterial communities did not cluster by treatment group, suggesting no global shifts in the bacterial populations as a result of the fecal microbiota transplantation. However, there was a significant day effect displayed during transplantation ($p < 0.001$).

To reduce animal-animal variation, a core measurable microbiome (CMM) was defined for the control and transplanted groups. For the transplant group, the CMM was composed of 306 OTUs (23.5% of total OTUs), which represented 81.1% of the rarefied quality-filtered reads. For the control group, the CMM was composed of 316 OTUs (25.3% of total OTUs), which represented 83.2% of the rarefied quality-filtered reads.

To further investigate potential bacterial community differences across the transplanted and control groups, differentially abundant OTUs across the CMM were identified using the LefSe algorithm.[430] A total of 30 significant, differentially abundant OTUs with LDA scores ≥ 2 were identified across comparisons of the two groups (**Figure 3.8**). The differential OTUs associated with the transplant group belong to the bacterial families *Veillonellaceae*, *Lachnospiraceae*, and *Ruminococcaceae*.

Additionally, the 16S rDNA bacterial community composition was analyzed based on the prevalence of disease in the PCVAD-affected (n = 9) and unaffected (n = 11) pigs. A two-way ANOVA was performed on the Chao1 and observed OTUs to determine the effect of subsequent disease phenotype on pre-challenge bacterial diversity. Both measures of bacterial diversity were lower in the affected group on 0 dpi (**Figure 3.9**); however, these differences were not statistically significant ($p \geq 0.14$ and $p \geq 0.24$, respectively). We also performed beta-diversity analysis to determine the effect of the disease phenotype on global microbial community diversity using PERMANOVA analysis. This analysis demonstrated no differences in community composition based on disease phenotype ($p \geq 0.46$), but detected a significant effect of day ($p < 0.001$) on the bacterial community composition.

Discussion

Although FMT has been accepted for centuries as a treatment for various gastrointestinal diseases, it has only been very recently that FMT has been recognized as an alternative therapeutic for diseases outside of the gastrointestinal tract, such as respiratory or neurologic diseases.[435,436] Moreover, using FMT as a prophylactic tool prior to the development of disease has been even less explored. The current study describes FMT efficacy when used as a prophylactic tool to prevent PCVAD in pigs infected with two important swine pathogens.

Additionally, the study was conducted in a manner in line with current swine industry standards, where pigs are typically handled at 3 weeks of age after weaning and without broad-spectrum antimicrobial therapy.

To identify beneficial characteristics of the FMT material, 2 diagnostic tests were used for characterization: 1) a pan-microbial array and 2) bacterial culture. Comparing these results in reveals several discrepancies between the two different detection methods. Interestingly, several bacteria cultured through standard methods, such as *Escherichia coli* and *Streptococcus* sp., are not detected on the pan-microbial array. Culture methods may promote growth of certain well-characterized bacterial species, even if the genome is present at a rate lower than that detectable by the microarray. Previous studies have reported similar results. For example, Sung et al. (2018) reported bacterial species detected in bronchoalveolar lavage fluid by both conventional culture techniques as well as next generation sequencing (NGS). Similar to the current study, they detected some species only by culture and other species only by sequencing, with increased diversity detected in the genome-based technique. Interestingly, the genera *Staphylococcus* and *Escherichia* were only detected by culture and not by NGS,[437] a result similar to our findings on the transplant material. However, with these two species being common environmental microbes, contamination of the bacterial culture must also be considered. With advantages and limitations to each diagnostic test, using culture and DNA-based techniques in combination can serve to provide a more comprehensive characterization of complex microbial communities.

Biphasic clinical disease after co-infection with PRRSV and PCV-2 has been described previously [116,118]. Clinical disease associated with PRRS is typically seen in the first 21 dpi, during peak PRRSV replication. In contrast, clinical disease associated with PCVAD is typically seen after 21 dpi and is associated with the peak and plateau in PCV-2 replication. Although

respiratory signs are common in both phases, clinical disease associated with PCVAD is typically more severe and associated with significant weight loss and muscle wasting. Compared to previous studies where PCV-2b was used, the current study used PCV-2d, which has been recently reported as the most common circulating PCV-2 genotype in U.S. swine [438]. This use of PCV-2d appeared to increase morbidity and mortality rates of co-infected pigs. The principal effect of FMT in the co-infection model was to decrease the number of PCVAD-affected pigs, as demonstrated by a significant reduction in morbidity and mortality. Specifically, a 70% reduction in mortality of transplanted pigs was demonstrated. Additionally, parenteral antimicrobial treatments prescribed for clinical disease were reduced by 60% in the FMT group. With increasing pressure to eliminate antimicrobial usage in food animal production, a 60% decrease in prescribed antimicrobials is a significant effect, important to both human and animal health. Interestingly, FMT did not appear to significantly impact clinical disease in the first half of the co-infection period, typically associated with PRRS. Nevertheless, further research is warranted to understand if FMT improves response to PRRS in a PRRSV-only infection model.

The mechanisms by which FMT is effective are poorly understood but thought to be associated with increasing microbial diversity and restoring normal microbial communities which provide both local and systemic benefits to the host.[439] How FMT protected nursery pigs from developing PCVAD in this study is unknown, but may be due to several possible mechanisms. First, FMT increased the relative abundance of several bacterial families associated with metabolism, including *Veillonellaceae, Lachnospiraceae,* and *Ruminococcaceae*. The members of the *Lachnospiraceae* family are fermentative, anaerobic, chemoorganotrophic and have the ability to hydrolyze many different substrates, such as xylanase, α- and ß-glucosidase, and α- and ß-galactosidase.[440] Bacteria in the *Ruminococcaceae* family are common gut

microbes of animals and humans, which help the host break down complex carbohydrates.[441] Both *Lachnospiraceae and Ruminococcaceae* have previously been associated with fatness traits in pigs[442] and our previous work detected a positive association between *Ruminococcaceae* species and growth after co-infection.[291] In the current study, the comparison of absolute weights over time in the two groups was significantly impacted by the high mortality rate in the control pigs; as such, the increase in weight in the remaining control pigs at the conclusion of the trial was likely the result of decreased competition for feed. Evaluating the weight gain of individual pigs, it is clear that the weight gain of the control pigs was impacted to a greater extent than the FMT pigs throughout the trial.

As a second possible mechanism, FMT may modulate the systemic immune response, increasing the function of immune cells or stimulating cytokine production. In the current study, evidence for an enhanced immune response to both PRRSV and PCV-2 was demonstrated by a reduction in pulmonary pathology as well as a more robust and prolonged antibody response to both viruses detected in the serum of transplanted pigs. It is also possible that FMT may enhance gastrointestinal health and provide competitive exclusion of pathogens. Supporting this possible mechanism in the current study was the documented reduction of two bacterial families thought to be primary pathogens, including *Spirocheataceae* and *Vibrionaceae*,[443-445] in transplanted pigs.

Perhaps surprising in the current study was the lack of significant global increases of microbiome diversity in pigs receiving the transplant. In humans, where FMT is most commonly used to treat recurrent *Clostridium difficile* infections, patients have almost always been treated with several standard doses of antibiotics, making an increase in microbiome diversity more likely with FMT therapy. Even in these human patients, however, Staley et al. (2016) reported

that successful resolution of *C. difficile* infections through FMT treatment did not require complete microbiota engraftment.[446] Similarly to the current study, it does not appear that complete microbiota engraftment occurred in transplanted pigs; nonetheless, significant beneficial effects occurred due to transplantation.

Compared to humans receiving FMT, an important concept to discuss for the current study is that pigs were not treated with antimicrobials and thus had normal microbiomes for their age at the time of transplantation. Pigs were weaned at 3 weeks and allowed normal contact with sows and a commercial environment after birth. The rationale behind this experimental design was to model commercial conditions, where antimicrobial stewardship practices have made it increasingly important to avoid the use of antimicrobials for prophylactic use, and to evaluate FMT as a preventative tool that may be applied to swine production in the field, including its use as a replacement for antimicrobials. However, this could be considered a limitation of the study, due to our inability to control the microbiota present at the time of transplantation, such as would be the case had microbiota-depleted or germ-free pigs been utilized.

A second consideration should be the lack of gender and donor diversity in the current study; the FMT material was collected from 2 older sows and transplanted into weaned barrows. Although the FMT donors were selected based on several specific characteristics and requirements, it should be noted that donation from other sows or boars may have resulted in a different outcome. Gender has been previously described as a factor affecting the success, failure or effect of FMT. For example, Meighani et al. (2016) reported that female sex was a significant predictor of FMT failure when treating humans with recurrent *Clostridium difficile* infection.[447] In contrast, Siegerstetter et al. (2018) demonstrated that female chickens trended towards having higher feed intake ($p = 0.087$) and weight gain ($p = 0.081$) after FMT; this same beneficial effect

of FMT was not seen in male chickens.[448] Regarding the current study, it is unknown how FMT collected from boars or administration to weaned gilts would have impacted the study outcome. Additional research is warranted to understand the effects of gender, both in collection and administration, on FMT efficacy in swine.

Decades of research into control of respiratory disease associated with PRRS have failed to produce a broadly protective vaccine or programs capable of long-term virus elimination from farms. Due to the significant economic and animal welfare impacts that respiratory disease continues to have on the swine industry, it is necessary to consider alternative strategies, such as FMT, for the control of respiratory disease in swine production. Very recently, microbiome therapeutics have been developed for the prevention and/or treatment of diseases in the respiratory tract of children. For example, in May 2017, a microbiome therapeutic utilizing a mixture of 4 gut bacteria, including *Faecalibacterium*, *Lachnospira*, *Veillonella*, and *Rothia* (FLVR), was announced for preventing childhood asthma and potentially other childhood allergic diseases.[449,450] Interestingly, two of those bacterial families were differentially expressed in FMT pigs post-transplantation. Utilizing beneficial gut microbes for the prevention and treatment of respiratory disease is an emerging and exciting area of study. As respiratory infections are a major cause of morbidity and mortality in swine and other livestock, FMT or other microbiome therapeutics provide a promising approach for control of these complex, often polymicrobial, and economically devastating diseases.

In conclusion, this study provides evidence of the significant relationship between the gut microbiome and outcome following systemic viral infections in swine. Most importantly, novel insight is provided into our ability to modulate the microbiome through FMT to improve the clinical outcome of pigs to common pathogens. Future research is necessary to understand the

mechanism behind this relationship and how large-scale microbiome modulation could be adapted to increase the health of PRRS-positive herds in the field.

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Table 3.1. Microorganisms detected in the fecal microbiota transplant material by the pan-microbial detection array*

Phylum‡	Family	Genus Species
Actinobacteria	*Bogoriellaceae*	*Georgenia sp.*
	Nocardiaceae	*Rhodococcus rhodnii*
Amoebozoa	*Entamoebidae*	*Entamoeba nuttalli*
Bacteroidetes	*Bacteroidaceae*	*Bacteroides graminisolvens, Bacteroidetes bacterium*
	Cyclobacteriaceae	*Algoriphagus marincola*
	Prevotellaceae	*Prevotella sp.*
	Rikenellaceae	*Rikenella microfusus*
Basidiomycota	*Ceratobasidiaceae*	*Rhizoctonia solani*
Euryarchaeota	*Methanobacteriaceae*	*Methanobrevibacter oralis, Methanobrevibacter smithii*
Firmicutes	*Carnobacteriaceae*	*Alkalibacterium sp.*
	Clostridiaceae	*Candidatus Clostridium anorexicamassiliense, Clostridiaceae bacterium, Clostridium sp.*
	Clostridiales	*Clostridiales bacterium*
	Lachnospiraceae	*Lachnospiraceae bacterium*
	Lactobacillaceae	*Lactobacillus amylovorus*
	Peptostreptococcaceae	*Clostridium bifermentans, Clostridium mangenotii*
Fusobacteria	*Fusobacteriaceae*	*Psychrilyobacter atlanticus*
Proteobacteria	*Anaplasmataceae*	*Candidatus Xenolissoclinum pacificiensis*
	Bradyrhizobiaceae	*Bosea sp., Bradyrhizobium sp.*
	Campylobacteraceae	*Campylobacter sp., Sulfurospirillum arcachonense*
	Desulfovibrionaceae	*Desulfovibrio alkalitolerans*
	Helicobacteraceae	*Helicobacter pametensis*
	Legionellaceae	*Legionella lansingensis*
	Piscirickettsiaceae	*Thiomicrospira kuenenii, Thiomicrospira sp.*
	Pseudomonadaceae	*Pseudomonas sp., Rhizobacter sp.*
	Sphingomonadaceae	*Sphingomonas sp.*
	Vibrionaceae	*Candidatus Photodesmus katoptron*
	Xanthomonadaceae	*Ignatzschineria larvae, Xanthomonadaceae bacterium*
Spirochaetes	*Spirochaetaceae*	*Borrelia parkeri, Spirochaeta sp., Treponema pedis, Treponema sp.*
Synergistetes	*Synergistaceae*	*Aminiphilus circumscriptus*
Tenericutes	*Acholeplasmataceae*	*Acholeplasma equifetale, Acholeplasma granularum*
	Mycoplasmataceae	*Mycoplasma conjunctivae, Mycoplasma fermentans, Mycoplasma iowae*

	Spiroplasmataceae	*Spiroplasma apis*
Virus	*Circoviridae*	*Fur seal faeces associated circular DNA virus*

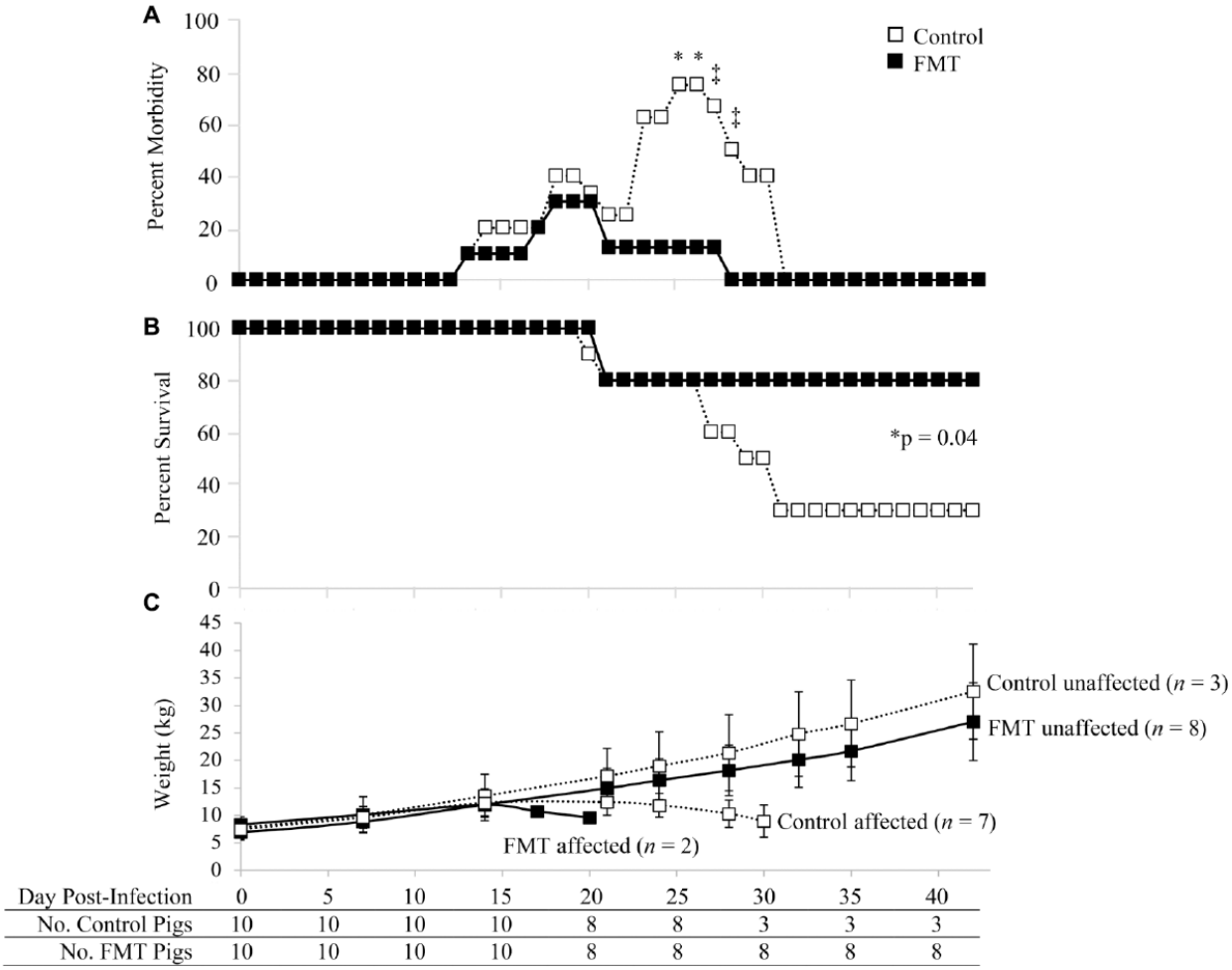
*Only those microbes identified at the phylum, family and genus level are included

‡ Organized alphabetically by phylum; order listed when family unidentifiable

Table 3.2. Effect of FMT on weight gain prior to co-infection*

Weight on arrival (-8 dpi)		Weight after 7 days of FMT (0 dpi)	
Control	FMT	Control	FMT
4.73	5.41	5.09	5.32
5.05	5.59	5.23	5.77
5.82	5.82	5.86	6.05
6.77	5.91	7.50	6.23
7.27	6.14	7.91	6.64
7.36	8.23	8.23	8.27
7.64	8.23	8.41	8.45
8.27	8.32	8.59	8.45
8.73	8.45	9.59	8.68
8.91	8.64	9.64	8.95
Mean: 7.05	Mean: 7.07	Mean: 7.60	Mean: 7.28
SD: 1.46	SD: 1.39	SD: 1.67	SD: 1.40
p = 0.99		p = 0.85	

*Data is shown in kg. Statistics performed by unpaired t-tests using repeated measures analysis.



Day Post-Infection	0	5	10	15	20	25	30	35	40
No. Control Pigs	10	10	10	10	8	8	3	3	3
No. FMT Pigs	10	10	10	10	8	8	8	8	8

Figure 3.1. Morbidity and mortality of pigs with and without fecal microbiota transplantation prior to co-infection with PRRSV and PCV-2d.

A. Percent morbidity over time; data is shown as the percent of pigs in each group with veterinary treatment prescribed due to moderate to severe clinical disease. Asterisks demarcate statistically significant differences (*$p < 0.05$ and ‡$p < 0.1$; Fisher's exact test). **B.** Survival curve shows a significantly higher survival rate in pigs administered the FMT. **C.** Weight gain is shown as the mean ± standard deviation of control and FMT groups considered unaffected (squares) and affected (circles) by PCVAD, as identified by mortality and clinical disease. The surviving pigs in each group are shown at the bottom of the figure over time.

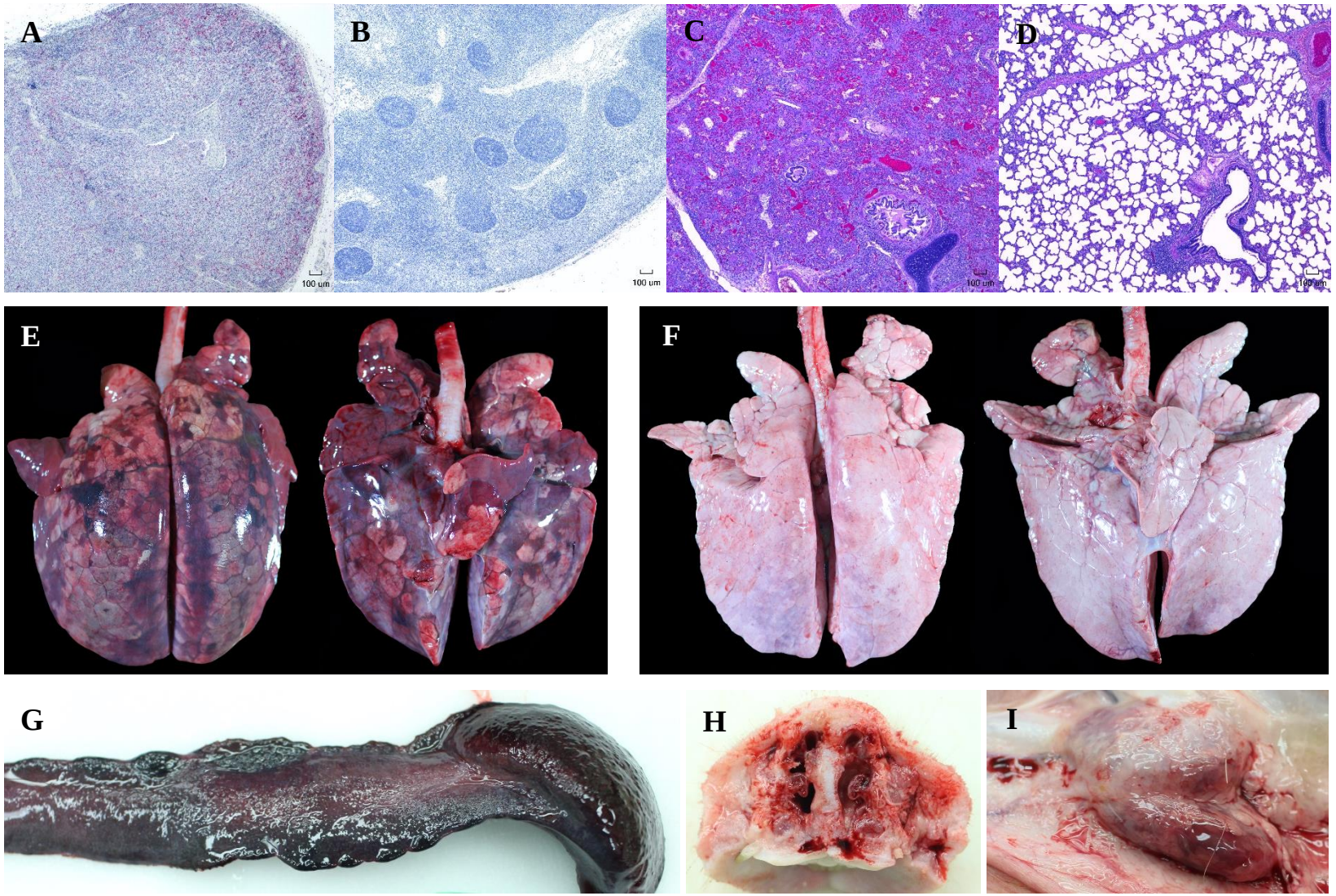


Figure 3.2. Representative gross and microscopic lesions associated with porcine circovirus associated disease (PCVAD).

Images shown are from representative PCVAD-affected pigs with severe clinical disease between 19 and 30 dpi or minimally-affected pigs for the purpose of comparison. **A)** Immunohistochemical staining of a tracheobronchial lymph node showing severe lymphoid

depletion associated with large amounts of PCV-2 antigen. **B)** Immunohistochemical staining of a tracheobronchial lymph node showing lymphoid follicles with minimal lymphoid depletion and no PCV-2 antigen staining. **C)** H&E-stained lung showing severe diffuse interstitial pneumonia affecting greater than 75% of lung. **D)** H&E-stained lung showing mild and multifocal interstitial pneumonia affecting less than 50% of lung. **E)** Dorsal and ventral gross lung showing severe consolidation, hemorrhage, and pneumonia affecting approximately 95% of lung. **F)** Dorsal and ventral gross lung showing minimal consolidation, hemorrhage, and pneumonia affecting approximately 12% of lung.

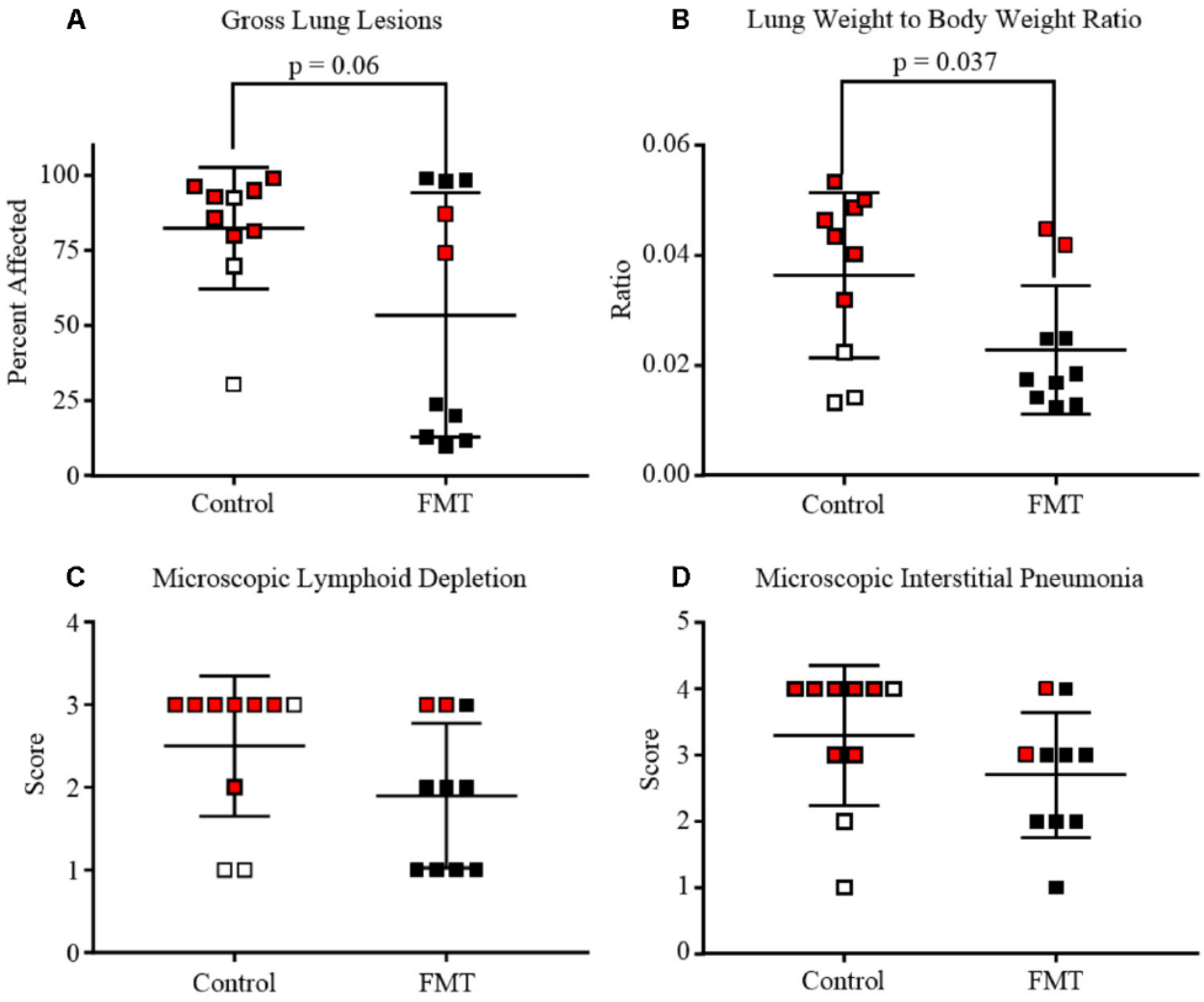


Figure 3.3. Degree of lung and lymphoid lesions in pigs after co-infection with PRRSV and PCV-2d.

Data is shown as individual scores at the time of death with horizontal lines representing the mean ± 1 standard deviation for each group. Pigs shown in red are those that died or were humanely euthanized due to severity of disease. **A.** Gross lung affected by pneumonia. Mean percent of lung affected was lower in FMT when compared to Controls (p = 0.06, unpaired t-test). **B**. Lung weight to body weight ratio at the time of necropsy showing the control pigs had significantly higher ratios (p = 0.037, unpaired t-test). **C.** Lymphoid depletion mean scores were higher in the control group (2.5 ± 0.3) when compared to the FMT group (1.9 ± 0.3), but differences were not statistically significant**. D.** Lung lesion mean scores were higher in the control group (3.3 ± 0.3) when compared to the FMT group (2.7 ± 0.3), but differences were not statistically significant.

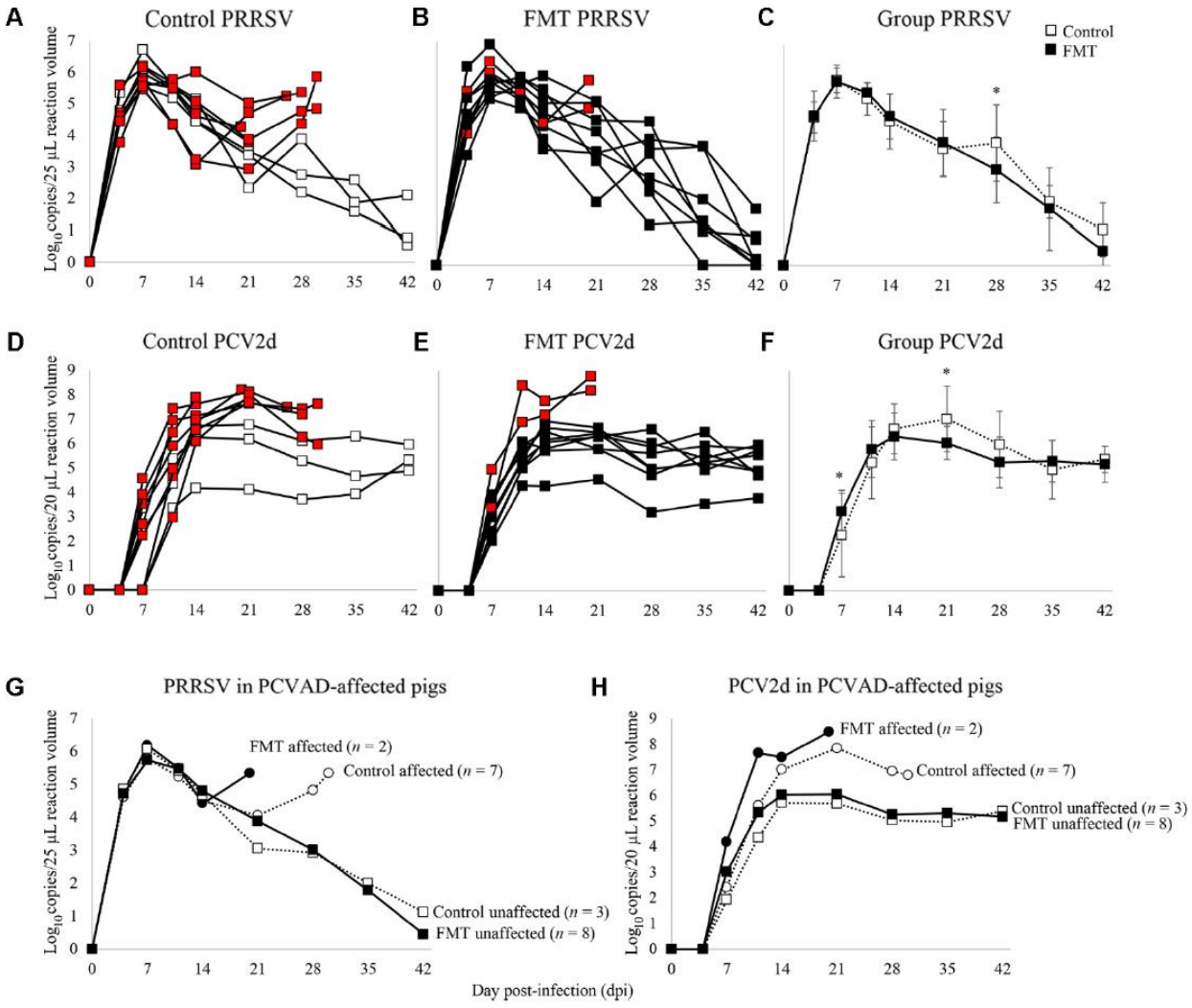


Figure 3.4. Time course of PRRSV and PCV-2d viremia.

Data is shown as the log_{10} copy number/PCR reaction volume for individual pigs in both the control and FMT groups (**A and** B for PRRSV; **D and E** for PCV-2d). Red boxes indicate pigs that died or were humanely euthanized during the course of the co-infection trial due to severity of disease. **C. and F**. Data is shown as mean log_{10} copy number/PCR reaction volume ± 1 standard deviation for each group. Asterisks demarcate statistically significant differences for PRRSV and PCV-2d. **G. and H**. Data is shown as PRRSV and PCV-2 viremia in PCVAD-affected (circles) and PCVAD-unaffected (squares) pigs within the control and FMT groups, as measured by mortality and clinical disease.

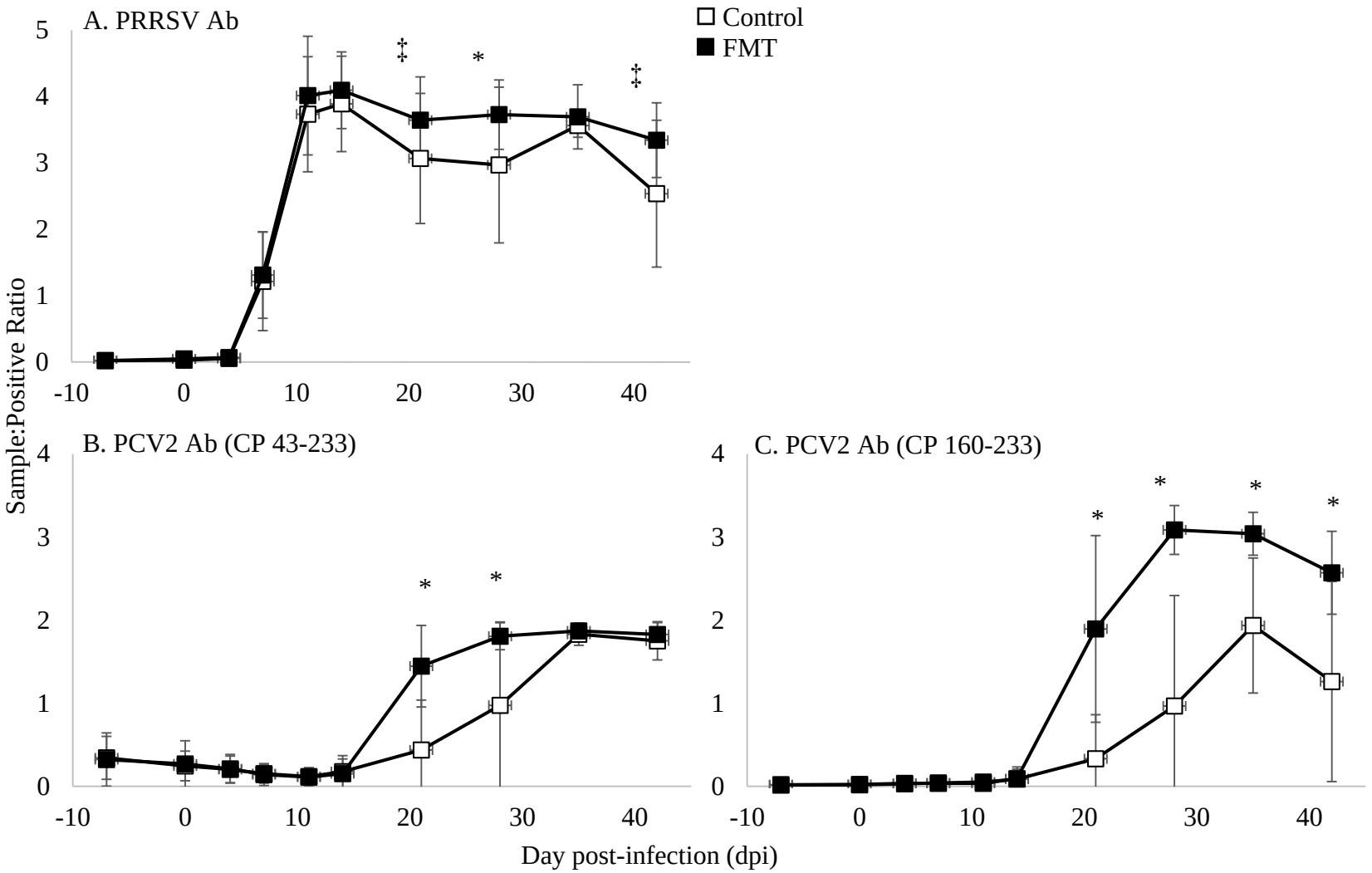


Figure 3.5. Detection of antibody in transplanted and control pigs.

Data is shown as the mean sample:positive ratio ± 1 standard deviation for PRRSV **(A)**, PCV-2 large epitope **(B)** and PCV-2 decoy epitope **(C).** Differences between the two group are shown as *$p < 0.03$ and ‡$p < 0.06$ (repeated measures analysis using multiple t-test

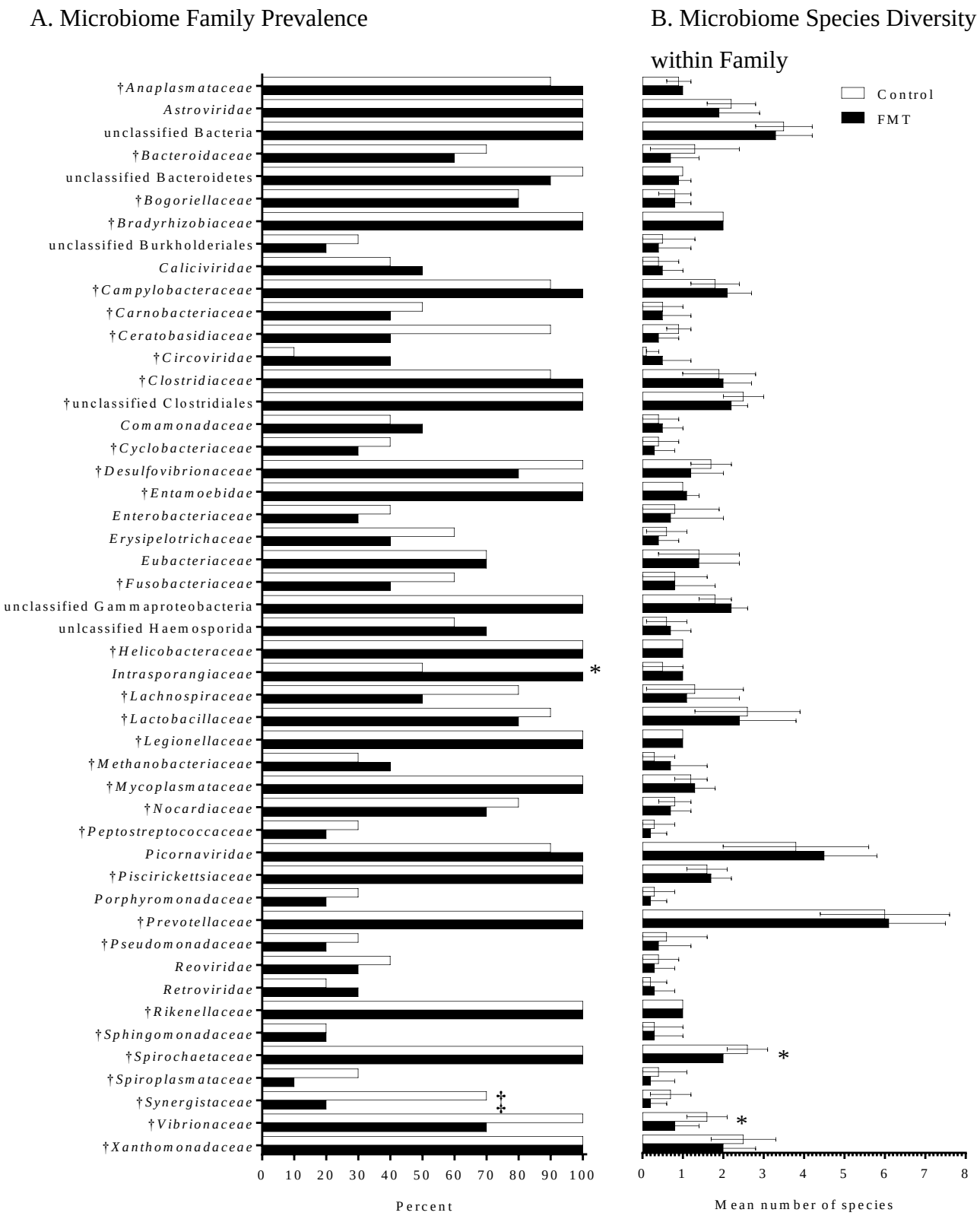


Figure 3.6. Fecal microbiome composition as detected by the pan-microbial array in FMT and control pigs after 7 days of transplantation.

A. Microbiome family composition is shown as the percent of FMT pigs (*n* = 10) and control pigs (*n* = 10) with each family detected on the pan-microbial array. Families with a total prevalence of less than 40% between the FMT and control groups are not shown. There was a significantly higher prevalence of a species within the family *Intrasporangiaceae* in the FMT group. A trend towards a higher percentage of control pigs having species within the family *Synergistaceae* was also detected (‡ p = 0.07; Fisher's exact test). **B.** Data is shown as the mean number of species detected ± one standard deviation in each family detected in FMT and control pigs. Within the families *Spirochaetaceae* and *Vibrionaceae*, there was greater species diversity in the control group compared to the transplanted group (*p = 0.01 and p = 0.02, respectively; Mann Whitney U test). †Indicates families found in the transplant material.

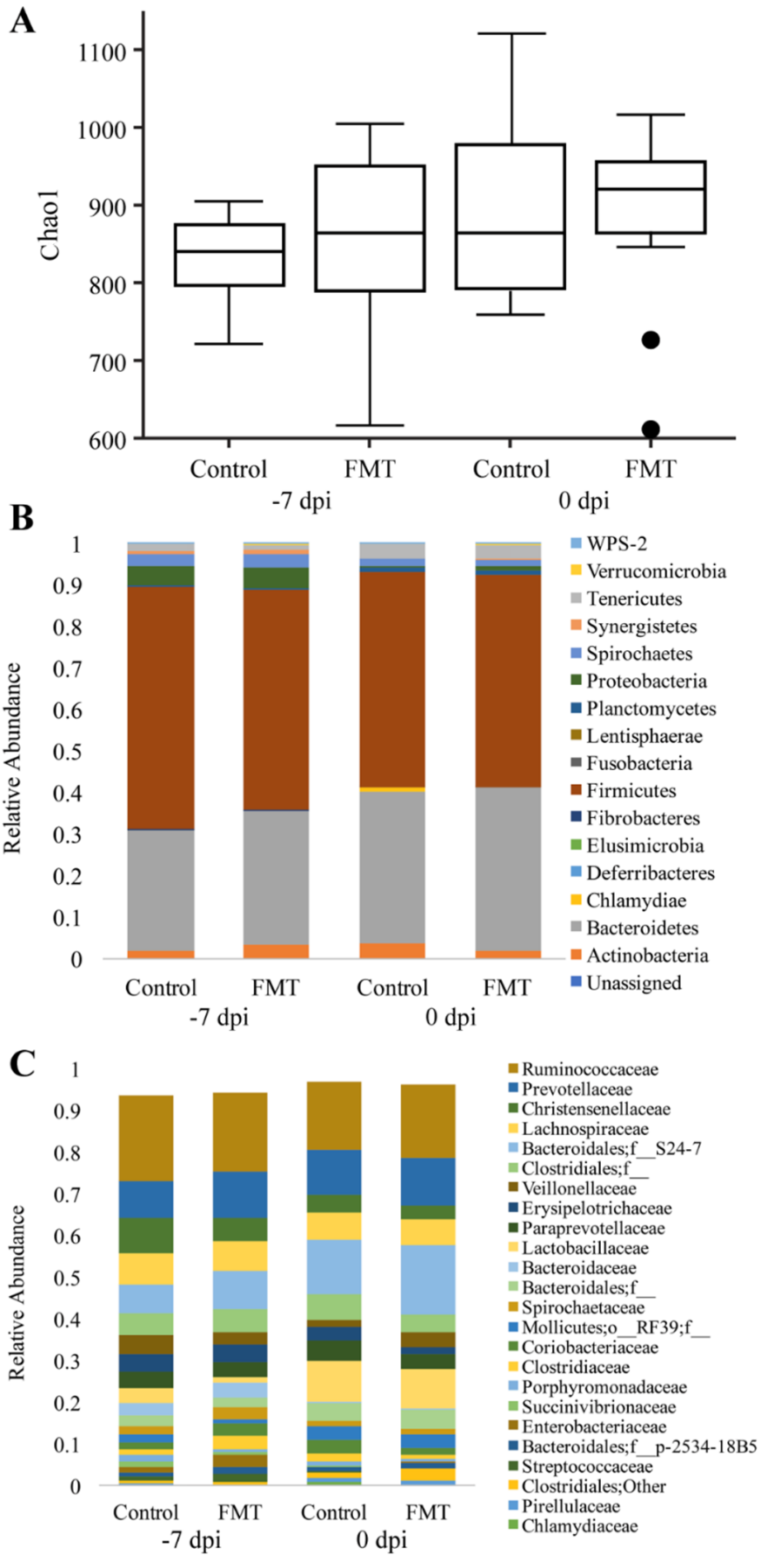

A
Chao1
1100
1000
900
800
700
600
Control
FMT
Control
FMT
-7 dpi
0 dpi
B
Relative Abundance
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
FMT
Control
FMT
-7 dpi
0 dpi
WPS-2
Verrucomicrobia
Tenericutes
Synergistetes
Spirochaetes
Proteobacteria
Planctomycetes
Lentisphaerae
Fusobacteria
Firmicutes
Fibrobacteres
Elusimicrobia
Deferribacteres
Chlamydiae
Bacteroidetes
Actinobacteria
Unassigned
C
Relative Abundance
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
FMT
Control
FMT
-7 dpi
0 dpi
Ruminococcaceae
Prevotellaceae
Christensenellaceae
Lachnospiraceae
Bacteroidales;f__S24-7
Clostridiales;f__
Veillonellaceae
Erysipelotrichaceae
Paraprevotellaceae
Lactobacillaceae
Bacteroidaceae
Bacteroidales;f__
Spirochaetaceae
Mollicutes;o__RF39;f__
Coriobacteriaceae
Clostridiaceae
Porphyromonadaceae
Succinivibrionaceae
Enterobacteriaceae
Bacteroidales;f__p-2534-18B5
Streptococcaceae
Clostridiales;Other
Pirellulaceae
Chlamydiaceae

Figure 3.7. 16S rDNA fecal microbiome analysis pre and post fecal microbiota transplantation.

A. Chao1 alpha diversity of the control and transplanted groups pre and post-transplantation (data is shown as the range of values with medians, quartiles and outliers). **B.** Bar graphs show the mean relative abundance of bacterial phyla for each group and time. **C.** Bar graphs show mean relative abundance of bacterial families making up 1% or more of all sequences detected in 1 or more sample subset.

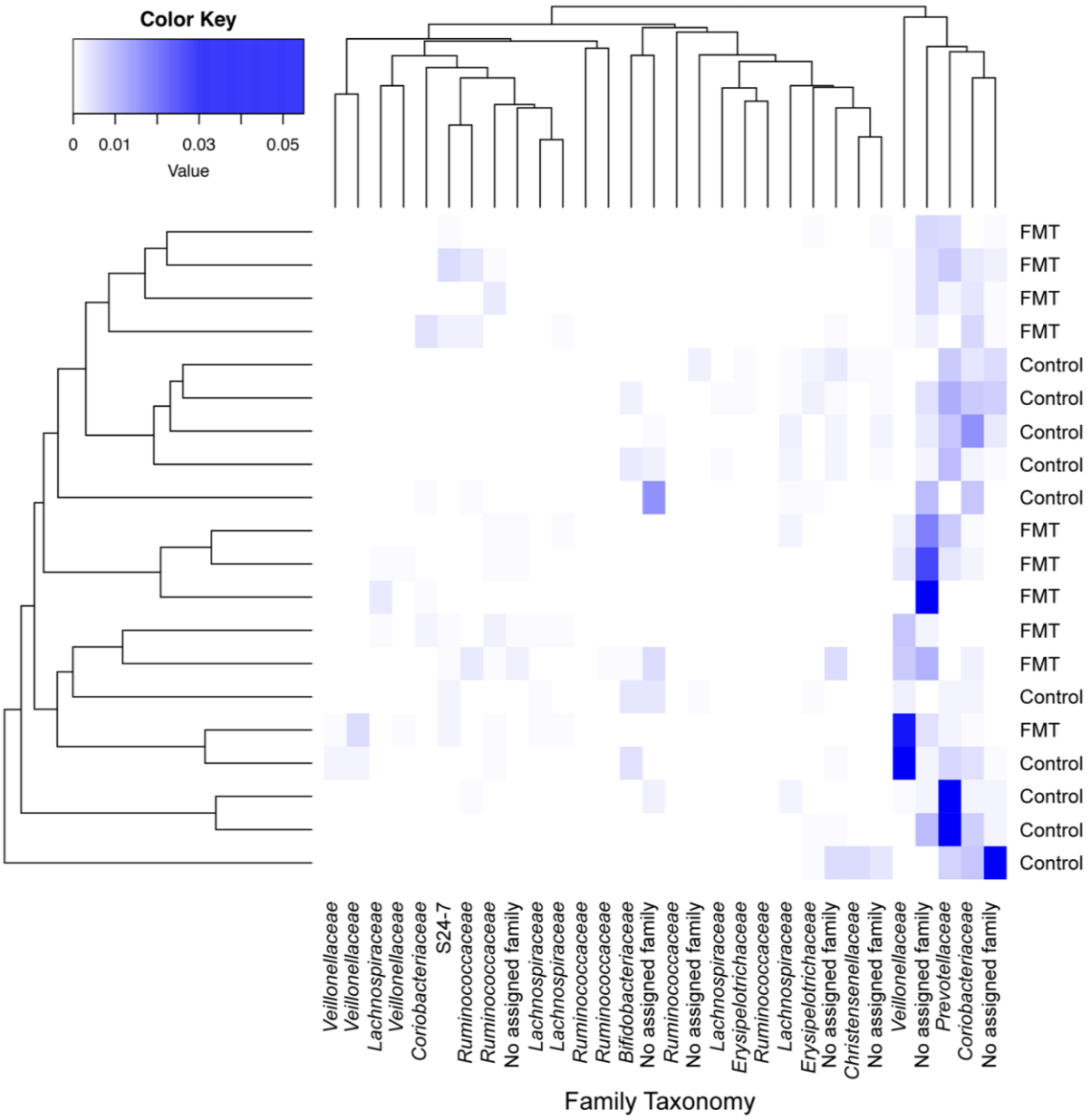


Figure 3.8. Differentially abundant operational taxonomic units (OTU) in the control and FMT groups after 7 days of fecal microbiota transplantation.

In the FMT group, 73.3% of the differential OTUs belong to the *Veillonellaceae, Lachnospiraceae, and Ruminococcaceae* families, and 13.3% of the OTUs were not classified at the family level. For the control group, 40% of the differential OTUs belong to *Erysipelotrichaceae*, *Lachnospiraceae* and *Ruminococcaceae* families and 33.3% of the differential OTUs were unclassified. Interestingly, the hierarchical clustering of the differential OTUs show two major clusters for each of the control and FMT groups.

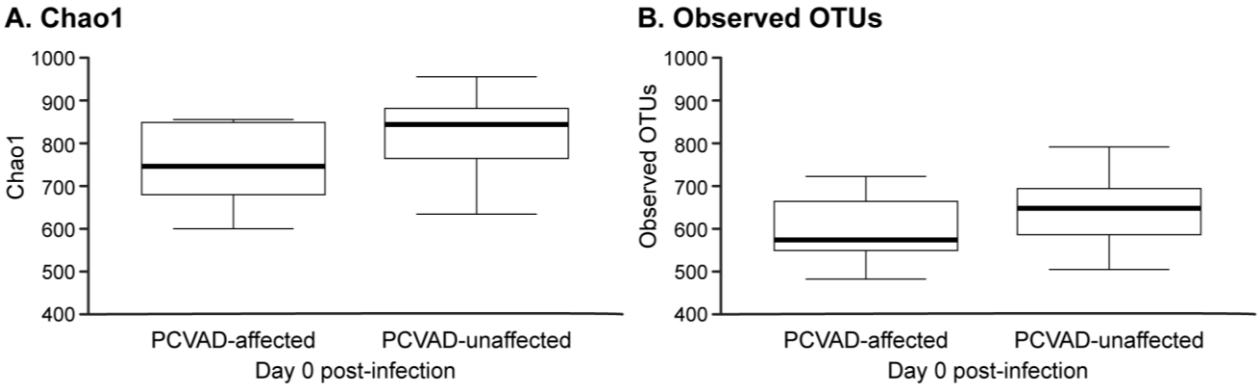


Figure 3.9. Fecal bacterial diversity in the PCVAD-affected and unaffected pigs at the time of challenge.

Data is shown as **A.** Chao1 alpha diversity and **B.** Observed OTUs for the affected and unaffected pigs after transplantation or mock-transplantation (data is shown as the range of values with medians, quartiles and outliers). PCVAD-affected pigs developed disease and were euthanized or died due to severity of clinical signs during the 42-day post-infection trial.

Chapter 4 - Gut microbiome associations with outcome following co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) in pigs immunized with a PRRS modified live virus vaccine

Published in Veterinary Microbiology

Constance LA, Thissen JB, Jaing CJ, McLoughlin KS, Rowland RRR, Serão NVL, Cino-Ozuna AG, Niederwerder MC. Gut microbiome associations with outcome following co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) in pigs immunized with a PRRS modified live virus vaccine. *Vet Microbiol.* 2021 Mar;254:109018. doi: 10.1016/j.vetmic.2021.109018. Epub 2021 Feb 16. PMID: 33639341.

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) are two of the most significant pathogens affecting swine. Co-infections are common and result in respiratory disease and reduced weight gain in growing pigs. Although PRRS modified live virus (MLV) vaccines are widely used to decrease PRRS-associated losses, they are generally considered inadequate for disease control. The gut microbiome provides an alternative strategy to enhance vaccine efficacy and improve PRRS control. The objective of this study was to identify gut microbiome characteristics associated with improved outcome in pigs immunized with a PRRS MLV and co-challenged with PRRSV and PCV2b. Twenty-eight days after vaccination and prior to co-challenge, fecal samples were collected from an experimental population of 50 nursery pigs. At 42 days post-challenge, 20 pigs were retrospectively identified

as having high or low growth outcomes during the post-challenge period. Gut microbiomes of the two outcome groups were compared using the Lawrence Livermore Microbial Detection Array (LLMDA) and 16S rDNA sequencing. High growth outcomes were associated with several gut microbiome characteristics, such as increased bacterial diversity, increased *Bacteroides pectinophilus*, decreased *Mycoplasmataceae* species diversity, higher Firmicutes:Bacteroidetes ratios, increased relative abundance of the phylum Spirochaetes, reduced relative abundance of the family *Lachnospiraceae,* and increased *Lachnospiraceae* species C6A11 and P6B14. Overall, this study identifies gut microbiomes associated with improved outcomes in PRRS vaccinated pigs following a polymicrobial respiratory challenge and provides evidence towards the gut microbiome playing a role in PRRS vaccine efficacy.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) are two of the most significant pathogens of swine worldwide. PRRSV, a single-stranded RNA virus in the family *Arteriviridae,*[451] is widely considered to cause the most costly disease of swine in the United States (U.S.), with estimated annual losses of $664 million due to diminished weight gain and respiratory disease in growing pigs.[410] PCV2, a single-stranded DNA virus in the family *Circoviridae*, [90] is estimated to cause economic losses up to $20/pig in unvaccinated herds[91] due to a group of syndromes termed porcine circovirus associated disease (PCVAD), which includes muscle wasting, weight loss and respiratory disease.[92] Both PRRSV and PCV2 result in systemic infections and modulation of host immunity,[121,122] reducing the rate of weight gain and increasing the likelihood of primary and secondary polymicrobial disease syndromes in swine.

PRRS modified live virus (MLV) vaccines are widely used in PRRS-endemic herds to reduce losses associated with PRRSV infection. In experimental and field settings, PRRS MLV immunization has the potential to improve weight gain, reduce viral replication, reduce pulmonary pathology, and decrease clinical disease after wild-type PRRSV exposure.[163] However, several challenges remain for PRRS MLV vaccine safety and efficacy, including the potential for reversion to virulence and recombination with wild-type strains,[164] potentiation of primary and secondary pathogens,[116] and incomplete protection against emerging wild-type strains.[165] As such, the currently available commercial vaccines are generally considered inadequate for disease control and improved vaccines or alternatives strategies for PRRS control are urgently needed.[170,171]

The gut microbiome, or community of microorganisms in the gastrointestinal tract, is an emerging alternative tool for improving the response of swine to PRRS.[280] Previous work has demonstrated several gut microbiome characteristics that are associated with several improved outcome parameters in pigs co-infected with PRRSV and PCV2. Specifically, increased gut microbiome diversity, increased *Ruminococcaceae* species, increased *Streptococcaceae* species, and fecal microbiota transplantation were associated with infection outcomes such as reduced virus replication, improved weight gain, and decreased morbidity.[118,291,338] The mechanism by which the gut microbiome impacts outcome in pigs exposed to respiratory pathogens is largely unknown, but is believed to be associated with modulation of immunity and through the metabolic products of microbes.[280]

Considering the proposed mode of action for the microbiome on modulating immunity,[452] extending the potential impact of gut microbes beyond primary infection to vaccine response is a rational approach. Although research focused on gut microbiome associations with PRRS

vaccine efficacy is limited to a study showing no effect of an oral single-strain probiotic on PRRS vaccine response,[342] the relationship between immune response to other infectious disease vaccines and gut microbiome characteristics has been described.[340,341,344] For example, improved immune response as defined by IgA seroconversion after oral immunization with a live human rotavirus vaccine has been correlated with fecal microbiome characteristics such as increased *Streptococcus bovis* and reduced species in the Bacteroidetes phylum.[345] Furthermore, pigs with high IgG production after immunization with a killed *Mycoplasma hyopneumoniae* vaccine were identified as having several differential microbiome characteristics, including increased *Lachnospiraceae*, *Prevotella* and *Fibrobacter* bacteria, when compared to pigs with low IgG production post-vaccination.[346]

In the current study, we took advantage of a larger host genetics study[173] to identify microbiome characteristics which may predispose outcome in PRRS MLV vaccinated pigs during co-challenge with PRRSV and PCV2. The results demonstrate that several gut microbiome characteristics, such as increased bacterial diversity and the presence of certain bacterial families and species, may play a role in subsequent growth after co-challenge. This data suggests that porcine gut microbes may serve as an alternative tool for improving the efficacy of PRRS MLV vaccines during polymicrobial respiratory disease.

Methods

Animals and housing. All use and experimentation of 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 Committee and Institutional Biosafety Committee. This study was conducted as a part of the

PRRS Host Genetics Consortium (PHGC) as described previously.[173] A subset of barrows (*n* = 50; mean age 23.4 ± 2.1 days) were obtained at weaning from a high health commercial herd negative for PRRSV. Piglets were not vaccinated for PCV2 and were used without regards to maternal antibody. Piglets were housed in a single environmentally controlled room at the Kansas State University Large Animal Research Center and maintained under BSL-2 conditions. Piglets were randomly distributed into six 13.4 m^2 pens, and housed in groups of 7-10 pigs per pen. Pigs were given access to food and water *ad libitum*.

Viruses. The PRRSV and PCV2b viral isolates used in this study originated from the lymph node of a pig with severe postweaning multisystemic wasting syndrome (PMWS) as previously described.[117,414] PRRSV (isolate KS62; GenBank accession no. KM035803) was isolated by propagation on MARC-145 cells and PCV2b (GenBank accession no. JQ692110) was isolated by utilizing the heat stability of the virus and preparing a lymph node suspension enriched for PCV2. The procedures used to isolate and titrate the viruses have been described previously in detail.[116,118,291] Analysis of the PCV2b tissue homogenate used for challenge detected two ubiquitous swine viruses, including torque teno sus virus (TTSuV) and porcine endogenous retrovirus (PERVs).[417] PRRSV was quantified on MARC-145 cells and swine testicle cells were used to quantify PCV2. The 50% tissue culture infectious dose per milliliter ($TCID_{50}$/mL) was calculated using the Reed and Muench method.[453]

Experimental design and sample collection. After 4 days of acclimation, all 50 pigs were vaccinated with a 2-ml dose of a commercial PRRS MLV vaccine (Ingelvac PRRS MLV; Boehringer Ingelheim Animal Health, Duluth, GA; GenBank accession no. AF159149). The vaccine was administered intramuscularly according to the vaccine label instructions. At 28 days post-vaccination (dpv) and approximately 8 weeks of age (mean age 55.4 ± 2.1 days), all pigs

were challenged with a combination of PRRSV and PCV2b. The challenge viruses were combined 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 administered intranasally and 1 ml administered intramuscularly. Rationale for co-infection has been described in detail[280] and includes the prevalence of PRRSV as a contributor to PCVAD and the potentiation of immunomodulation.

Individual body weights were determined on 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 dpv. Blood samples were collected on 0, 4, 7, 11, 14, 21, 28, 32, 35, 39, 42, 49, 56, 63, and 70 dpv. Fecal samples were collected from all 50 pigs during the week prior to co-challenge for microbiome analysis. At 42 days post-challenge (dpc) or 70 dpv, 20 pigs were retrospectively identified as having high (n = 10) or low (n = 10) growth rates during the co-challenge period. To select these groups, average daily gain (ADG) was calculated as the change in weight over the 42-day period and reported in kg. Any pig displaying clinical signs which required veterinary medical treatment (as described below) were excluded from the study. At 42 dpc, 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 Histological Evaluation. Pigs were visually examined by a veterinarian or veterinary assistant on each day of the study for clinical signs associated with PRRSV/PCV2 co-challenge, including respiratory signs (e.g., dyspnea, coughing, nasal discharge, ocular discharge and open mouth breathing), lethargy, depression, diarrhea, pyrexia, lameness, joint effusion, decreased body condition, muscle wasting and aural cyanosis. Any pig showing moderate to severe clinical disease was treated or euthanized under the direction of a veterinarian. Examples of clinical signs in which treatment was administered included 1) dyspnea, 2) mucopurulent nasal discharge, 3) lameness with associated joint effusion, 4) pallor with muscle wasting, and 5)

lethargy or depression with pyrexia. Clinically affected pigs were administered parenteral antibiotics, including ceftiofur hydrochloride (Excede®; Zoetis, Parsippany, NJ), oxytetracycline (Liquamycin® LA-200®; Zoetis, Parsippany, NJ), or enrofloxacin (Baytril®; Bayer HealthCare LLC, Shawnee Mission, KS). Pigs with overt clinical disease and rectal temperatures of ≥ 104°F were administered a nonsteroidal anti-inflammatory drug, such as flunixin meglumine (Banamine®; Merck Animal Health, Madison, NJ) or meloxicam. Any pig with documented clinical disease requiring veterinary medical treatment was excluded from selection for fecal microbial analysis.

At 42 dpc, all pigs were humanely euthanized using pentobarbital sodium (Fatal-Plus®; Vortech Pharmaceuticals, Dearborn, MI). A masked board certified veterinary pathologist performed complete necropsies and histopathologic evaluations. Tonsils were collected and 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). Lymphoid depletion was scored on a scale of 0-3 as previously described.[118] Briefly, scores were given 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 and viral load. Methods utilized to measure PRRSV and PCV2 viremia have been described in detail previously.[116,118,291] Briefly, viral DNA and RNA were extracted simultaneously from 50 μL of serum using Ambion's MagMAX 96 Viral Isolation Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) 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, Rockville, MD)

according to the manufacturer's instructions. The PRRSV PCR assay results were reported as $\log_{10}$ RNA starting quantity (copy number) per 50-µl reaction volume. PCV2 DNA was quantified using SsoAdvanced Universal SYBR green supermix (Bio-Rad, Hercules, CA). The forward and reverse PCR primers were 5'-AATGCAGAGGCGTGATTGGA-3' and 5'-CCAGTATGTGGTTTCCGGGT-3', respectively. Standard curves and positive and negative controls were included on each plate. The PCV2b PCR assay results were reported as $\log_{10}$ DNA starting quantity (copy number) per 20 µL reaction volume. Total viral load for PRRSV and PCV2 were calculated by Riemann sums of the total area of the trapezoids under the line segments connecting weekly or biweekly viremia measurements. Viral load was calculated during the vaccination period (0-28 dpv) for PRRSV MLV vaccine replication and during the challenge period (28-70 dpv) for PRRSV and PCV2 replication.

Microsphere immunoassay for detection of PRRSV and PCV2 antibodies. PRRSV nucleocapsid protein and PCV2b capsid protein polypeptide fragments (43-233 and 160-233) were cloned into the pHUE vector, as previously described in detail [338]. Proteins were expressed, purified and measured prior to being coupled to carboxylated Luminex MagPlex® polystyrene microspheres (Luminex Corporation, Austin, TX) according to the manufacturer's instructions. For the assays, approximately 2500 antigen-coated beads, suspended in 50 µL PBS with 0.05% Tween-20 and 4% goat serum (PBST-GS), were placed in each well of a 96-well polystyrene round bottom plate (Corning® Costar® Corporation, Cambridge, MA). Sera were diluted 1:400 in PBST-GS and 50 µL was added to each well. An adhesive foil plate sealer was applied and the plate was incubated for 30 min at room temperature with gentle shaking. After incubation, the plate was placed on a magnet and beads were washed three times with 190 µL of PBS-GS. For the detection of IgG, 50 µL of biotin-SP-conjugated affinity purified goat anti-swine secondary

antibody (IgG, Jackson ImmunoResearch, West Grove, PA) was diluted to 2 µg/mL in PBST-GS and 100 µL was added to each well. The plate was incubated at room temperature for 30 min and washed three times followed by the addition of 50 µL of streptavidin-conjugated phycoerythrin (2 ug/ml in PBST-GS; SAPE). After 30 min, the plate was washed and microspheres resuspended in 100 µL of PBST-GS. Microspheres were analyzed using a MAGPIX instrument (Luminex Corporation, Austin, TX) and Luminex® xPONENT 4.2 software. A minimum of 50 microspheres was used for the calculation of mean fluorescence intensity (MFI). The sample to positive (S/P) ratio was calculated as the MFI of the sample minus the MFI of the negative control divided by the MFI of the standard positive control minus the MFI of the negative control.

Microarray Analysis of fecal microbiome. The Lawrence Livermore Microbial Detection Array (LLMDA) was used to analyze microbiome composition and diversity of fecal samples collected prior to co-challenge. This specific array detects annotated sequences of microbes within GenBank®, the National Institute of Health genetic sequence database. The version 7 of the LLMDA in the 4plex 180K probe format was used in this study, which detects a total of 10,612 microorganisms including 5,457 bacteria, 4,377 viruses, 327 archaebacteria, 319 fungi, and 132 protozoa. Probe lengths on the array is around 60 nt and have roughly equivalent affinities for their complementary target DNA molecules [415]. Probes were designed to detect all sequenced microbial families with a large number of probes per sequence (average of 30 probes) to improve sensitivity. 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.[118,416-418]

The PowerViral™ Environmental RNA/DNA Isolation Kit (MO BIO, San Diego, CA) was used to extract DNA and RNA from the fecal samples. For each sample, approximately 0.25 g of feces was added to 600 µl of PV1/β-mercaptoethanol in a glass bead tube included in the kit. Samples were homogenized and lysed by vortexing tubes for 10 minutes at maximum speed. Samples were further processed using the PowerViral™ Kit protocol. All samples were eluted into 100 µl of RNase-Free water. The purified nucleic acids were quantified using the Thermo Scientific™ Nanodrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA). For each sample, 10 µl of the extracted DNA and RNA was amplified using the random amplification procedure as previously described [418]. The amplified cDNA and DNA was purified with the Qiaquick PCR purification columns (Qiagen, Hilden, Germany) and quantified using the Nanodrop™ spectrophotometer.

Approximately 1 µg of amplified cDNA and DNA were fluorescently labeled using a one-coloring labeling protocol (Roche NimbleGen, Madison, WI). Briefly, the samples were labeled using nick translation with Cy3-labeled random nonamer primers (TriLink Biotechnologies, San Diego, CA) and Klenow DNA polymerase (New England Biolabs, Ipswich, MA) at 37°C for 2 hr. The labeled DNA was precipitated in isopropanol, and the pellet was washed, and dried. The pellet was then reconstituted in 50 µl of RNase-Free water and quantified using the Nanodrop™ spectrophotometer.

The Agilent Technologies Oligo aCGH/ChIP-on-Chip Hybridization kit (Santa Clara, CA) was used to hybridize samples to the arrays. For each sample, 10 µg of fluorescently labeled DNA was mixed with 10x aCGH blocking agent, 2x HiRPM hybridization buffer and nuclease free water. The samples were then denatured at 95˚C for 3 min, and incubated at 65˚C for 3 min. Each sample was then immediately loaded onto the array and hybridized for approximately 40 hr

at 65˚C in a microarray rotator oven (Agilent Technologies Inc., Santa Clara, CA) set to a speed of 20. Microarrays were washed using the standard manufacturer's protocol with Oligo aCGH/ChIP-on-chip Wash Buffer 1 for 5 min at room temperature and Oligo aCGH/ChIP-on-chip Wash Buffer 2 for 1 min at 37°C (Agilent Technologies Inc., Santa Clara, CA). Using the SureScan Microarray Scanner (Agilent Technologies Inc., Santa Clara, CA), all arrays were scanned to a resolution of 3 μm.

Microarray data was generated from the microbe sequences using the CLiMax method developed at Lawrence Livermore National Laboratory, at a detection threshold of ≥ 99%. 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. Diversity of the fecal samples was measured by calculating the number of families and species detected in each sample. The mean number of families and species were compared between high and low growth rate groups. Microbiome composition was compared between the two groups at the level of phylum, family and species. The Firmicutes to Bacteroidetes (Firmicutes/Bacteroidetes) ratio was determined by dividing the total number of Fimicutes bacterial species by the total number of Bacteroidetes bacterial species detected for each pig.

16S rDNA Analysis of Fecal Microbiome. DNA was extracted from fecal samples as described above for microarray detection analysis prior to submission to the University of Minnesota Genomics Center (UMGC) for 16S library preparation of the V4 region using standard diagnostic protocols and a two-step PCR protocol as described previously.[454] Briefly, a dual-indexing protocol was utilized that uses a single pair of PCR primers with 5′ adaptor tails to

amplify samples in a 'primary' amplification, while a 'secondary' PCR adds flow cell adaptors and indices.

The primary amplification was done in a qPCR reaction, using ABI7900. The following recipe was used: 3 µl of template DNA, 0.48 µl of nuclease-free water, 1.2 µl of 5× KAPA HiFi buffer (KAPA Biosystems, Woburn, MA), 0.18 µl of 10 mM dNTPs (KAPA Biosystems, Woburn, MA), 0.3 µl of DMSO (Fisher Scientific, Waltham, MA), 0.12 µl of ROX (25 µM) (Life Technologies, Carlsbad, CA), 0.003 µl of 1,000× SYBR Green, 0.12 µl of KAPA HiFi polymerase (KAPA Biosystems, Woburn, MA), 0.3 µl of forward primer (10 µM), and 0.3 µl of reverse primer (10 µM). Cycling conditions were as follows: 95°C for 5 min, followed by 35 cycles of 98°C for 20 s, 55°C for 15 s, and 72°C for 1 min. The primers for the primary amplification contained both 16S-specific primers (V4 515F and V4 806R) and adaptor tails for adding indices and Illumina flow cell adaptors in a secondary amplification. The following primers were used (16S-specific sequences in bold):

V4_515F_Nextera:

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGCCAGCMGCCGCGGTAA**

V4_806R_Nextera:

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGG**GACTACHVGGGTWTCTAAT**

The amplicons from the primary PCR were diluted 1:100 in sterile, nuclease-free water, and a second PCR reaction was set up to add the Illumina flow cell adaptors and indices. The secondary amplification was done using the following recipe: 5 µl of template DNA, 1 µl of nuclease-free water, 2 µl of 5× KAPA HiFi buffer (KAPA Biosystems, Woburn, MA), 0.3 µl of 10 mM dNTPs (KAPA Biosystems, Woburn, MA), 0.5 µl of DMSO (Fisher Scientific, Waltham, MA), 0.2 µl of KAPA HiFi Polymerase (KAPA Biosystems, Woburn, MA), 0.5 µl of forward

primer (10 μM), and 0.5 μl of reverse primer (10 μM). Cycling conditions were as follows: 95°C for 5 min; ten cycles of 98°C for 20 s, 55°C for 15 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The following indexing primers were used (p5 and p7 flow cell adapters are in bold; X indicates the positions of the 8-bp Illumina indices):

1) Forward indexing primer:

   AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCAGCGTC

2) Reverse indexing primer:

   CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGTGGGCTCGGPCR

Products were quantified using a PicoGreen dsDNA assay kit (Life Technologies, Carlsbad, CA), normalized and pooled the samples, and concentrated approximately 1 μg of material to 10 μl using 1.8× AMPureXP beads (Beckman Coulter, Brea, CA). The pooled sample was then size-selected at 427 bp ± 20%, on a Caliper XT DNA 750 chip (Caliper Life Science, Hopkinton, MA). The size-selected material was cleaned up using AMPureXP beads and eluted in 20 μl of EB buffer (10 mM Tris-HCl, pH 8.5). The final pooled sample was quantified using the PicoGreen dsDNA assay. The sample pools were diluted to 2 nM on the basis of the PicoGreen measurements, and 10 μl of the 2 nM pool was denatured with 10 μl of 0.2 N NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 20% PhiX, heat-denatured at 96 °C for 2 min and immediately sequenced with a MiSeq 600 cycle v3 kit (Illumina, San Diego, CA).

For data processing, UMGC's bioinformatics pipeline which implements QIIME[455] version 1.9.1 analysis software was used. Raw fastq files were filtered for primer and adapter dimer sequences, removing contaminating host sequences and chimeric sequences, clustering sequences into OTUs using the QIIME open-reference OTU calling method with the greengenes 16s reference. Sequencing adapter sequences were trimmed from the 3' ends of reads using

Trimmomatic [456]. PandaSeq (version 2.7)[457] was used to remove primer sequences from the beginning of reads and to stitch the overlapping paired reads together. Reads without primer sequences and reads that could not be stitched together were discarded. Stitched reads whose length were outside the expected length of the targeted variable region were discarded. FASTQ files were converted to QIIME FASTQ format using a custom script. Individual sample FASTA files were concatenated into one FASTA file, chimera detection was run and chimeric sequences were removed using ChimeraSlayer's usearch61 method.[458] Contaminating host sequences were identified and discarded by aligning stitched reads to the HOST reference genome using BWA/BOWTIE2 [if a bwa or bowtie2 index was provided to the pipeline]. For OTU picking, we used QIIME's pick_open_reference_otus.py script with usearch61. Taxonomy was assigned using QIIME's assign_taxonomy.py script, which uses the RDP classifier and the Greengenes reference database clustered at 97% identity. Reference-based OTUs were then collapsed according to taxonomy at the genus level using QIIME's summarize_taxa.py script.

Statistical Analysis. Statistical analysis of 16S rDNA data was conducted with the Dynamic Assessment of Microbial Ecology (DAME) server.[459] DAME is an open source platform that uses the R environment to analyze and visualize microbial sequencing data. Alpha (α-) and beta (β-) diversity as well as relative abundance were analyzed between high and low growth rate groups. The α-diversity and β-diversity indices were calculated based on the rarefied OTU counts. A Mann-Whitney *U* test was used to compare α-diversity between the two groups; specifically Chao1 was used to determine species richness and the Shannon's index was used to determine species evenness. Permutational multivariate analysis of variance (PERMANOVA) was performed on β-diversity measures using the adonis2() function from the vegan package with a setting of 500 permutations. Bray-Curtis analysis was used to analyze differences between

the two groups. Negative binomial regression[460] using the R package DESeq2[461] was used to determine differential abundance of individual taxa. Likelihood ratio tests for overall experimental group comparisons was used to compare differential abundance between the two groups.

All remaining statistical analyses were performed using GraphPad Prism® 7.01 software (La Jolla, CA). Mean weekly weights was measured by repeated measures two-way ANOVA. ADG, mean viremia, viral load, lymphoid depletion, antibody levels, the mean Firmicutes/Bacteroidetes ratio, and the mean number of Proteobacteria were compared between groups using the unpaired *t*-test. Mean arrival age, mean microbiome diversity and mean number of species within each family were compared between groups using the Mann-Whitney *U* test. Proportions of each group with individual species and families detected were compared using Fisher's exact test.

Results

Weight gain divergence after PRRS vaccination and PRRSV/PCV2 co-challenge led to groups of high and low growth rate pigs. Of the 50 pigs that had fecal samples collected prior to co-challenge, twelve pigs were excluded from the microbiome study due to the presence of clinical disease and/or mortality. Therefore, 38 pigs were considered for selection into high and low growth rate groups for fecal microbiome analysis. These pigs supported subclinical infections or had mild and transient clinical disease that did not require veterinary intervention or antimicrobial therapy. ADG between 0 and 42 dpc was utilized as the selection criteria for the two groups in this study (**Figure 4.1A**). Mean ADG for the high growth rate group was 0.95 ± 0.06 kg, with a range of 0.87 kg and 1.03 kg. Mean ADG for the low growth rate group was 0.71 ± 0.11 kg, with a range of 0.49 kg and 0.83 kg. No overlap occurred between the ADG values of

individual pigs within the two groups and the mean ADG was significantly different over the 42 day co-challenge period between the two groups ($p < 0.001$; unpaired t-test). Mean weekly weights between the two groups were similar during the vaccination period ($p > 0.05$; repeated measures two-way ANOVA); however, ADG over the entire 28-day vaccine period was significantly greater in the high growth rate group. Mean ADG prior to challenge was 0.47 ± 0.08 kg and 0.36 ± 0.09 kg for the high and low growth rate groups, respectively ($p = 0.008$; unpaired t-test). A significant divergence in the mean absolute weekly weights of the two groups occurred at 7 dpc; mean weights of 25.5 ± 3.8 kg and 19.5 ± 3.4 kg were measured in high and low growth rate groups, respectively ($p = 0.01$; repeated measures two-way ANOVA). Weekly weights continued to be significantly different between the two growth rate groups for the remaining 5 weeks of the post-challenge period (**Figure 4.1B**). Over the course of the 70 day study, the growth rate slopes of the two groups were significantly different ($p = 0.004$; linear regression). At the conclusion of the study, the high growth rate group weighed an average of 59.2 kg compared to 44.6 kg in the low growth rate group ($p < 0.0001$; repeated measures ANOVA).

Viremia, antibody production and lymphoid depletion of high and low growth rate groups. Virus replication on weekly to bi-weekly serum sampling days and total viral load in serum were determined for both PRRSV and PCV2b. PRRSV viremia had a bimodal distribution with peaks associated with vaccine virus replication and subsequent challenge virus replication (**Figure 4.2A**). Vaccine virus replication was similar between the two growth rate groups, with peak MLV replication occurring at 11 dpv in both groups ($p > 0.05$; repeated measures analysis). After challenge with wildtype PRRSV, high growth rate pigs had a more rapid incline in virus replication followed by a more rapid decline compared to low growth rate pigs. On 32 dpv, mean

PRRSV viremia was 3.1 and 2.5 $\log_{10}$ copies/PCR reaction for the high and low growth rate groups, respectively ($p = 0.086$; repeated measures analysis using multiple t-tests). High growth rate pigs peaked PRRS challenge virus replication at 35 dpv whereas low growth rate pigs peaked 4 days later at 39 dpv. At 39 dpv, there was a trend towards a significant reduction in the PRRS viremia of high growth rate pigs, with a mean of 2.6 $\log_{10}$ copies/PCR reaction being detected compared to 3.2 $\log_{10}$ copies/PCR reaction in the low growth rate group ($p = 0.076$; repeated measures analysis using multiple t-tests). This trend continued at 42 dpv, where high growth rate pigs (mean 2.1 $\log_{10}$ copies/PCR reaction) had reduced PRRSV detected in serum compared to the low growth rate group (2.7 $\log_{10}$ copies/PCR reaction; $p = 0.091$, repeated measures analysis using multiple t-tests). Overall, PRRSV viral load during the vaccination and challenge periods were similar between the two growth rate groups (**Figure 4.2B**; $p = 0.78$ and $p = 0.95$, respectively; unpaired *t*-test).

PCV2 viremia curves were similar between the two growth rate groups post-challenge, with peak virus replication occurring at 49 dpv followed by a generalized plateau of continued virus detection in the serum until 70 dpv (**Figure 4.2C**). On 39 dpv, there was a trend towards high growth rate pigs having higher PCV2 viremia compared to low growth rate pigs ($p = 0.054$; repeated measures analysis using multiple t-tests); PCV2 detection was approximately 1 $\log_{10}$ copies/PCR reaction greater in high growth rate pigs. Overall, PCV2 total viral loads were similar between the two groups; 57.0 ± 35.5 for high growth pigs and 46.9 ± 29.9 for low growth pigs (**Figure 4.2D;** $p = 0.50$; unpaired *t*-test).

Antibody production against PRRSV N protein, PCV2 whole capsid protein (CP 43-233), and PCV2 decoy epitope (CP 160-233) were quantified at 28 dpv (prior to co-challenge) and 63 dpv (35 dpc; **Figures 4.3A-C**). Although antibody levels were greater numerically in the high

growth rate group against PRRSV N protein after vaccination and after challenge, no significant differences were detected ($p > 0.1$; unpaired t-test). Antibody levels directed at the PCV2 capsid protein were also similar between the two groups. However, low growth rate pigs had significantly higher levels of baseline antibodies directed against PCV2 whole capsid protein (CP 43-233); 0.12 ± 0.05 S:P ratio in high growth pigs compared to 0.27 ± 0.20 S:P ratio in low growth pigs ($p = 0.04$; unpaired *t*-test). These low levels of detectable antibodies prior to challenge are likely associated with passive maternal transfer while nursing.

Tonsillar tissues were examined for lymphoid depletion associated with porcine circovirus associated disease at 70 dpv and compared between high and low growth rate groups (**Figure 3D**; representative histopathologic images shown in **Figures 4.3E and F**). Of the 20 pigs included in the study, 16 pigs (80%) had some degree of lymphoid depletion. Overall, mean lymphoid depletion scores indicated more severe pathology in the low growth rate group; 1.4 ± 0.7 and 1.2 ± 1.0 in low and high growth rate groups, respectively. However, no significant difference between the groups was detected ($p = 0.58$; Mann-Whitney U test).

Improved growth rates were associated with increased fecal microbiome diversity and shifts in microbial composition. Overall, the LLMDA identified 184 uniquely classified microbes across all 20 pigs. Identified bacterial species most commonly fell within the phyla Proteobacteria or Firmicutes. At the family level, fifty-nine unique classification groups were identified, with most being identified at the family level ($n = 52$) and seven additional higher classifications which could not be further identified. From the fifty-nine classifications, most were bacterial ($n = 52$), but other represented groups included archaea ($n = 1$), eukaryotes ($n = 3$) and viruses ($n = 3$). Microbiome diversity was calculated using the LLMDA data as the number of families and species detected in the feces of each pig (**Figures 4.4A and 4B**). The mean

number of families detected were similar in both growth rate groups; 29.2 ± 5.5 and 29.1 ± 2.6 in high and low growth rate groups, respectively (p = 0.96; Mann-Whitney *U* test). Similarly, no significant difference was detected in the mean number of species between the two groups (p = 0.73, Mann-Whitney *U* test). The Firmicutes/Bacteriodetes ratio was calculated by dividing the number of Firmicutes species by the number of Bacteroidetes species. High growth pigs had a higher numerical mean Firmicutes/Bacteroidetes ratio (2.6 ± 1.0 and 1.9 ± 0.7 in high and low growth rate groups, respectively), with a trend towards significance detected (p = 0.07; unpaired *t*-test; **Figure 4.4C**).

At the level of family microbial composition detected by the LLMDA, most microbial families were detected at similar prevalence rates among the two growth rate groups (**Figure 4.5A**). Several microbial families were detected in all 20 pigs, including *Anaplasmataceae*, unclassified Bacteria, *Bradyrhizobiaceae*, unclassified Clostridiales, *Prevotellaceae*, and *Spirochaetaceae*. Although not statistically significant, *Mycoplasmataceae* was detected in less than half of high growth rate pigs (4/10) compared to a greater detection rate in low growth rate pigs (8/10; p = 0.17, Fisher's exact test). Additionally, *Streptococcaceae* was detected at a higher level in high growth rate pigs (7/10) compared to low growth pigs (3/10), albeit a lack of statistical significance (p = 0.18, Fisher's exact test). Species diversity within each family was analyzed for differences associated with growth; two significant differences were detected (**Figure 4.5B**). First, high growth rate pigs had increased species diversity within the group of unclassified bacteria (p = 0.046; Mann-Whitney *U* test). Second, within the *Mycoplasmataceae* family, there was a trend towards less species diversity in high growth pigs; 0.5 ± 0.7 species in high growth pigs and 1.3 ± 0.8 species in low growth pigs (p = 0.059; Mann-Whitney *U* test).

Increased species diversity was also noted in low growth pigs within the family *Prevotellaceae*, although no significant difference was detected ($p = 0.12$; Mann-Whitney *U* test).

At the species level on the LLMDA, three bacterial species were identified at greater rates in high growth pigs (data not shown). *Bacteroides pectinophilus* was detected in half of high growth rate pigs (5/10) while no low growth rate pigs had this species present ($p = 0.03$; Fisher's exact test). Furthermore, two bacterial species in the family *Lachnospiraceae* trended towards having higher prevalence in high growth rate pigs. *Lachnospiraceae* bacterium C6A11 and *Lachnospiraceae* bacterium P6B14 were found in 6 and 7 of the high growth rate pigs, respectively, compared to 1 and 2 of the low growth rate pigs, respectively ($p = 0.057$ and $p = 0.069$, respectively; Fisher's exact test). Although not significant, half the number of high growth pigs (4 versus 8 in low growth group) had *Mycoplasma conjunctivae* and two times the number of high growth pigs (8 versus 4 in low growth group) had *Ruminococcaceae* bacterium AE2021 detected in the feces ($p = 0.169$; Fisher's exact test). These bacterial species may play a role in promoting or deterring growth performance after vaccination and co-challenge.

In addition to the LLMDA, fecal bacteriomes were further analyzed using 16S rDNA sequencing. Two of the samples from high growth rate pigs were excluded from the analysis due to failed quality control after 16S rDNA sequencing at UMGC. Therefore, 16S rDNA sequencing results were compared between 8 high growth rate pigs and 10 low growth rate pigs. Both bacterial and archaeal microbes were identified through 16S rDNA sequencing of fecal samples, with several bacterial families being detected across all 18 pigs, such as *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Lactobacillaceae*, *Prevotellaceae*, *Streptococcaceae*, and *Veillonellaceae*, *Coriobacteriaceae*, and *Erysipelotrichaceae*.

Based on the relative abundance of OTUs, the two most abundant phyla were Bacteroidetes and Firmicutes in the fecal samples from the 18 pigs; no significant differences in relative abundance of these two phyla were identified between the growth rate groups (adjusted $p > 0.05$; Likelihood Ratio Test; **Figure 4.6A**). However, the relative abundance of bacteria in the phylum Spirochaetes trended toward being significantly higher in the high growth group (adjusted $p = 0.06$; Likelihood Ratio Test). The relative abundance of Spirochaetes was 1.5% in high growth pigs while only 0.3% in low growth pigs. At the family level (**Figure 4.6B**), a lower relative abundance of *Lachnospiraceae* was detected in high growth rate pigs (5.8%) compared to low growth rate pigs (8.2%; adjusted $p = 0.021$; Likelihood Ratio Test).

Alpha diversity between the two groups was compared using Chao1 and Shannon Index metrics (**Figure 4.6C**). The Chao1 diversity metric based on OTU was significantly higher in high growth pigs compared to low growth pigs ($p = 0.026$; Mann-Whitney *U* test). Further, the observed OTU diversity was also significantly greater in high growth rate pigs ($p = 0.023$; Mann-Whitney *U* test; data not shown). This data suggests gut microbiome diversity may be beneficial for growth under the study conditions. Although the mean Shannon Index was numerically higher in high growth rate pigs, no significant difference was detected between the two groups ($p = 0.145$; Mann-Whitney *U* test). Beta diversity analysis did not detect any significant differences at the phyla or family level between the two groups ($p > 0.05$; PERMANOVA; data not shown).

Overall, microbial testing modalities utilized in the current study identified several gut microbiome characteristics associated with improved growth after vaccination and co-infection, including increased bacterial diversity, increased *Bacteroides pectinophilus*, decreased *Mycoplasmataceae* species diversity, higher Firmicutes:Bacteroidetes ratios, increased relative

abundance of the phylum Spirochaetes, reduced relative abundance of the family *Lachnospiraceae,* and increased *Lachnospiraceae* species C6A11 and P6B14.

Discussion

There is a growing body of evidence for the role of the gut microbiome in response to vaccination for infectious diseases of humans and livestock.[340,341] Despite PRRS MLV vaccines being widely used to reduce PRRS-associated losses in endemic herds, the currently available commercial vaccines are inadequate for disease control[171] and additional tools are necessary to reduce the effects of PRRS on swine production. Through the gut-lung axis, a bi-directional communication pathway between the gastrointestinal tract and pulmonary tissues,[462] beneficial gut microbes provide an opportunity to improve immunity and efficacy of PRRS MLV vaccines.

Herein, several microbiome characteristics were identified in the post-vaccination/pre-challenge feces of pigs that had subsequent improvements in growth during the co-challenge period. These characteristics may predispose more rapid weight gain in the presence of wild-type PRRSV after vaccination. First, increased gut microbiome diversity was detected in high growth rate pigs. Microbiome diversity has also been associated with reduced clinical disease and high growth rates of co-infected pigs in the absence of PRRS vaccination.[118,291] Moreover, associations between microbiome diversity and improved outcome following challenge with bacterial respiratory pathogens in swine have been reported.[287] Further, antibody production after vaccination for *Mycoplasma hyopneumoniae* and PCV2 has been linked to increased gut microbiome diversity in pigs.[463] However, reduced gut microbiome diversity was associated with enhanced antibody production in pigs immunized with cholera toxin subunit B orally and tetanus toxoid intramuscularly.[464] Although increased gut microbiome diversity has shown to be

beneficial after respiratory infection with diverse pathogens,[280] further studies are necessary to clarify effects on infectious and noninfectious immunizations.

In addition to respiratory and immunological outcomes, PRRSV reduces nutrient digestibility and feed efficiency of growing pigs.[288] Here, the primary differentiator between high and low growth rate groups was indeed weight gain, as no significant differences were detected in humoral immunity, clinical disease or tonsillar pathology. As such, an interesting second microbiome characteristic associated with high growth included an increased Firmicutes to Bacteroidetes ratio. In early gut microbiome research evaluating lean versus obese humans, increased Firmicutes abundance and decreased Bacteroidetes abundance were considered to have enhanced nutrient extraction capabilities.[289,290] The trend of Firmicutes:Bacteroidetes ratios being increased in high growth nonvaccinated pigs after PRRSV/PCV2 co-infection has also been reported.[291] Moreover, a positive correlative relationship between Firmicutes abundance and weight gain[292,293] and a negative correlative relationship between Bacteroidetes abundance and weight gain[294] have been demonstrated in swine. Whereas, others reported no significant differences in the relative abundance of these two phyla between high and low growing pigs.[295]

Perhaps surprising was the current finding of high growth pigs having an increased relative abundance of bacteria in the phylum Spirochaetes, as this family is generally considered pathogenic.[465] In contrast, previous work reported decreased species diversity within the family *Spirochateaceae* in association with improved clinical disease outcome after fecal transplantation and PRRSV/PCV2 co-infection.[338] Further, in non-disease challenged conditions, high body weight pigs had significantly less Spirochaetes phylum abundance in feces compared to low body weight pigs.[295] However, another study demonstrated that increased relative abundance of bacteria within the Spirochaetes phyla is positively correlated to hemicellulose digestibility in

pigs, increasing the availability of energy from dietary plant material.[466] Differences between these studies underscore the need for further clarification of *Spirochateaceae* species function in health-challenged and growing swine.

Three bacterial species, including *Bacteroides pectinophilus*, *Lachnospiraceae* bacterium C6A11 and *Lachnospiraceae* bacterium P6B14, were detected at greater prevalence rates in high growth rate pigs. First, *Bacteroides pectinophilus* is an obligate anaerobe known to be an inhabitant of the human gut,[467] which aids in the breakdown of pectin, an otherwise indigestible portion of plant cell walls. *B. pectinophilus* is considered unique from other *Bacteroides* species based on 16S rRNA genetic diversity and the presence of novel protein families[468] and can diminish in gastrointestinal diseases such as irritable bowel syndrome.[469] *Lachnospiraceae* bacterium C6A11 and Lachnospiraceae bacterium P6B14 are unclassified anaerobic fermentative species in the Firmicutes phylum originally isolated from a cow rumen in New Zealand[470] that play an important role in metabolism of plant material.

Interestingly, *Lachnospiraceae* results reported herein differed based on assay; specifically, high growth rate pigs had increased prevalence of the two species described above per the LLMDA and decreased relative abundance of the family per 16S rDNA sequencing. In other studies of swine, fecal microbiota transplantation increased the relative abundance of *Lachnospiraceae* and was associated with improved growth and health outcomes.[338,471] Data published by Tran et al. (2014, 2018) found that pigs consuming feed with spray-dried porcine plasma had increased ADG and decreased relative abundance of *Lachnospiracaeae* in fecal samples; further, a significant negative correlation was detected between one *Lachnospiraceae* species (*Blautia marasmi*) and body weight.[472,473] Consistent with this dilemma and the need for further understanding the role of *Lachnospiraceae* bacteria, a recently published review

describes the inconsistency of *Lachnospiraceae* as both beneficial for health and metabolism while also being associated with certain disease conditions as a challenge in human microbiome research.[474]

Many diverse studies have demonstrated a role for gut microbes in host outcome following respiratory disease and growing evidence suggests gut microbes modulate the response to infectious disease immunizations. Described herein, several gut microbiome characteristics, such as increased diversity and shifts in microbial composition, were associated with a significant increase in ADG of pigs after PRRSV vaccination and PRRSV/PCV2 co-infection. These microbiome characteristics may contribute to improved outcome of pigs exposed to either attenuated vaccine or wild-type PRRS viruses. Further research aimed to expand these findings and identify beneficial gut microbes that may improve the health of pigs in PRRS endemic herds and aid in the efficacy of current PRRS vaccines is warranted.

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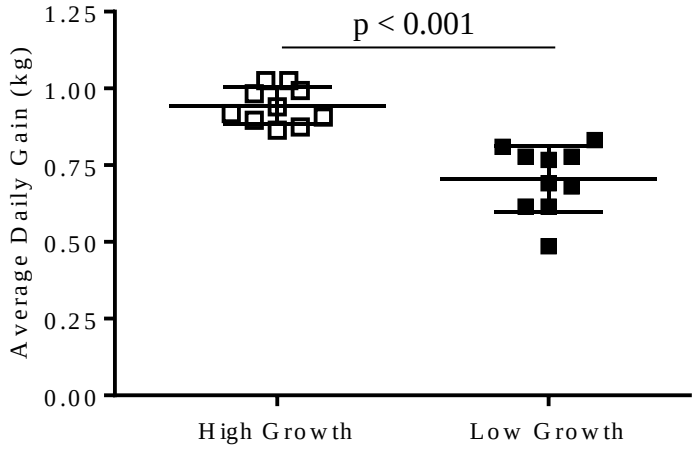
A. Average daily gain post-challenge



B. Weekly body weights

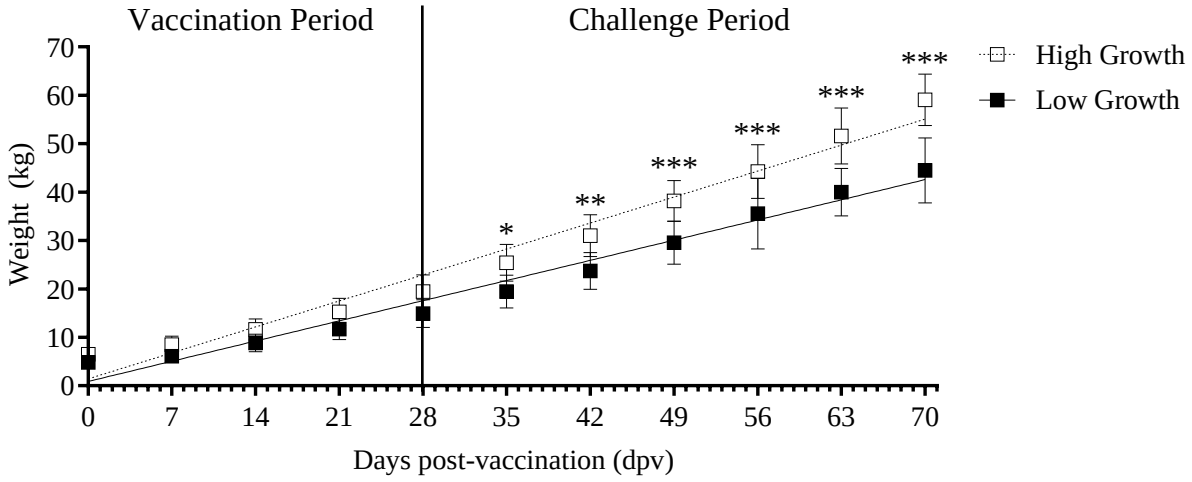


Figure 4.1. Weight gain in high and low growth pigs after PRRSV MLV vaccination and co-challenge with PRRSV and PCV2b.

A) Data shown as mean ADG in kg ± one standard deviation for high and low growth rate groups post-challenge. Mean ADG was significantly greater in high growth pigs ($p < 0.001$; unpaired t-test). **B)** Data shown as the mean weight in kg ± one standard deviation with regression lines. Asterisks identify statistically significant differences in mean weights between the two groups (*$p \leq 0.01$, **$p \leq 0.001$ and ***$p \leq 0.0001$; two-way ANOVA with repeated measures). The slope of weight gain in high growth rate pigs was significantly greater than low growth rate pigs ($p = 0.004$; linear regression analysis).

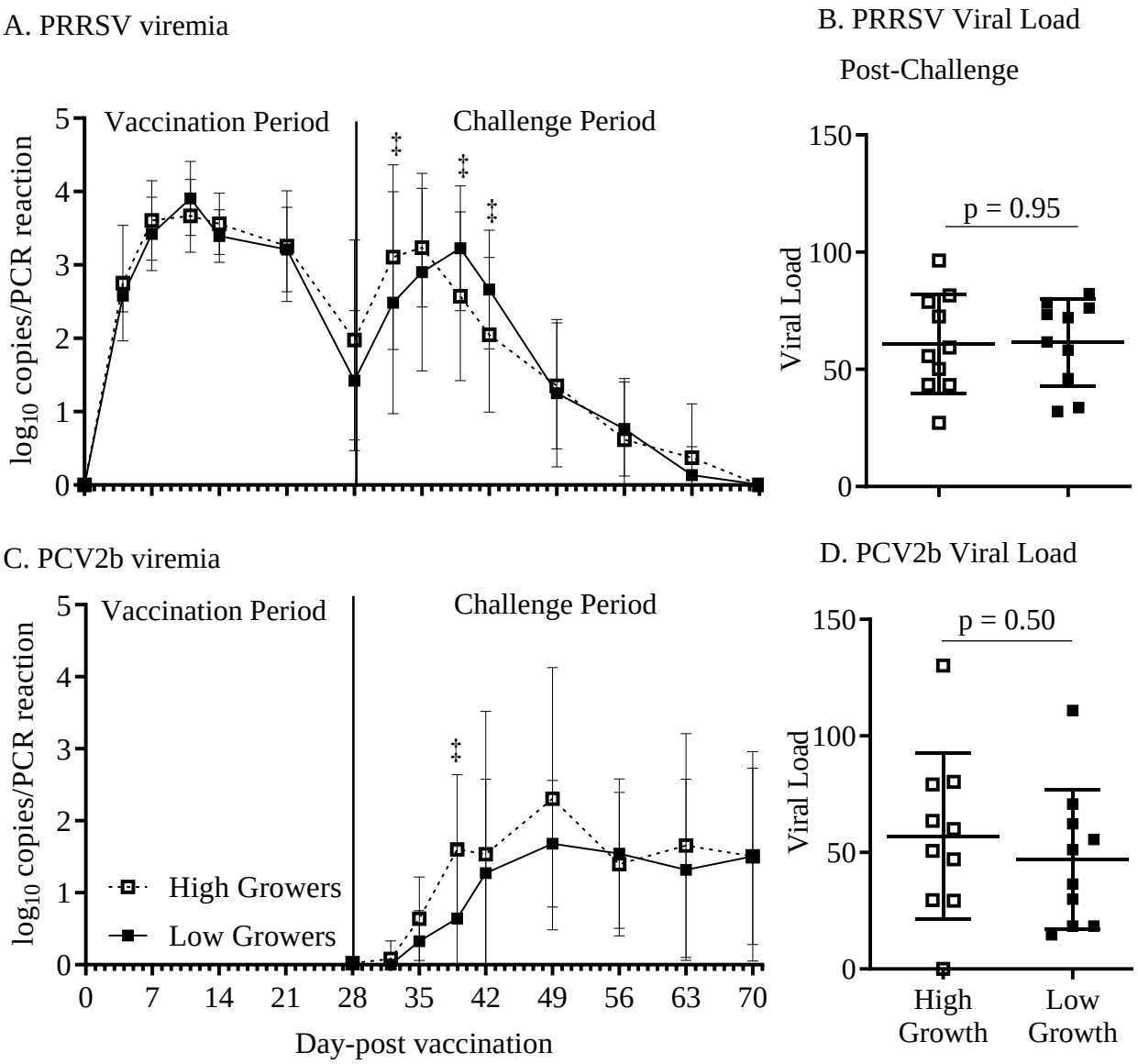


Figure 4.2. PRRSV and PCV2 virus replication in high and low growth rate pigs after PRRS MLV vaccination and PRRSV/PCV2 co-challenge.

Data shown as the mean log10 copies/PCR reaction ± standard deviation for each virus and growth rate group (**A and C**). On 32 dpv, the high growth rate group had a trend towards significantly higher PRRSV replication, while on 39 and 42 dpv, the low growth rate group had a trend towards significantly higher PRRS viremia. On 39 dpv, the high growth rate group had a trend towards significantly higher PCV2 viremia. Trends towards significance (‡$p < 0.1$; repeated measures analysis using multiple t-tests) are highlighted. **B and D**) Data shown as the post-challenge total viral loads for each pig with horizontal lines representing mean ± one standard deviation. No significant differences in PRRSV or PCV2 viral load was noted post-challenge (unpaired t-test).

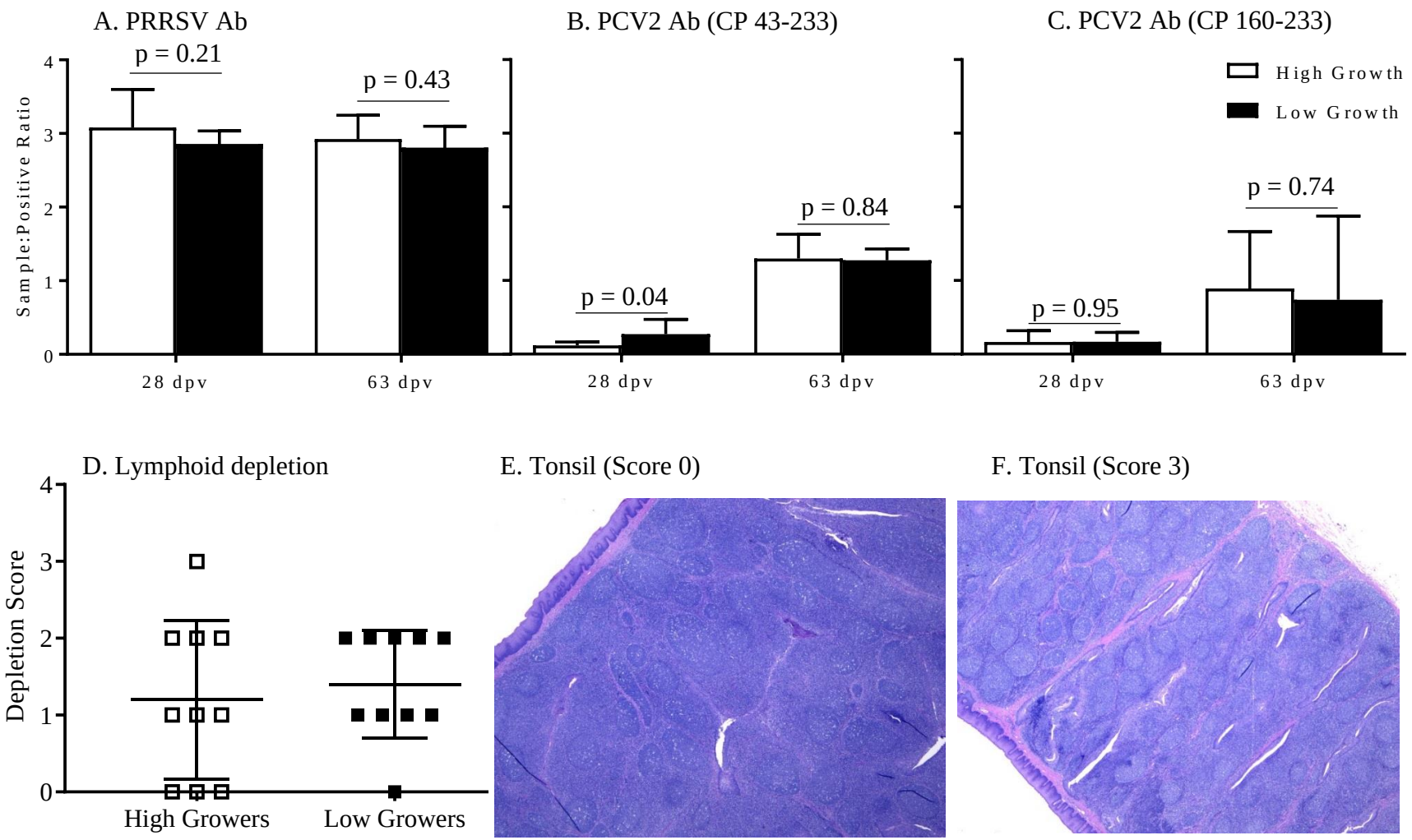


Figure 4.3. Antibody response and lymphoid depletion in high and low growth rate pigs after PRRS MLV vaccination and co-challenge with PRRSV and PCV2

Data shown as the mean Sample:Positive (S/P) ratio ± one standard deviation in each group for PRRSV **(A)**, PCV2 CP 43-233 epitope **(B)**, and PCV2 CP 160-233 decoy epitope **(C)** on 28 and 63 dpv with p-values (unpaired t-tests). **D)** Data shown as individual lymphoid depletion scores for each of the 20 pigs with horizontal lines representing mean scores ± one standard deviation. Representative histopathologic images of 2X H&E stained tonsils are shows as follows: score 0, no lymphoid depletion (E) and score 3, severe lymphoid depletion (F). No significant difference was detected in the severity of lymphoid depletion scores between the two groups ($p = 0.58$, Mann-Whitney U test).

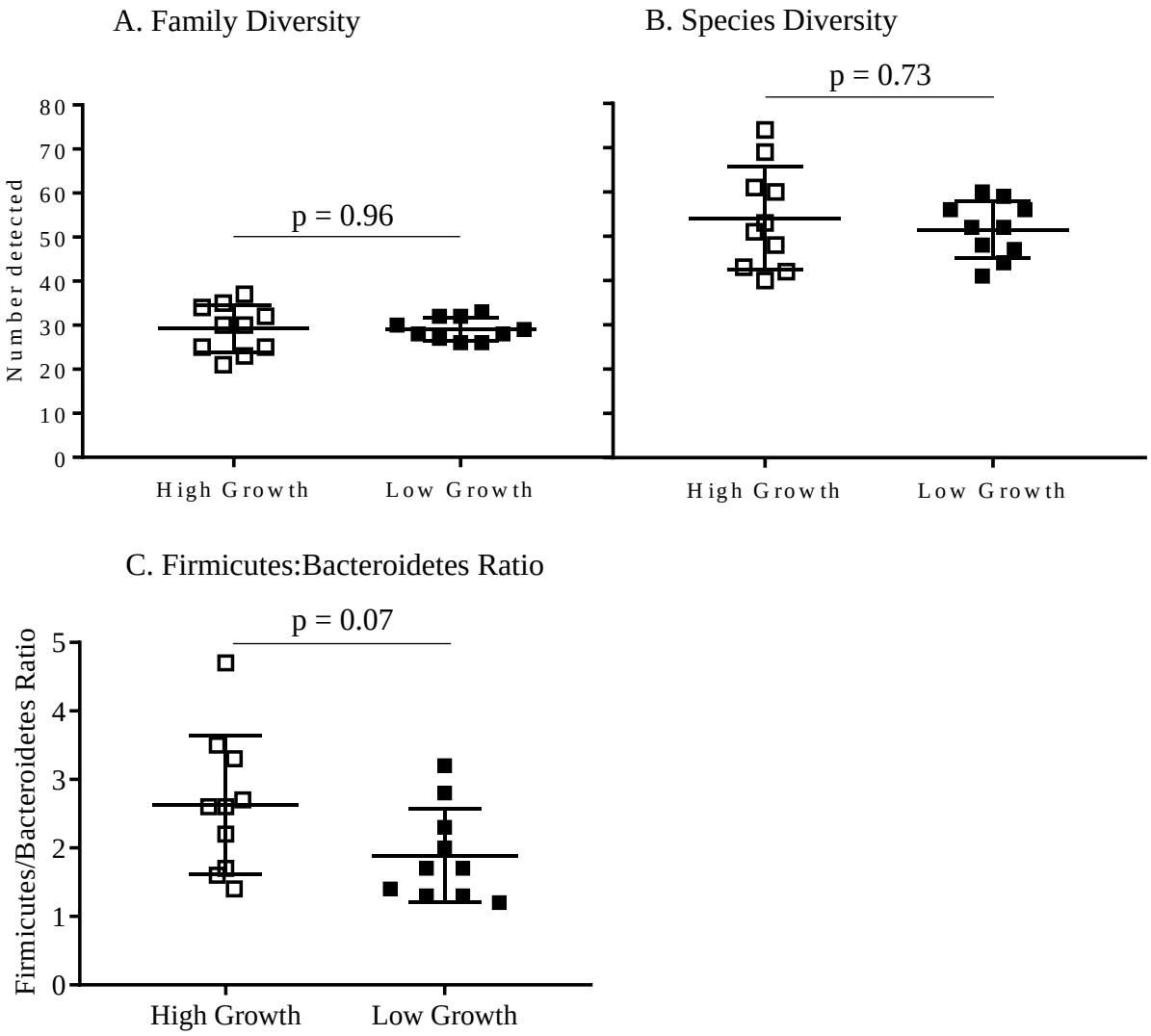


Figure 4.4. Fecal microbiome diversity in pigs with high and low growth rates after PRRS MLV vaccination and subsequent co-infection with PRRSV and PCV2.

Data shown as the total number of microbial families **(A)** and microbial species **(B)** detected by the LLMDA prior to co-challenge for individual pigs. Group means and standard deviations are represented by horizontal lines. No significant difference in microbiome diversity was detected on a family or species level between the two groups ($p > 0.05$; Mann-Whitney U test). **C)** Data shown as the Firmicutes:Bacteroidetes ratio for each pig in the two groups. Horizontal lines represent mean ± one standard deviation. High growth pigs had a trend towards significantly higher ratios when compared to low growth pigs (unpaired t-test).

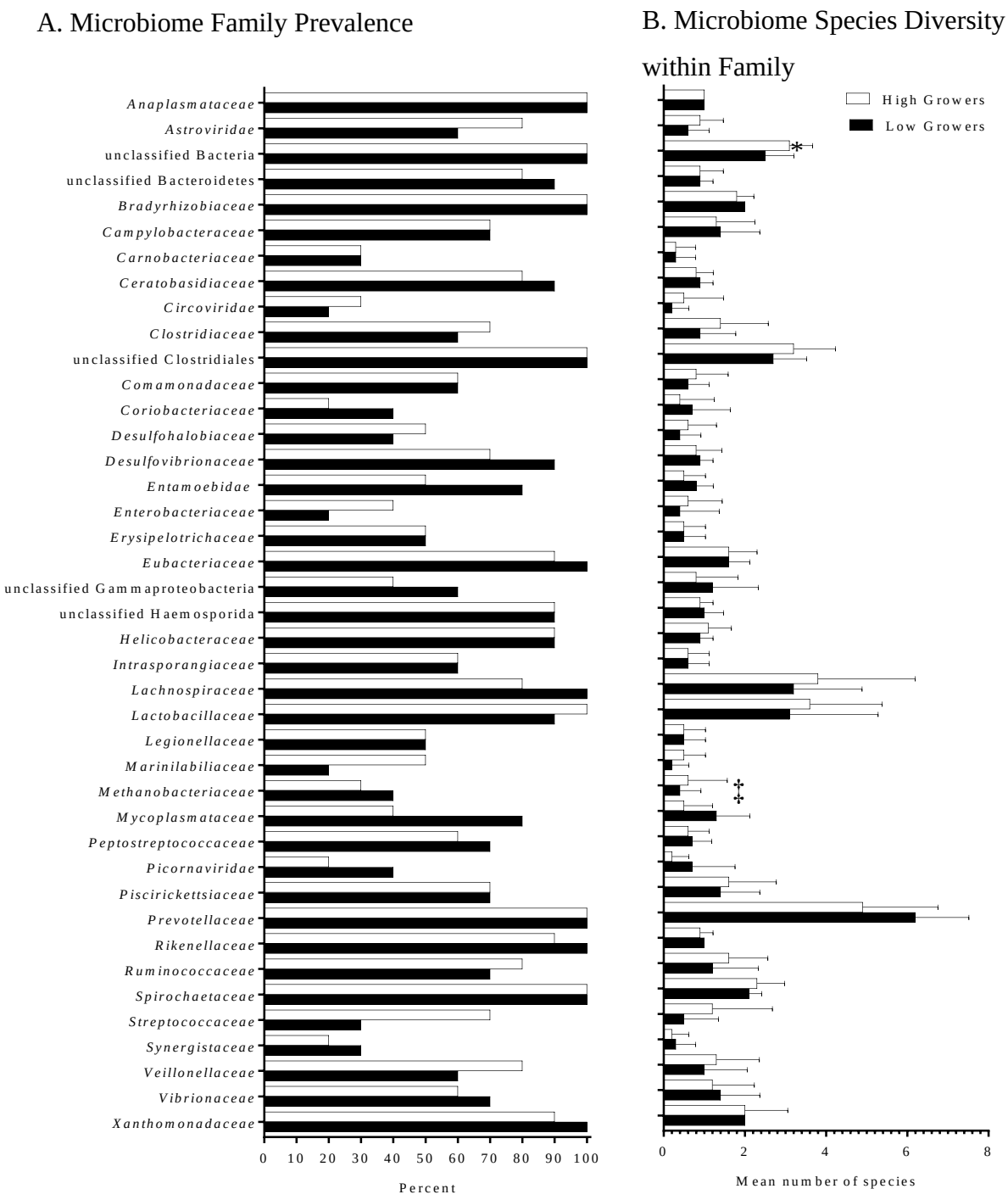


Figure 4.5. Fecal microbiome composition in pigs with high and low growth rates after PRRS MLV vaccination and subsequent co-infection with PRRSV and PCV2.

A) Microbiome family prevalence is shown as the percent of high growth (n = 10) and low growth (n = 10) rate pigs with each family detected on the LLMDA. Families detected in less than 40% of all 20 pigs are not shown. No significant differences in family prevalence were detected between growth rate groups**. B)** Data shown as the mean number of species detected ± one standard deviation in each family identified in high and low growth rate pigs. Statistical significance (*$p < 0.05$) and trends towards significance (‡$p < 0.1$) are highlighted.

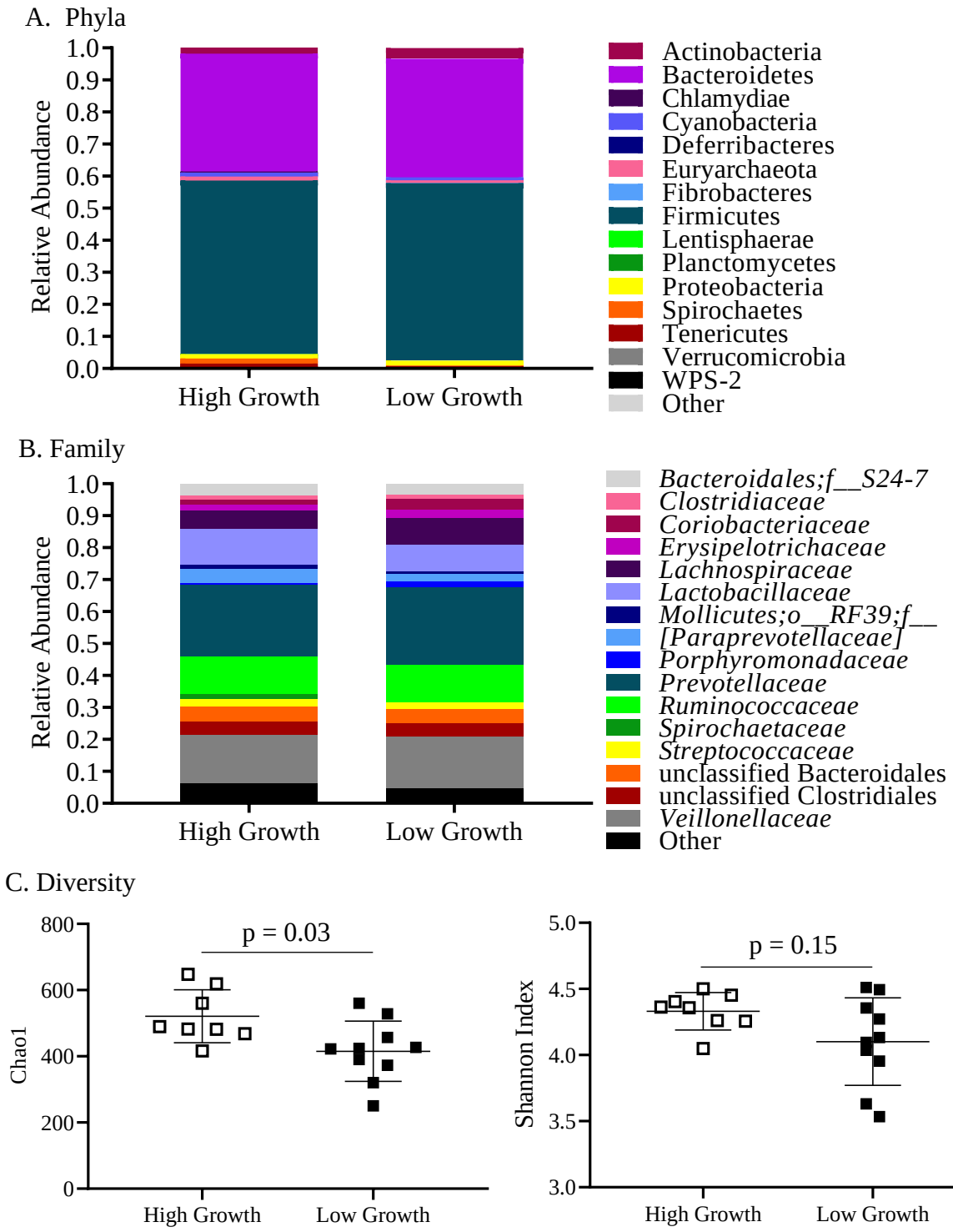


Figure 4.6. Fecal microbiome analysis by 16S rDNA sequencing in high and low growth rate pigs.

A) Data shown as the mean relative abundance of bacterial phyla for high and low growth groups. **B)** Data shown as the mean relative abundance of bacterial families for high and low growth groups. Families making up 1% or less of all sequences detected in 1 or more sample

subsets are grouped together and classified as “Other”. **C)** Data shown as the α-diversity metric (Chao1 or Shannon Index) for individual pigs in each group. Group means and standard deviations are represented by horizontal lines. Chao1 α-diversity was significantly greater in the high growth group (Mann Whitney U test).

Chapter 5 - Fecal Microbiota Transplantation Reduces Viremia after Porcine Reproductive and Respiratory Syndrome (PRRS) Modified Live Virus (MLV) Vaccination

Abstract

Fecal microbiota transplantation (FMT) is the process by which the fecal microbiota from a healthy individual is transplanted into an immature or diseased individual. Benefits of FMT are thought to be the result of enhanced numbers of beneficial microbial populations. Our previous study established that FMT improved outcome after co-infection with PRRSV and porcine circovirus type 2d. The objective of this study was to determine the effects of FMT on PRRSV modified live virus (MLV) vaccination. Pigs were split into four groups; two groups of pigs (FMT; $n = 20$) were administered a fecal microbiota transplant while two control groups ($n = 20$) were administered a sterile mock-transplant for 7 days prior to vaccination. One FMT and one control group were then vaccinated with the PRRSV MLV vaccine and allowed to mount an immune response over the next 28 days. Then all pigs were infected with PRRSV and followed for 42 days. Throughout the duration of the study FMT and vaccination status had no effect on morbidity and treatment rates, nor PRRSV-associated pathology. During the 28 day vaccination period transplanted pigs had lower, however not significant, viremia levels. Over the 42-day post-infection period, while PRRS MLV vaccination decreased viremia and increased antibody load, there was no effect seen due to transplantation. FMT resulted in overall decreases in microbial diversity; however, shifts in microbial composition were consistent to previous studies; increased microbes within taxonomic groups Bacteroidetes, *Prevotellaceae*, *Turcibacteraceae*, *Ligilactobacillus ruminis* and *Limosilactobacillus pontis,* as well as a decrease of microbes

within the taxonomic group Micrococcales. Overall, this study supports the idea that FMT improves PRRSV MLV vaccination by reducing vaccine-associated viremia.

Introduction

Porcine reproductive and respiratory (PRRS) modified live virus (MLV) vaccines are widely used to reduce PRRS-associated losses. PRRS, caused by the PRRS virus (PRRSV) within the *Arteriviridae* family, triggers increased morbidity and mortality in growing pigs, due to respiratory disease, decreased reproductive performance, and weight gain reductions. PRRSV has an inherently high mutation rate attributable to the high error frequency during RNA replication,[28,29] resulting in a diverse genetic population. This substantial genetic variation creates challenges in stimulating long-term and broadly protective immunity, either through natural exposure or through modified live virus vaccination.[14,15] In experimental and field settings, PRRS MLV immunization has the potential to improve weight gain, reduce viral replication, pulmonary pathology, and clinical disease after wild-type PRRSV exposure.[163] However, several challenges remain for PRRS MLV vaccine safety and efficacy, including the potential for virulent reversion and wild-type strain recombination,[164] pathogen potentiation,[116] and incomplete protection against emerging wild-type strains.[165] Available commercial vaccines generally provide insufficient disease control and superior or alternatives PRRS control strategies are necessary.[170,171]

The gut microbiome is a collection of microorganisms, composed of bacteria, viruses, fungi, archaea and protozoa, that inhabit the gastrointestinal tract. Within the gut, there is abundant immunomodulation, due to microbial interaction with host epithelial and immune cells, secretory immunoglobulins, and metabolites that cause changes not only locally within the gut, but also to distant places such as the respiratory tract. Pigs without a microbiome, also known as

gnotobiotic, have decreased immune function, T-lymphocytes,[315] fecal secretory immunoglobulin A,[317] and less developed Peyer's patches.[475] Since the gut microbiome plays such a significant role in immunological development and regulation, it is likely that it also contributes to vaccine-induced immune response.

Research focused on gut microbiome associations with PRRS vaccine efficacy is limited. While one study shows no effect of *Lactobacillus casei* on PRRS vaccine response,[342] another showed that after PRRSV vaccination, increased *Lactobacillus* relative abundance was associated with increased antibody titers and milder pathogenic damage.[343] After PRRSV challenge, there were also positive correlations between *Lactobacillus* and *Prevotella* bacteria and decreased rectal temperatures. Another study from the same group showed that increased *Prevotella* and *Ruminococcus* abundance pre-vaccination best predicted increased antibody titers in association with vaccine response. The relationship between the gut microbiome and other porcine respiratory diseases has been illustrated.[340,341,344] For example, high immunoglobulin G (IgG) production, after *Mycoplasma hyopneumoniae* immunization in pigs, was connected with increased *Lachnospiraceae* and *Prevotella* bacteria, when compared to pigs with low IgG production.[346] In a study investigating the immune response to influenza A virus vaccination, pigs with increased microbiome diversity, and increased *Prevotella* and *Muribaculaceae* abundance were associated with a stronger immune response, measured by virus-specific IgG and hemagglutination inhibition assays, while weaker response was associated with increased abundance of *Helicobacter* and *Escherichia-Shigella* bacteria.[476]

Together with the known effects of gut microbiome on outcome after PRRSV infection, PRRSV has also been shown to effect the gut microbiome. In one study, PRRSV infection caused immune modulation associated with the gut microbiome.[477] PRRSV infected pigs had

higher *Treponema* and *Methanobrevibacter* abundance, and reduced *Prevotella* abundance. Decreased bacteria within the taxonomic groups *Methobrevibacter, Spirochaeta, Micrococcaceae* and *Spirochaetaceae,* as well as increased bacteria within the taxonomic groups Firmicutes, *Lachnospiraceae*, and *Prevotellaceae* were associated with decreased PRRS-associated clinical signs, gross lesions and viremia. Increased *Micrococcaceae* and *Spirochaetaceae* in addition to decreased Firmicutes bacteria were associated with increased interferon-gamma (IFN-γ), interleukin 6 (IL-6) and haptoglobin. Dynamically shifting these microorganisms could lead in more appropriate immune responses to PRRSV infection and vaccination.

Fecal microbiota transplantation (FMT) was originally described as the process by which fecal material, containing microorganisms, from a healthy individual is transplanted into a diseased individual. Fecal transplantation was described as early as the 4th century in China for the human diarrheal treatment[407] and in the 17th century in Italy for ruminant disease treatment[368] suggesting its possible role in microbe modulation leading to clinical and immunological changes. These treatments can be implemented without antibiotic usage, which has enormous implications in the face of ever growing antimicrobial resistance concerns.

Previous work in pigs has demonstrated that FMT improved clinical disease outcome and antibody response, and decreased lung pathology response after PRRSV and porcine circovirus type 2d co-infection.[478] Improved outcome was associated with increased microbial family numbers, higher *Intrasporangiaceae* and *Synergistaceae* species prevalence, lower *Spirochaetaceae* and *Vibrionaceae* species diversity, and increased *Veillonellaceae*, *Lachnospiraceae,* and *Ruminococcaceae* relative abundance. With respect to other porcine respiratory disease, one study showed that an oral microbial inoculum obtained from healthy

boars prior to *Mycoplasma hyopneumoniae* challenge resulted in decreased coughing and lung pathology.[287] The objective of the current study was to determine if FMT from high-parity healthy sows could improve the response of the PRRSV MLV vaccine. The results showed that FMT lowered PRRSV MLV vaccine-associated viremia. This was associated with increased microbial abundance within taxonomic groups Bacteroidetes (Bacteroidia, Bacteroidiales, and *Bacteroides zylanisolvens*), *Prevotellaceae*, *Turicibacteraceae* (unclassified *Turicibacter sp.*), *Ligilactobacillus ruminis* and *Limosilactobacillus pontis,* together with decreaseed microbial abundance within the taxonomic group Micrococcales (*Microbacteriaceae*). Altering these specific bacterial groups could lead to improved PRRSV MLV vaccine response.

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. The dates of the study were between May 14, 2018 and July 31, 2018. Twenty pairs of Yorkshire x Landrace barrow siblings (n = 40; 18.6 ± 0.5 days of age upon arrival) were obtained at weaning from a single high health, closed colony commercial source negative for PRRSV, *Mycoplasma hyopneumoniae*, pseudorabies virus, porcine epidemic diarrhea virus (PEDV) and acute malignant hypothermia (porcine stress syndrome). Sibling pairs were from 10 different sows. The piglets were not vaccinated for PCV2 and were utilized without regards to maternal antibody. No prophylactic or therapeutic antibiotics were administered at weaning or within 1 week of arriving at Kansas State. All pigs were housed in one environmentally controlled room

at the Kansas State University Large Animal Research Center and maintained under biosafety level 2 (BSL-2) conditions. Pigs were housed in groups of 10 in a 98.2 sq ft pen on concrete flooring with mats. All pigs were given approximately 9 hours to acclimate to their new environment. Pen order was maintained throughout the study to avoid cross-contamination of fecal microbiota; personnel entered pens in the following order: 1) control non-vaccinated pen, 2) FMT non-vaccinated pen, 3) control vaccinated pen, and 4) vaccinated FMT pen; personal protective equipment was changed between non-vaccinated, FMT pigs and vaccinated, control pigs. Pigs were given access to food and water ad libitum.

Fecal microbiota transplant (FMT). *FMT preparation and administration:* The fecal transplant material was obtained in the same method and is described previously.[478] Briefly, two sows from a commercial farrow-to-wean farm in Kansas were selected as donors for the transplant material. This herd was negative for PRRSV and had recently undergone a *Mycoplasma hyopneumoniae* elimination program. The two sows were selected based on several characteristics, including older age, high parity, large litters with a high percentage of born alive piglets, low pre-weaning mortality, no history of fetal mummification, and no antibiotic treatment received within at least the last 15 months prior to donation. Feces were collected during lactation and sows had not yet weaned their respective litters at the time. Feces were initially screened and confirmed as negative for gastrointestinal parasites using a fecal float qualitative exam by the Kansas State Veterinary Diagnostic Laboratory (KSVDL).

To prepare the FMT, fresh feces was collected naturally during defecation or manually from the rectum of the two sows. Feces were processed within approximately 3 hours after collection, during which the fecal microbiota was concentrated and stored using a protocol adapted from the human FMT literature.[412] Specifically, feces were weighed into 50 gram

aliquots and mixed in a standard commercial blender (Oster, Sunbeam Products Inc.) with 250 mL of sterile saline (0.9% sodium chloride irrigation USP, Braun Medical) until homogenized. The fecal slurry was then passed progressively through 2.0, 1.0, 0.5, and 0.25 mm stainless steel sieves into a sieve receiver (Fisherbrand™). The filtered liquid was collected, aliquoted into 50 ml tubes, and centrifuged at 6,000xg for 15 minutes. The supernatant was discarded and each bacterial pellet was resuspended in approximately 20 mL of sterile saline. All resuspensions were gently vortexed prior to mixing the concentrated microbiota in a large beaker. Glycerol (molecular biology reagent grade, MP Biomedicals™) was added to create a 10% glycerol suspension and the transplant material was stored at -80°C until the day of transplantation. On the day of transplantation, the FMT was thawed for 2 hours on ice and kept cold prior to administration.

FMT Characterization: Prior to administration, the FMT material was analyzed for microbial composition and pathogenic organisms. The FMT material was submitted to KSVDL for routine bacterial culture, including aerobic culture, anaerobic culture and *Salmonella* enrichment. Species identification was attempted for all bacteria cultured. The Lawrence Livermore Microbial Detection Array (LLMDA) was used to analyze microbiome composition and diversity of the transplant material. This array detects annotated sequences of microbes associated with infection of vertebrates within GenBank®, the National Institute of Health genetic sequence database. The version 7 of the LLMDA in the 4plex 180K probe format was used in this study. This version of the array targets 4,377 viruses, 5,457 bacteria, 327 archaebacteria, 319 fungi, and 132 protozoa. The LLMDA oligonucleotide probes vary between 50 and 65 nucleotides in length and have roughly equivalent affinities for their complementary target DNA molecules [415]. Probes 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.[118,416-418]

The methods have been discussed previously;[478] however, they will be briefly described. The PowerViral™ Environmental RNA/DNA Isolation Kit (MO BIO, San Diego, CA) was used to extract DNA and RNA from the fecal samples using the kit's protocol. For each sample, 10 µl of the extracted DNA and RNA was amplified using the random amplification procedure.[418] The amplified cDNA and DNA was purified with the Qiaquick PCR purification columns (Qiagen) and quantified using the Nanodrop™ spectrophotometer. Approximately 1 µg of amplified cDNA and DNA were fluorescently labeled using a one-coloring labeling kit (Roche NimbleGen, Madison, WI). The samples were labeled using nick translation with Cy3-labeled random nonamer primers (TriLink Biotechnologies, San Diego, CA) and Klenow DNA polymerase at 37°C for 2 hr. The labeled DNA was precipitated in isopropanol, centrifuged for 10 min, and the pellet was washed and dried. The pellet was then reconstituted in 50 µl of RNase-Free water and quantified using the Nanodrop™ spectrophotometer. The Agilent Technologies Oligo aCGH/ChIP-on-Chip Hybridization kit (Santa Clara, CA) was used to hybridize samples to the arrays. For each sample, 10 µg of fluorescently labeled DNA was mixed with blocking agent, hybridization buffer and nuclease free water. The samples were then denatured at 95˚C for 3 min, and incubated at 65˚C for 3 min. Each sample was then immediately loaded onto the array and hybridized for approximately 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 for 5 min at room temperature and Oligo aCGH/ChIP-on-chip Wash Buffer 2 for 1 min at 37°C (Agilent Technologies Inc., Santa Clara, CA). Using the SureScan Microarray Scanner (Agilent Technologies Inc., Santa Clara, CA), all arrays were scanned to a resolution of 3 µm. Microarray data was generated from the microbe sequences using the CLiMax method developed at Lawrence Livermore National Laboratory,[416] at a detection threshold of ≥ 99%. 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.

FMT microbial composition: Fecal floatation for parasites confirmed feces were negative for parasites through standard diagnostic testing at KSVDL. Aerobic and anaerobic culture identified several culturable bacteria known to inhabit the gastrointestinal tract, including non-hemolytic *Escherichia coli*, *Bacillus altitudinis*, *Streptococcus alactolyticus*, *Enterococcus hirae*, non-hemolytic *Staphylococcus* sp., *Bacteroides vulgatus*, and *Clostridium perfringens*. Several additional anaerobic bacteria were cultured but unable to be identified at the genus or species level; these bacteria included gram negative coccobacilli, gram positive long rods, and large gram positive boxy rods. Salmonella enrichment culture was negative. The pan-microbial array detected 12 phyla, 33 microbial families and 49 microbial species detected. Microbes were from the phyla Actinobacteria, Amoebozoa, Bacteroidetes, Basidiomycota, Euryarchaeota, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes, and Tenericutes. Additionally, a single virus was detected. The majority of species detected fell within the Proteobacteria phylum (16/49; 32.7%) with the second highest number of species falling with the Firmicutes phylum (9/49; 18.4%) and the third highest number of species falling within the Tenericutes phylum (6/49; 12.2%). Known swine pathogens were not detected with the LLMDA.

PRRS Virus. The PRRS virus used to prepare the inoculum for this study originated from the lymph node of a pig with severe postweaning multisystemic wasting syndrome (PMWS) as previously described.[117] PRRSV (isolate KS62; GenBank accession no. KM035803) was isolated by propagation on MARC-145 cells. PRRSV titration was also performed on MARC-145 cells as previously described [116]. Briefly, PRRSV infectivity was titrated through serial 10-fold dilutions of PRRS stock virus in minimal essential medium (MEM; Corning) supplemented with 7% fetal bovine serum (FBS; Sigma-Aldrich), penicillin-streptomycin (Pen Strep; 80 U/mL and 80 µg/mL, respectively; Gibco), 3 µg/mL amphotericin B (Fungizone; Gibco), and 25 mM HEPES (Life Technologies). The dilutions were 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 median tissue culture infective dose ($TCID_{50}$/mL) was calculated using the Spearman-Karber method.[411] To prepare the inocula for pigs, the stock virus was made to yield a 2-mL dose consisting of 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.

Experimental design and sample collection. The experimental design was a 2 x 2 factorial design with FMT administration and PRRS vaccination. Four treatments each employing a group of pigs were applied: (a) pigs treated with FMT and vaccinated against PRRS (vaccinated, FMT), (b) pigs vaccinated against PRRS and not treated with FMT (vaccinated, control), (c) pigs treated with FMT only (non-vaccinated, FMT), and (d) pigs neither vaccinated against PRRS nor treated with FMT (non-vaccinated control). Groups were balanced according to arrival weight and housed in separate pens. Approximately 9 hours after arriving at Kansas State University on May 14th, pigs were administered a fecal microbiota transplant (FMT) or a

mock transplant (CON). Mock transplants were made of 10% glycerol in sterile saline. To administer the FMT or mock-transplant, 5 mL doses were delivered orally through flexible dispensing tips (6.4 mm Flexoject^{TM} Dispensing Tips, Innovet). Solutions were delivered slowly on the tongue or in the cheek pouch, allowing the pig to chew on the tip and naturally consume the material over 30 seconds to 1 minute. Transplants or mock-transplants were administered daily for seven consecutive days prior to co-infection. After one week of transplantation, and approximately three and half weeks of age (mean age of 25.6 ± 0.5 days), two pens were vaccinated (Vx) with a 2-mL dose of a commercial PRRS vaccine (Ingelvac PRRS MLV; Boehringer Ingelheim Animal Health; Gen Bank accession no. AF159149) administered intramuscularly according to the vaccine label instructions. Unvaccinated pigs (Non-Vx) were of a similar age (25.6 ± 0.5 days). At 28 days post-vaccination (dpv), and approximately seven and half weeks of age (mean age of 53.6 ± 0.5 days), all pigs were infected with PRRSV and PCV2d. Individual body weights were collected upon arrival (-7) and on 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 dpv. Average daily gain (ADG) was calculated by the change in weight (kilograms) over the change in time (days), and was reported in kilograms (kg). ADG was determined for the entire 42-day study. Blood samples were collected from all pigs on 0, 4, 7, 11, 14, 21, 28, 32, 35, 39, 42, 49, 56, 63, and 70 dpv. Fecal samples were collected from all 39 pigs weekly on -7, 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 dpv. Pigs were humanely euthanized under the direction of the attending laboratory animal veterinarian if 1) pigs had greater than or equal to 20% weight loss, 2) pigs were moribund or nonresponsive to veterinary treatment, or 3) pigs had severe dyspnea or clinical disease that compromised animal welfare. At 42 dpi, all remaining pigs were humanely euthanized in accordance with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals and complete necropsies were performed.

Challenge Inoculum. PRRSV (isolate NVSL; GenBank accession no. AY545985) was isolated by propagation on MARC-145 cells. The PRRS virus shares 88.06% identity with the MLV at the peptide sequence level of GP5. PRRSV titration was performed on MARC-145 cells as previously described.[116] Briefly, PRRSV infectivity was titrated through serial 10-fold dilutions of PRRS stock virus in minimal essential medium (MEM; Corning) supplemented with 7% fetal bovine serum (FBS; Sigma-Aldrich), penicillin-streptomycin (Pen Strep; 80 U/mL and 80 µg/mL, respectively; Gibco), 3 µg/mL amphotericin B (Fungizone; Gibco), and 25 mM HEPES (Life Technologies). The dilutions were 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 median tissue culture infective dose ($TCID_{50}$/mL) was calculated using the Spearman-Karber method.[411] To prepare the inocula for pigs, the stock virus was mixed to yield a 2-mL dose consisting of 10^5 $TCID_{50}$ PRRSV in MEM. The 2-mL dose was split, with 1 mL being delivered intranasally and the remaining 1 mL delivered intramuscularly.

Clinical and pathological evaluation. Pigs were evaluated daily throughout the 77 day trial by a veterinarian or veterinary assistant for the presence of clinical signs associated with PRRS, including tachypnea, dyspnea, coughing, sneezing, mucoid rhinorrhea, open mouth breathing, conjunctivitis, aural cyanosis, altered joint conditions (altered ambulation or joint effusion), diarrhea, decreased body condition, and altered mentation (lethargy or depression). Any pig showing clinical signs of PRRS was restrained for a physical examination and rectal temperature measurement was taken by the attending veterinarian. A standardized health evaluation form was developed and utilized during both trials. This form was completed for all pigs showing clinical signs that warranted veterinary evaluation. Included on the health

evaluation form were clinical signs characterized by presence or absence in addition to several clinical signs scored based on severity. Examples of clinical signs documented as a binomial variable included diarrhea, pyrexia, tachypnea, mucoid rhinorrhea, coughing, sneezing, dyspnea, open-mouth breathing, ocular discharge, conjunctivitis, altered ambulation, and joint effusion. Examples of clinical signs scored based on severity included altered mentation and response to stimuli, and body condition. However, in the calculation of clinical signs for comparing the two viruses in the current study, all clinical signs were characterized as binomial variables. For example, attitude was considered normal for any pig characterized as bright, alert, and responsive whereas attitude was considered abnormal for any type of decreased mentation (e.g., quiet, alert and responsive; depressed, alert, responsive; moribund). In addition to presence of clinical signs, the frequency along with duration of each clinical sign were calculated for the two populations of pigs.

Pigs with clinical disease were prescribed parenteral veterinary treatment as deemed appropriate by the attending veterinarian. Examples of clinical presentations where parenteral treatment was administered included 1) dyspnea or persistent coughing, 2) mucoid rhinorrhea, 3) altered ambulation with or without joint effusion, 4) diarrhea with pyrexia, and 5) lethargy or depression with pyrexia. Antimicrobials, including oxytetracycline (Liquamycin®; LA-200®) or ceftiofur hydrochloride (Excenel®), were administered for altered ambulation or clinical signs of respiratory disease. Typical antimicrobial therapy included once daily intramuscular injection for a total of 3 days. The non-steroidal anti-inflammatory medication flunixin meglumine (Banamine®) was administered for altered ambulation and/or pyrexia, with a typical treatment regimen of once daily intramuscular injection for a period of three days. Pigs were monitored for progression or resolution of clinical signs, including daily rectal temperature measurements

during treatment and a 3-day post-treatment evaluation period for all pigs recovering from clinical illness. Pigs that were non-responsive to veterinary treatment or had progressive clinical disease and compromised animal welfare were humanely euthanized by the attending veterinarian.

Mortality and morbidity were assessed throughout the 42-day study. Any pig that died or was humanely euthanized because of PRRS was counted as a mortality. Any clinical signs warranting a veterinary evaluation and/or a mortality throughout the 42-day study were counted as a morbidity. Morbidity rates over time were determined by summing the total number of pigs with clinical signs on a given day divided by the total number of pigs alive on that day. Mean duration of specific clinical signs was calculated by summing the number of days individual pigs had a given clinical sign throughout the study divided by the total number of pigs that had the given clinical sign.

At 42 dpi, all surviving 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. First, whole body weights were collected post-mortem. Second, lungs and trachea were removed *in toto* immediately after euthanasia and total lung weights were measured. The trachea was excised immediately distal to the larynx. Wet lung weight to body weight ratio was calculated as a measure of pulmonary pathology. Dorsal and ventral aspects of the whole lung were photographed (Canon EOS Rebel T6 DSLR) and digital images were evaluated after gross necropsy using a photo scoring system. Gross anatomical photo scores were reported as the percentage of whole lung affected by pneumonia (ranging from 0 to 100%). Scores were combined from 5 sections of the lung as previously described [413], including 1) right dorsal lung – 25%, 2) left dorsal lung – 25%, 3) right ventral lung – 22.5%, 4)

left ventral lung – 22.5%, and 5) accessory lung lobe – 5%. The photos were evaluated by a board certified veterinary pathologist who was blinded to the source of the lung pictures.

Tissues collected for histopathology included lung (1 section from each lobe), tracheobronchial lymph node, thymus, tonsil, duodenum, jejunum, ileum, mesenteric lymph node, spiral colon, cecum, spleen, nasal turbinates, kidney and skin. Additional tissues were collected at the pathologist's discretion by evidence of gross lesions. 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 [116,118]. 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.

Viremia. Blood samples were collected from all pigs at 15 points (0, 4, 7, 11, 14, 21, 28, 32, 35, 39, 42, 49, 56, 63, and 70 dpv) to determine PRRSV viremia, total viral load, and viremic duration. Methods utilized to measure PRRSV and PCV2 viremia have been described in detail previously (Niederwerder et al., 2015; Niederwerder et al., 2016; Ober et al., 2017). Briefly, viral DNA and RNA were extracted simultaneously from 50 μL of serum using Ambion's MagMAX 96 Viral Isolation Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) 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, Rockville, MD) according to the manufacturer's instructions. The PRRSV PCR assay results were reported as

log10 RNA starting quantity (copy number) per 50-µl reaction volume. PCV2 DNA was quantified using SsoAdvanced Universal SYBR green supermix (Bio-Rad, Hercules, CA). The forward and reverse PCR primers were 5'-AATGCAGAGGCGTGATTGGA-3' and 5'-CCAGTATGTGGTTTCCGGGT-3', respectively. Standard curves and positive and negative controls were included on each plate. The PCV2b PCR assay results were reported as log10 DNA starting quantity (copy number) per 20 µL reaction volume. Total viral load for PRRSV was calculated as the area under the curve of viremia in Graphpad Prism®. Total viral load for PRRSV was calculated by Riemann sums of the total area of the trapezoids under the line segments connecting weekly or biweekly viremia measurements. Viral load was calculated for PRRSV MLV vaccine replication during the vaccination period (0-28 dpv) and for wild-type PRRSV replication during the challenge period (28-70 dpv).

Microsphere immunoassay for detection of PRRSV antibodies. Blood samples were collected from all pigs at 15 points (0, 4, 7, 11, 14, 21, 28, 32, 35, 39, 42, 49, 56, 63, and 70 dpv) to determine antibody levels and antibody load. PRRSV nucleocapsid protein were cloned into the pHUE vector, as previously described in detail [338]. Proteins were expressed, purified and measured prior to being coupled to carboxylated Luminex (Luminex Corporations; Austin, TX) MagPlex® polystyrene microspheres according to the manufacturer's instructions. For the assays, approximately 2500 antigen-coated beads, suspended in 50 µL PBS with 0.05% Tween-20 and 4% goat serum (PBST-GS), were placed in each well of a 96-well polystyrene round bottom plate (Costar®; Corning®; Tewksburg, MA). Sera were diluted 1:400 in PBST-GS and 50 µL was added to each well. The plate was sealed and incubated for 30 min at room temperature with gentle shaking. After incubation, the plate was placed on a magnet and beads were washed three times with 190 µL of PBS-GS. For the detection of IgG, 50 µL of biotin-SP-conjugated affinity

purified goat anti-swine secondary antibody (IgG, Jackson ImmunoResearch; West Grove, PA) was diluted to 2 µg/mL in PBST-GS and 100 µL was added to each well. The plate was incubated at room temperature for 30 min and washed three times followed by the addition of 50 µL of streptavidin-conjugated phycoerythrin (2 ug/ml in PBST-GS; SAPE). After 30 min, the plate was washed and microspheres resuspended in 100 µL of PBST-GS. Microspheres were analyzed using a MAGPIX instrument (Luminex Corporations; Austin, TX) and Luminex® xPONENT 4.2 software. A minimum of 50 microspheres was used for the calculation of mean fluorescence intensity (MFI). The sample to positive (S/P) ratio was calculated as the MFI of the sample minus the MFI of the negative control divided by the MFI of the standard positive control minus the MFI of the negative control. Total antibody load for PRRSV antibodies was calculated by Riemann sums of the total area of the trapezoids under the line segments connecting weekly or biweekly viremia measurements. Antibody load was calculated for PRRSV MLV vaccine associated antidbodies during the vaccination period (0-28 dpv) and for PRRSV wild-type virus associated antibodies during the challenge period (28-70 dpv).

Microbiome Characterization. Feces was collected after 7 days of FMT and prior to vaccination to characterize predictive differences in the DNA-based microbiome using Illumina-based next generation sequencing, and the RNA-based microbiome using RNA-seq.

DNA and RNA extraction. The DNA was extracted using Qiagen's AllPrep PowerViral DNA/RNA Kit by following the manufacturer's instructions. For the optional steps, in order to modify the protocol to retain mostly DNA, ß-ME was not added to Solution PM1 and phenol-chloroform-isoamyl alcohol was not added to the PowerBead Tube. RNA was extracted using Qiagen's RNeasy PowerMicrobiome Kit by following the manufacturer's instructions. Phenol-chloroform-isoamyl alcohol was added to the PowerBead Tube to assist in protecting the

integrity of the RNA during homogenization of the sample. Bead beating was utilized in both protocols.

UMGC Illumina NexteraXT methods (NovaSeq).

Sample Quality Assessment: Upon submission, the DNA samples were quantified using a fluorimetric PicoGreen assay. The purity of the samples were also assessed via a Nanodrop. For a sample to pass QC, it needs to quantify greater than 0.2 ng/ul. If the samples pass QC they entered the TruSeq NexteraXT DNA library preparation queue.

Library Creation: gDNA samples were converted to Illumina sequencing libraries using Illumina's NexteraXT DNA Sample Preparation Kit (Cat. # FC-131-1096). Please visit www.illumina.com for a detailed list of kit contents and methods. In summary, 1 ng of gDNA is simultaneously fragmented and tagged with a unique adapter sequence. This "tagmentation" step is mediated by a transposase. The tagmented DNA is simultaneously indexed and amplified 12 PCR cycles. Final library size distribution were validated using capillary electrophoresis and quantified using fluorimetry (PicoGreen). Libraries were then normalized and pooled.

Cluster generation and sequencing: Pooled libraries were denatured and diluted to the appropriate clustering concentration. The libraries were then loaded onto the NovaSeq paired end flow cell and clustering occurs on board the instrument. Once clustering was complete, the sequencing reaction immediately began using Illumina's 2-color SBS chemistry. Upon completion of read 1, 2 separate 8 or 10 base pair index reads were performed. Finally, the clustered library fragments were re-synthesized in the reverse direction thus producing the template for paired end read 2.

Primary analysis and de-multiplexing: Base call (.bcl) files for each cycle of sequencing were generated by Illumina Real Time Analysis (RTA) software. The base call files and run

folders were streamed to servers maintained at the Minnesota Supercomputing Institute. Primary analysis and de-multiplexing were performed using Illumina's bcl2fastq v2.20. The end result of the bcl2fastq workflow was de-multiplexed FASTQ files.

UMGC Illumina RNA-Seq methods Total RNA Prep Ligation with Ribo-Zero Plus

Sample Quality Assessment: Total eukaryotic RNA isolates were quantified using a fluorimetric RiboGreen assay. Total RNA integrity was assessed using capillary electrophoresis (e.g., Agilent BioAnalyzer 2100), generating an RNA Integrity Number (RIN). For samples to pass the initial QC step, they need to quantify higher than 200 nanograms and have a RIN of 7 or greater, then they were converted to Illumina sequencing libraries.

Library Creation: Total RNA samples were converted to Illumina sequencing libraries using Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus (cat#: 20040525). Please see www.illumina.com for a detailed list of kit contents and methods. In summary, a normalized input mass of total RNA was enzymatically depleted of rRNA using sequence specific Ribozero capture probes. The reduced RNA was then fragmented and reverse transcribed (via random hexamers) into cDNA and then underwent second strand synthesis. The resulting cDNA fragments were blunt-ended, adenylated, and ligated to universal pre-index anchors. A final PCR amplification enriched for the anchor-ligated DNA fragments and incorporated primer sequences for cluster generation and unique barcodes for each library. Final library size distribution was validated using capillary electrophoresis and quantified using fluorimetry (PicoGreen). Indexed libraries were then normalized and pooled equimolar.

Cluster generation and sequencing: Pooled libraries were denatured and diluted to the appropriate clustering concentration. The libraries were then loaded onto the NovaSeq paired end flow cell and clustering occurred on board the instrument. Once clustering was complete, the

sequencing reaction immediately began using Illumina's 2-color SBS chemistry. Upon completion of read 1, 2 separate 8 or 10 base pair index reads were performed. Finally, the clustered library fragments were re-synthesized in the reverse direction thus producing the template for paired end read 2.

Primary analysis and de-multiplexing: Base call (.bcl) files for each cycle of sequencing were generated by Illumina Real Time Analysis (RTA) software. The base call files and run folders were streamed to servers maintained at the Minnesota Supercomputing Institute. Primary analysis and de-multiplexing were performed using Illumina's bcl2fastq v2.20. The end result of the bcl2fastq workflow was de-multiplexed FASTQ files.

Microbiome data analysis. DNA and RNA-sequencing data was used to analyze microbiome composition and diversity of fecal samples collected prior to PRRSV MLV vaccination. Genomes from NCBI Genbank for the archaea, bacteria, fungi, protozoa, and viruses' subdirectories were downloaded from the web on 9-13-2021 (https://ftp.ncbi.nlm.nih.gov/genomes). Genomic fasta (.fna) files for all downloaded genomes were filtered for completed genomes only, and joined to produce a database file for each subdirectory. This included a 1.1GB database for archaea that included 439 species, 3.4Gb database for 165 fungal species, 435 Mb database for 19 protozoa species, and 107.3Gb database for 27,158 bacterial species. For viruses, we included cds fasta (.fna) files for those genomes that were available, and for the viruses that did not have a cds fasta, we kept the genomic fasta. Overall, the viral database file was 2.4Gb for 42,061 viral species. To analyze the gDNA, we combined all these database genomes together to produce a 112Gb reference database for alignment. To analyze the RNA-seq data, we used the virus only database file for alignment.

Both database files were indexed using *minimap2*[479] using *-k 21 -w 11*, parameters for kmer length and window size parameters respectively.

Raw fastq reads for both sets of data (gDNA and RNA) were aligned to the reference database using *minimap2* with default parameters. Each pig's alignment file (.sam) was processed to produce a bam file, and the resulting bam file was filtered using map quality score $\geq$ 30 to retain high quality, unique alignments using *Samtools*.[480] Resulting high quality alignments were used to produce a final .txt file per pig that included the genus and species information and number of reads that aligned using a custom *Perl* script for both datasets.

Output data, consisting of genus and species information, was compared in excel. Overall reads were filtered to include only species that are present at >0.01% relative abundance. Higher taxonomy (phylum, class, order, and family) was determined through the NBCI Taxonomy and classifications at the genus and species level were updated as necessary (e.g. *Lactobacillus reuteri* is now classified as *Limosilactobacillus reuteri*).[481] Diversity of the fecal samples was measured by calculating the number of phyla, classes, orders, families and species detected in each sample. Relative abundance of phyla, class, order, family and species was determined by dividing the number of aligned reads by the total number of aligned reads per pig. The Firmicutes to Bacteroidetes (Firmicutes:Bacteroidetes) ratios were determined by dividing the overall relative abundance of Firmicutes species by the overall relative abundance of Bacteroidetes species, as well as by dividing the total number of Fimicutes bacterial species by the total number of Bacteroidetes bacterial species detected for each pig.

Statistical Analysis. All statistical analyses were performed using GraphPad Prism 9.0 software (La Jolla, CA). Clinical sign duration, viremia duration, plus macroscopic and microscopic lung lesions scores were compared using Kruskal-Wallis tests with associated

Dunn's multiple comparisons tests. Daily morbidity and treatment rates were compared using the chi-square test. Vaccine period viral and antibody loads were compared using unpaired *t*-tests. Challenge period viral and antibody loads were compared using a two-way ANOVA with associated Tukey's multiple comparisons tests. Three-way ANOVAs were utilized to compare weight gain between the four groups; pairwise comparisons were done if significance was found within the ANOVA using Tukey's multiple comparison tests. Two-way ANOVAs were utilized to compare PRRSV viremia and antibody levels during the vaccination period, in addition to lung to body weight ratios; if significance was found, pairwise comparisons were done using Šidák's multiple comparisons. Three-way ANOVAs were utilized to compare PRRSV viremia and PRRS antibody levels during the challenge period; if significance was found, pairwise comparisons were done using Tukey's multiple comparisons tests. Simple linear regression was used to determine the slopes of PRRSV viral decay after peak challenge viremia. Unpaired *t*-tests were used to screen for potential microbial associations between groups. Where at least one comparison between groups was significant ($p < 0.05$; unpaired t-tests) and had at least one group with a relative abundance greater than 0.1%, a Kruskal-Wallis test was applied for more stringent analysis. Microbiome diversity and Firmicutes:Bacteroidetes ratios (both relative abundance and frequency) were compared using Mann-Whitney *U* and Kruskal-Wallis tests with associated Dunn's multiple comparisons tests.

Results

FMT had no effect on pigs prior to vaccination. Upon arrival to Kansas State University, control and FMT pigs appeared clinically within normal limits. Morbidity levels, during the transplant or mock transplant period, were similar between the four groups and was most associated with post-weaning diarrhea (**Figure 5.1**). Mean weight of the control, non-

vaccinated group was 5.2 ± 1.1 kg, mean weight of the control, vaccinated group was 5.2 ± 1.2 kg, mean weight of the FMT, non-vaccinated group was 5.4 ± 1.1 kg and the mean weight of the FMT, vaccinated group was 5.2 ± 1.2 kg ($p > 0.99$; three-way ANOVA with Tukey's multiple comparison tests; **Figure 5.2A**). No significant difference in weight gain was noted during the transplantation or mock-transplantation time period, suggesting no detrimental effect of FMT on weight gain in unchallenged conditions; mean weights on 0 dpi for the control, non-vaccinated group was 6.3 ± 1.1 kg, for the control, vaccinated group was 6.4 ± 1.6 kg, for the FMT, non-vaccinated group was 6.2 ± 1.2 kg and for the FMT, vaccinated group was 5.9 ± 1.3 kg ($p > 0.99$; three-way ANOVA with Tukey's multiple comparison tests; **Figure 5.2A**).

FMT pigs had similar morbidity as control pigs with and without PRRSV MLV vaccination. Pigs were assessed daily throughout the 42-day study for clinical signs associated with PRRSV infection; pigs showing clinical signs were assessed and treated as needed. There was one mortality that was unrelated to PRRSV infection (intestinal torsion and adhesions); this pig was excluded from further analysis. One pig in the control vaccinated group had tarsal swelling that was present prior to challenge and throughout the study; this was found to be cystic in nature with no purulent material within the joints on necropsy and was therefore excluded from morbidity rates.

Morbidity was assessed for the four groups of pigs and are shown in **Figure 5.1**. Overall morbidity was calculated during the PRRSV MLV vaccine period in two vaccinated groups (control and transplanted). During this period, 100% of control, vaccinated pigs developed clinical signs while 90% of transplanted, vaccinated pigs developed clinical signs; this was not statistically significant ($p > 0.99$; Fisher's exact test; data not shown) and was most likely due to post-weaning stress. Of the pigs with clinical signs, only one pig (in the control non-vaccinated

group) needed treatment. During the vaccination period, clinical sign duration, calculated by the total number of days each pig had to be assessed, was calculated in the vaccinated groups. Clinical signs were similar between the two groups; on average 4.0 ± 3.6 days in length for control, vaccinated pigs while they were 4.0 ± 3.0 days in length for transplanted, vaccinated pigs ($p = 0.89$; Mann-Whitney *U* test; data not shown). These data suggest that FMT did not affect clinical outcome during PRRSV MLV vaccination.

Overall morbidity was also calculated during the PRRSV challenge period. Of the 39 pigs infected with PRRSV, 37 pigs (94.5%) pigs had PRRSV-associated clinical signs requiring assessment. Of these pigs, nine (9/10) were in the control non-vaccinated group, nine (9/9) were in the transplanted non-vaccinated group, ten were in the control vaccinated group (10/10), and nine were in the transplanted vaccinated group (9/10); this was not significantly different ($p = 0.99$; chi-square test; data not shown). A total of six out of the 37 (16.2%) pigs that were assessed required treatment with antibiotics and/or NSAIDs. Of the pigs that were treated two were in the control non-vaccinated group, two were in the transplanted non-vaccinated group, one was in the control vaccinated group, and one was in the transplanted vaccinated group; this was also not significantly different ($p = 0.3$; chi-square test; data not shown). During the challenge period, clinical sign duration was calculated in all four groups. The control non-vaccinated pigs had a clinical sign duration of 5.6 ± 6.3 days, transplanted, non-vaccinated pigs had a clinical sign duration of 6.6 ± 3.6 days, and control, vaccinated pigs had a clinical sign duration of 6.8 ± 5.2 days, and transplanted; interestingly, transplanted, vaccinated pigs had a lower clinical sign duration than the other groups at 4.5 ± 6.1 days, however, this was not statistically significant ($p = 0.3$; Kruskal-Wallis test; data not shown). These data suggest that

neither FMT, nor PRRSV MLV vaccination, significantly affected clinical outcome during PRRSV challenge.

FMT pigs had similar weights compared to control pigs during the PRRSV MLV vaccination and PRRSV challenge periods while PRRSV MLV vaccination improved ADG. Weights were measured throughout the study, and used to determine average daily gain (ADG) in the four groups; results are shown in **Figure 5.2**. Weight steadily increased throughout the study in all groups. There was significant variation associated with time and time by vaccination ($p < 0.0001$ and $p = 0.04$; three-way ANOVA). Pairwise comparisons were conducted between groups to further analyze these differences; however, weights were found to be similar throughout the vaccination and challenge period ($p > 0.1$; Tukey's multiple comparison test; **Figure 5.2A and B**).

Since no significant findings were found in weight between groups, ADG was examined for more global changes during the vaccination and challenge period. During the 28 day vaccination period mean ADG was similar in all groups at approximately 0.6 ± 0.1 kg ($p = 0.11$; two-way ANOVA; **Figure 5.2C**); notably, there was significant variation due to individual animals ($p = 0.002$; two-way ANOVA). Pairwise comparisons did not find a significant difference between groups during the vaccination period ($p > 0.36$; Tukey's multiple comparisons test). Mean ADG after PRRSV challenge was significantly different based on treatment ($p = 0.002$; two-way ANOVA), along with inter-individual variation ($p = 0.009$; two-way ANOVA). Within the control groups, non-vaccinated pigs had significantly lower ADG of 0.84 ± 0.12 kg compared to vaccinated pigs, which had ADG of 0.98 ± 0.12 kg ($p = 0.03$; Tukey's multiple comparison test). Within the transplanted groups, non-vaccinated pigs also had significantly lower ADG at 0.82 ± 0.09 kg in contrast to vaccinated pigs with ADG of $0.96 \pm$

0.17 kg ($p = 0.02$; Tukey's multiple comparison tests). While some vaccinated pigs in the transplanted group had higher ADG than their control counterparts, there was not a significant difference between the two groups ($p > 0.8$; Tukey's multiple comparison test). These data suggest that while the PRRSV MLV vaccine improves weight gain during PRRSV challenge, that the FMT did not have a significant effect on weight gain.

FMT reduced viremia after PRRSV MLV vaccination and challenge. Serum PRRSV virus replication was determined bi-weekly during the vaccination period and used to calculate total viral load (**Figure 5.3**). In the two groups of vaccinated pigs, PRRSV viremia had a bimodal distribution with peaks associated with vaccine and challenge virus replication. During the vaccination period, there was significant time and pig variation ($p < 0.0001$ and $p = 0.003$, respectively; two-way ANOVA; **Figure 5.3A**). Therefore, we further evaluated differences on a daily basis. On 0 dpv, vaccine viremia was not present (0.0 $\log_{10}$ copies/PCR reaction) in both the control and transplanted animals. Vaccine virus replication steadily increased and peaked at 7 dpv. At this time, viremia in transplanted pigs deviated from control pigs; mean PRRSV viremia was 3.2 and 3.6 $\log_{10}$ copies/PCR reaction for the transplanted and control groups, respectively ($p = 0.76$; Šidák's multiple comparison test; **Figure 5.3B**). This difference was larger, however not significant, on 11 dpv with mean PRRSV viremia was 2.9 and 3.4 $\log_{10}$ copies/PCR reaction for the transplanted and control groups, respectively ($p = 0.27$; Šidák's multiple comparison test). At 14 dpv, there continued to be a be differences, albeit not significant, with mean viremia in transplanted pigs 2.8 $\log_{10}$ copies/PCR reaction compared to 3.3 $\log_{10}$ copies/PCR reaction in the control group ($p = 0.56$; Šidák's multiple comparison test). During the rest of the vaccination period, viremia in the transplanted and control pigs were similar ($p > 0.99$; Šidák's multiple comparison test). With regards to viral load, transplanted pigs had a lower viral load than control

pigs during the vaccination period; however, this difference was not significant ($p = 0.24$; unpaired *t*-test; **Figure 5.3C**).

After PRRSV challenge, serum PRRSV virus replication was determined weekly to bi-weekly and used to calculate total viral load (**Figure 5.3**). There was significant variation caused by time, time by vaccination, and time by transplantation ($p < 0.0001$, $p < 0.0001$, and $p = 0.01$, respectively; three-way ANOVA; **Figure 5.3A**); we therefore, investigated differences between individual groups. On the day of challenge (28 dpv), both non-vaccinated groups had 0 log_{10} PCR copies/reaction compared to their vaccinated counterparts which had 2.3 and 2.9 log_{10} copies/PCR reaction; this difference was significant ($p < 0.0001$; Tukey's multiple comparisons test; **Figure 5.3B**). This difference was not apparent at 32 dpv ($p = 0.9$ and $p = 0.2$ in control and transplanted groups, respectively; Tukey's multiple comparisons test). Challenge virus replication peaked at 35dpv in all groups. While non-vaccinated pigs often had higher viremia than vaccinated pigs during the challenge period, this difference was not significant ($p > 0.1$; Tukey's multiple comparisons test). After peak viremia (35 dpv), slopes of viral decay were found to be significantly different between the groups ($p = 0.048$; linear regression; data not shown), with decay trending towards significantly faster in transplanted groups than control groups. While vaccinated pigs initially had higher viremia, they were surpassed by non-vaccinated pigs later in challenge, leading to similar viral loads between the four groups (**Figure 5.3C**; $p > 0.1$; two-way ANOVA with Tukey's multiple comparisons test). Viremia duration, or the mean number of days each pig had viremia, was similar between the groups ($p = 0.84$; Kruskal-Wallis test; data not shown). These data suggest that while FMT does not decrease viremia, viral load nor viral duration, that it leads to faster viral decay.

Antibody production and load was higher after PRRSV MLV vaccination but similar between control and transplanted animals. Antibody production against PRRSV nucleocapsid (N) protein was quantified bi-weekly during the vaccination period (**Figure 5.4**). During the vaccination period, there was significant antibody level variation as a result of time and individual pigs ($p < 0.0001$; two-way ANOVA; **Figure 5.4A**); however, there were no significant differences associated with transplantation status ($p = 0.96$; two-way ANOVA; **Figure 5.4B**). In contrast, after PRRSV challenge, there was significant variation attributable to multiple factors; time, vaccination status, and time by vaccination status ($p < 0.0001$; three-way ANOVA; **Figure 5.4A**); additionally there was a trend towards significance for variation due to transplantation status ($p = 0.07$; three-way ANOVA). Pairwise comparisons were conducted to look for differences between the groups on a daily basis. On the day of challenge (28 dpv) antibody levels were significantly higher in vaccinated compared to the non-vaccinated groups; approximately 0 sample to positive (S:P) ratio in both non-vaccinated groups, while the vaccinated, control group was 2.2 ± 0.2 S:P ratio and the vaccinated, transplanted group was 2.4 ± 0.3 S:P ratio ($p < 0.0001$; Tukey's multiple comparison tests; **Figure 5.4B**). Significant differences were also found between the non-vaccinated and vaccinated groups on 32 dpv ($p < 0.0001$; Tukey's multiple comparisons tests). Throughout the rest of the challenge period, PRRSV antibody levels were similar between all of the groups.

To assess for global changes in antibodies we looked at antibody load. There was no difference found in antibody load between the control and transplanted groups ($p = 0.85$; unpaired *t*-test; **Figure 5.4C**). After challenge, there was significant variation associated with treatment and individual pigs ($p = 0.02$ and $p = 0.0015$, respectively; two-way ANOVA). Antibody load was higher in vaccinated pigs than non-vaccinated pigs, most likely associated

with antibody production early in the challenge period. Within control groups, non-vaccinated pigs had a lower antibody load of 74.2 ± 9.6, compared to vaccinated pigs which had an antibody load of 83.6 ± 7.2 ($p = 0.03$; Tukey's multiple comparisons test). Within transplant groups, non-vaccinated pigs had an antibody load of 70.7 ± 8.5 and vaccinated pigs had an antibody load of 81.1 ± 9.4 ($p = 0.02$; Tukey's multiple comparison test).

Lung pathology was similar with respect to vaccination and transplantation status. Gross lung tissue images were captured during necropsy and subsequently scored for severity of lesions. There was no significant difference found between any groups ($p = 0.32$; Kruskal-Wallis test). Within control pigs, non-vaccinated pigs had a mean of 43.8 ± 42.8% lung affected while vaccinated pigs had a mean of 36.9 ± 29.6% lung affected ($p > 0.99$; Dunn's multiple comparisons test; data not shown). Within FMT pigs, non-vaccinated pigs had a mean 57.8 ± 29.8% lung affected, while vaccinated pigs had a mean of 58.8 ± 37.6% lung affected ($p > 0.99$; Dunn's multiple comparisons test; data not shown). At necropsy, a wet lung weight to body weight ratio was calculated for each pig. Pigs in all four groups had a similar lung to body weight ratio at approximately 0.01 ($p > 0.1$; two-way ANOVA with Tukey's multiple comparisons tests; data not shown). Microscopic lung lesions were also assessed through histopathology. Lesions included lymphoplasmacytic and histiocytic interstitial pneumonia, suppurative bronchopneumonia, and interlobular septal edema and hemorrhage. Degree of interstitial pneumonia was scored in all 39 pigs, with 1/20 control and 1/19 FMT pigs having severe diffuse interstitial pneumonia with >75% lung lobe involvement (data not shown). Control pigs has slightly higher, but not significant, severity of microscopic lung lesions ($p = 0.32$; Kruskal-Wallis test; data not shown).

Fecal microbiome diversity decreased after transplantation but also lead to shifts in microbial composition. Feces was collected after transplantation, but prior to vaccination to look for predicative associations between the gut microbiome and vaccine efficacy. The pig that was excluded from clinical data due to non-PRRS related morbidity and euthanasia was excluded from further analysis. Additionally, one pig had poor sample quality after RNA isolation and was excluded from further analysis. For genomic DNA, libraries for 39 pigs were sequenced generating nearly 881 million paired-end (2x151bp) reads, for 11.3 million reads per pig. Nearly 3 billion paired-end (2x151bp) RNA-seq reads were generated from fecal samples for 38 pigs, resulting in an average of nearly 38.1 million PE reads per pig using a NovaSeq at University of Minnesota. On average, ~1.5 million high quality gDNA reads uniquely mapped to the bacterial, fungal, viral, archaea, and protozoa genomes, and ~2 million high quality RNA-seq reads uniquely mapped to the viral genomes per pig.

Microbial diversity was determined by calculating the mean number of microbial classification (phylum, class, order, family) in the four groups. On average approximately 12 phyla, 19 classes, 25 orders, 46 families and 157 species were detected (**Figure 5.5**). There was a trend towards significant difference in the phyla diversity between the groups ($p = 0.07$; Kruskal-Wallis test; **Figure 5.5, top left panel**), with significance at the class, order and family level ($p = 0.04$, $p = 0.02$, and $p = 0.008$, respectively; Kruskal-Wallis test; **Figure 5.5, top right, bottom left and right panels**). Pairwise comparisons were done to analyze potential changes associated with vaccination and transplantation status. There was a decrease in the phyla, order and family diversity within the vaccinated groups, between control and transplanted pigs ($p = 0.02$, $p = 0.04$, and $p = 0.06$, respectively; Mann-Whitney *U* tests; **Figure 5.5**). There was also a decrease in class and family diversity within non-vaccinated groups, between control and transplanted pigs

($p = 0.05$ and $p = 0.03$, respectively; Mann-Whitney *U* tests). Firmicutes:Bacteroides ratios, based on relative abundance, were found to be similar ($p = 0.3$; Kruskal-Wallis test; data not shown), while ratios based on bacterial species frequencies was significantly different ($p = 0.02$; Kruskal-Wallis test; data not shown); however, pairwise comparisons did not find significant differences between the groups ($p > 0.14$; Dunn's multiple comparisons test; data not shown). These data together suggest that FMT decreased overall microbial diversity and had no apparent effect on Firmicutes:Bacteroidetes ratios.

There were microbial groups detected in all 39 pigs; these are shown in **Table 5.1**. Microbial organisms included one archaeal species (*Methanobrevibacter smithii*) with remaining microorganisms (n =47) falling within bacterial groups. Bacteria within the phyla Firmicutes were most represented (n =32), with bacteria in this phyla most often in the classes Bacilli, Clostridia and Erysipelothrichia. Bacteria were also found in the phyla Actinobacteria, Bacteroidetes, and Spirochaetes. At the species level, there were multiple *Bacteroides*, *Phocaeicola*, *Eubacterium*, *Blautia*, *Clostridium*, and *Lachnoclostridium* species present. One additional unclassified bacteria was observed (an uncultured human bacteria). These data suggest that these bacterial groups represent a core set of microbial species within pigs.

Metagenomic sequencing uncovered that microbial species most commonly fell within the phyla Firmicutes, Euryarchaeota, and Bacteroidetes (**Figure 5.6, top left panel**). There was a significant difference between the four groups within the phyla Bacteroidia and Spirochaetia ($p < 0.05$; Kruskal-Wallis test). At the class level, 29 uniquely classified groups were identified, with a majority falling within the classes Clostridia, Methanobacteria, and Bacilli (**Figure 5.6, top right panel)**. There was a significant difference between the four groups within the classes Bacteroides and Spirochaetes ($p < 0.05$; Kruskal-Wallis test). Within orders, there were 42

identified microbial groups, with Methanobacteriales, Clostridiales, Lactobacillales and Eubacteriales being the most commonly identified (**Figure 5.6, bottom left panel**). There was a significant difference between the four groups within the orders Bacteroidiales, Bifidobacteriales and Spirochaetales ($p < 0.05$; Kruskal-Wallis test). At the family level, 90 unique classification groups were identified, with most being identified at the family level (n = 73; **Figure 5.6, bottom right panel**). From the 73 classifications, most were bacterial (n = 61), but other represented groups included archaea (n = 2), eukaryotes (n = 2) and viruses (n = 8). The most commonly represented families were *Methobacteriaceae* (archaea), *Lactobacillaceae* (bacteria), and *Coriobacteriaceae* (bacteria). There was a significant difference between the four groups within the families *Oscillospiraceae*, *Bacteroidaceae*, *Turicibacteraceae*, *Bifidobacteriaceae*, *Prevotellaceae*, *Treponemataceae*, and *Podoviridae* ($p < 0.05$; Kruskal-Wallis test). Metagenomic sequencing also identified 411 uniquely classified microbes across the 39 pigs (data not shown). The most prevalent species in all four groups was *Methanobrevibacter smithii*; prevalence ranged from 15.2 – 26.1 %. Some of the more prevalent species were *Bacteroidies*, *Clostridium*, *Eubacterium*, *Lactobacillus*, *Limisolactobacillus*, and *Lachnospiracaeae* species.

In order to examine biologically relevant microbial groups, we focused on significant differences in overall relative abundance with groups consisting of greater than or equal to one percent ($\geq$1%) relative abundance. If global differences were detected, pairwise comparisons were studied to identify individual group variations. At the level of phyla microbial composition detected by metagenomics, most phyla had similar abundance among the four groups. However, microbial groups within the most abundant phyla were most likely to have significant differences and are, therefore, shown in **Figure 5.7**. Two phyla were found to be significantly different; Bacteroidetes and Spirochaetes ($p = 0.046$ and $p = 0.03$; Kruskal-Wallis tests). This significance

persisted, due to only one lineage being represented, at the class level within Bacteroidia and Spirochaetia ($p = 0.046$ and $p = 0.03$; Kruskal-Wallis tests), as well as within the orders Bacteroidales and Spirochaetales ($p = 0.046$ and $p = 0.03$; Kruskal-Wallis tests). Within the order Spirochaetales, the family *Treponemataceae* showed significant differences between the groups ($p = 0.03$; Kruskal-Wallis test), continuing to the species level with *Treponema succinifaciens* with significant differences between the groups ($p = 0.03$; Kruskal-Wallis test). While these global changes were found in the Spirochaetes lineage, none of these resulted in biologically relevant changes between the groups ($p > 0.1$; Dunn's multiple comparisons test). However, within the phyla Bacteroidetes, there was a trend towards significant changes between the transplanted groups, with non-vaccinated pigs having a lower relative abundance compared to vaccinated pigs ($p = 0.09$; Dunn's multiple comparisons test; **Figure 5.7A**); this difference persisted at the class and order level ($p = 0.09$; Dunn's multiple comparisons test; **Figure 5.7B and C**). Additionally, within the order Bacteroidales, the family *Prevotellaceae* showed significant differences between the groups ($p = 0.01$; Kruskal-Wallis tests). Significant changes were seen within the transplanted groups; non-vaccinated pigs had significantly lower *Prevotellaceae* relative abundance compared to vaccinated pigs ($p = 0.04$; Dunn's multiple comparison test; **Figure 5.7D**). Following the Bacteroidetes taxonomic group, within the family *Bacteroidaceae*, which showed no significance ($p = 0.49$; Kruskal-Wallis test; **Figure 5.7D**), *Bacteroides xylanisolvens* was found to be significantly different between the groups ($p = 0.02$; Kruskal-Wallis test). The differences were associated with the transplanted groups; non-vaccinated pigs had lower relative abundance of *Bacteroides xylanisolvens* than vaccinated pigs ($p = 0.055$; Dunn's multiple comparisons test). Within the Actinomycetia phylum, while the other three groups contained less than 0.0001% *Micrococcales,* and therefore

Microbacteriaceae, the control, vaccinated group had a relative abundance of 1.3 ± 2.4 % ($p = 0.03$; Kruskal-Wallis test); this resulted in trends towards significant differences between this group and control non-vaccinated in addition to the transplanted, vaccinated group ($p = 0.08$ and $p = 0.08$, respectively; Dunn's multiple comparisons test; **Figure 5.7C and D**). Within the Erysipelotrichiales order, there were groups differences within the *Turicibacteraceae* ($p = 0.0006$; Kruskal-Wallis test). These differences were associated with changes between control and transplant groups. Between the non-vaccinated groups, control pigs had lower relative abundance of *Turicibacteraceae* than their transplanted counterparts ($p = 0.04$; Dunn's multiple comparisons test). This difference persisted in an unclassified species in the genus *Turicibacter* ($p = 0.0005$; Kruskal-Wallis test). Differences were again associated with transplantation status, with control pigs having a lower relative abundance of *Turicibacter sp.* compared to transplanted pigs, both non-vaccinated and vaccinated ($p = 0.04$ and 0.009; respectively; Dunn's multiple comparisons test; **Figure 5.7E**). Within the family *Lactobacillaceae*, which was not significantly different between groups ($p = 0.45$; Kruskal-Wallis test; **Figure 5.7D**), there were significant differences found between groups within the species *Ligilactobacillus ruminis* and *Limosilactobacillus pontis* ($p = 0.0002$ and $p = 0.001$, respectively; Kruskal-Wallis test). Similar to *Turicibacter sp.*, *Ligilactobacillus ruminis* changes in relative abundance were associated with transplantation. Control pigs had a lower relative abundance of *Ligilactobacillus ruminis* compared to transplanted pigs in both non-vaccinated and vaccinated ($p = 0.01$ and 0.0499; respectively; Dunn's multiple comparisons test; **Figure 5.7E**). Similar changes were found in non-vaccinated pigs with respect to *Limosilactobacillus pontis*; control pigs were found to have a lower relative abundance compared to transplanted pigs ($p = 0.03$; Dunn's multiple comparisons test).

For RNA-seq, reads were filtered to identify RNA viruses. RNA-seq identified 10 uniquely classified viruses across the 38 pigs (**Figure 5.8**). Identified microbial species were only found within two classified phyla; Pisuviricota and Negarnaviricota (**Figure 5.8**, **top left panel**). Within each class only one order (**Figure 5.8**, **top right panel**) was detected; these orders were represented by the families *Picornaviridae*, *Astroviridae* and *Paramyxoviridae* (**Figure 5.8**, **bottom left panel**). Species that represented greater than one percent (1%) relative abundance included porcine enterovirus 9, enterovirus G, porcine kobuvirus, posavirus, porcine astrovirus, and wild boar astrovirus (**Figure 5.8**, **bottom right panel**). Porcine parainfluenza influenza virus 1 was also found at a low relative abundance. A virus within Picornavirales order, posavirus, was found to be significantly different between the groups ($p = 0.02$; Kruskal-Wallis test; **Figure 5.8, bottom right panel**). Specifically, when comparing between vaccinated groups, the transplanted pigs had higher levels of posavirus than control pigs ($p = 0.03$; Dunn's multiple comparisons test).

Overall, metagenomics and RNA-seq in the current study identified several gut microbiome characteristics in transplanted pigs associated with lower viremia after vaccination, including increased *Turicicbacteraceae* bacteria, specifically an unidentified *Turcibacter sp.* as well as decreased Micrococcales bacteria. Additionally, two other species within the family *Lactobacillaceae*, *Ligilactobacillus ruminis* and *Limosilactobacillus pontis* were found at a higher abundance in transplanted versus control pigs. Beneficial gut microbes were also associated with vaccination; vaccinated had a higher abundance of bacteria within the phylum Bacteroides, the order Micrococcales, and the family *Prevotellaceae.*

Discussion

PRRS MLV vaccines are beneficial for improved weight gain, reduced PRRS viral replication, pulmonary pathology, and clinical disease. However, the currently available commercial vaccines are inadequate for disease control[171] as a result of vaccine shedding, reversion to virulence and lack of broadly protective immunity against genetically distinct strains.[14,15] The gut-lung axis, communication between gut microbes to and from the respiratory tract, [462] could be modulated to improve immunity and efficacy of currently available commercial PRRS MLV vaccines. In the current study we demonstrated that, although not significantly, FMT lowered viremia associated with PRRSV MLV vaccination. PRRS viremia has been previously correlated with viral shedding;[482] therefore, decreased viremia could potentially lead to decreased virus shedding. With smaller numbers of pigs we were limited in differences that can been seen between groups; however, if decreases persist with larger studies, FMT could be used as an adjunct therapy for PRRS MLV vaccination. Additionally, decreased viremia could be advantageous in PRRSV/PCV2 co-infections as PRRS MLV vaccine-associated viremia has been demonstrated to potentiate PCV2 replication and associated pathology.[116]

Clinically, pigs in all groups were similar with comparable morbidity, clinical sign duration and treatment rates. Lung pathology, both macro- and microscopic, was also similar between groups. This is interesting because previous work has shown the vaccine to decrease PRRSV-associated gross and histological changes.[163,478] While weekly weight differences were not seen with respect to vaccination nor transplant status, the vaccine did result in increased ADG after challenge. Vaccination also led to PPRS viremia changes; while viremia was higher initially due to sustained vaccine viremia, vaccination led to lower, while not significant, viremia later in infection. These findings are consistent with previous findings that the vaccine improves

weight gain and decreases viral replication.[116] Interestingly, while there were no apparent effects of transplantation on viremia or weight gain during the challenge period, transplantation led to increased viral decay after peak viremia. Therefore, it is possible that FMT could be used to decrease overall PRRSV-associated viremia.

We found it noteworthy that, in the current study, FMT decreased microbial diversity. Decreases in microbial diversity were found at all taxonomic levels, but were most apparent at the family level. This is in contrast to our past FMT study which showed the microbial diversity was similar between control and transplant groups.[478] We also did not find a significant increase in the Firmicutes:Bacteroidetes ratio in transplanted pigs compared to our previous studies. Other studies by our group indicated that increased microbial diversity and Firmicutes:Bacteroidetes ratio is associated with improved outcome after PRRSV infection;[291,328,483] however, these were experimental PRRSV/PCV2 co-infections. Thus, future studies should investigate if these changes are more beneficial in a co-infection model.

While microbial diversity was decreased in transplanted pigs, we found significant shifts in the gut microbiome composition. Specific microbial groups were found at different relative abundance between non-vaccinated and vaccinated in addition to control and transplanted pigs. In the current study, while broad analysis suggested biological implications, we did not find significant differences within the Spirochaetes phylum between the groups. There has been conflicting data about the role of these bacteria related to the gut microbiome. In the current study, while not significant, vaccinated and transplanted pigs had higher relative abundance; the highest abundance was found in vaccinated, transplanted pigs. In our previous FMT study we found decreased microbiome species diversity within the *Spirochaetaceae* family was associated with improved outcome after PRRSV and PCV2 co-infection.[478] A study by Argüello et al. found

similar results, with decreased bacteria within the class Spirochaeta associated with improved outcome after PRRSV infection.[477] This study also found that decreased bacteria within the families *Spirochaetaceae* were associated with decreased IFN-γ, IL-6, and haptoglobin.[477] In addition to *Spirochaetaceae*, Argüello found similar immunological associations with decreased bacteria in the *Micrococcaceae*. The current study had similar findings within vaccinated pigs; transplanted pigs had significantly lower levels of bacteria both at the family and order level. Another bacteria found at a higher abundance in vaccinated, transplanted pigs was *Bacteroides xylanisolvens*. Not much is known about this species within pigs, however, it has been previously isolated in swine feces.[484] It has also been associated with improved outcome in a murine lung cancer model, showing increases in CXCL9 and IFN-γ.[485] *Turicibacteraceae* together with an unidentified species within this family were found at a higher abundance in transplanted pigs, both non-vaccinated and vaccinated. In one study, where pigs were given a symbiotic, consisting of a probiotic and xylo-oligosaccharides, increased jejunal IL-10, interferon-α, and secretory IgA were associated with increased relative abundance of *Turicibacter* species within the jejunum.[486] It is possible that bacteria within this group could be beneficial for improved immune response associated with the PRRS MLV vaccine. Finally, *Lactobacillaceae* bacteria make up a considerable proportion of the swine gut microbiome.[274] In the current study they consisted of, on average, 21% of the pig's microbiome. Specifically for the two species, *Ligilactobacillus ruminis* (basionym *Lactobacillus ruminis*) and *Limosilactobacillus pontis* (basionym *Lactobacillus pontis*), there was a higher relative abundance in transplanted than control pigs. *Ligilactobacillus ruminis* is within the *Ligilactobacillus salivarius* (basionym *Lactobacillus salivarius*) clade; bacteria within these clade have been shown to have antiviral activity against porcine epidemic diarrhea virus.[487] In terms of PRRSV vaccination, previous work found

increased relative abundance of *Lactobacillus* species was associated with increased antibody titer, milder pathogenic damage and shorter viral clearance time, following PRRSV vaccination, plus decreased rectal temperatures after PRRSV challenge.[343] However, another study showed no effect of an oral single-strain probiotic on PRRS vaccine response.[342] We did not find increased antibody titers, nor histopathological differences; however, we did see decreased viremia during vaccination as well as increased viral decay during challenge. It is possible that different members of this family have diverse effects and should be further investigated.

Metagenomics have been previously used to describe the pig gut microbiome.[274,488,489] However, this is first study, to the authors knowledge, to also use RNA-seq identification of gut-associated RNA viruses. One previous study utilized RNA-seq to characterize boar semen;[490] however, their findings were purely bacterial in nature. RNA viral families with in the swine gut were identified in a previous study using polymerase chain reaction.[491] Families identified in this study were consistent with the current study; *Astroviridae*, *Picornaviridae*, *Caliciviridae* and *Coronaviridae*, the two later which we identified at very low relative abundance compared to the previous study. Our data demonstrated significant differences between the treatment groups within one virus, posavirus. This virus, contained within the Picornaviridae family, stands for "POrcine Stool Associated Virus".[492] Past studies have identified this species within swine feces;[491,493] however, not much else is known about its gastrointestinal nor extra-gastrointestinal effects. In 2017, Oude Munnink et al. hypothesized that because of the lack of viral antibodies present in pigs, that it may inhabit other gut commensals rather than the gut itself.[492] Within the current study, we found this virus was significantly more abundant in transplanted pigs; control pigs had minimal virus reads (approximately 1000 reads were detected in one control, non-vaccinated pig) compared to, on average, 233,647 reads in transplanted pigs. The pigs in this

study were relatively young (18.6 ± 0.5 days of age upon arrival) compared to the sows that donated fecal material for the transplant (average age 4.8 years); therefore, the data suggests that this virus might be obtained later on in life from either the sow or the environment. Future studies should investigate whether this virus is beneficial to give to younger pigs to aid in improved vaccine outcome.

Compared to other virus, research to understand the bacteriophage's role within the gut microbiome is relatively new. As indicated by a Pubmed search of "bacteriophage", research on these microorganisms originally increased in the mid-1900s, however, a newer uptick occurred in the mid-2010s. Bacteriophages are known to inhabit the mucous layer of the gut, infecting specific bacteria, thereby preventing overgrowth of gut bacterial populations.[494] With the growing antimicrobial resistance concern, these organisms are now being studied for their use in antibacterial therapy, not only in gastrointestinal disease but respiratory disease as well.[495] *Podoviridae*, a family of bacteriophages identified to be different between the groups, has been demonstrated to infect multiple bacterial species. While the changes were not significant in pairwise comparisons, vaccinated, transplanted pigs had higher levels of this virus family. Interestingly, this family contains bacteriophages known to infect swine respiratory pathogens, including *Bordatella bronchiseptica*[496] and *Pasteurella multocida.*[497] These two bacterial species are common species associated with PRRS-associated secondary bacterial infection.[75] Therefore, future studies should study this viral family, as it might aid in treatment of PRRS-associated disease.

This study supports the concept that gut microbiome modulation could be used to decrease viremia associated with PRRSV MLV vaccination, however, further studies are needed to confirm these initial findings. Our data is consist with other studies, indicating that certain

microbial taxonomic groups are beneficial for outcome after vaccination. In the future, studies are necessary to identify the biochemical and molecular pathways involved in benefits observed. By understanding these mechanisms we can learn to apply large-scale microbial modulation to increase herd health and decrease negative vaccine effects.

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Table 5.1. Microorganisms detected in all pigs by next generation sequencing metagenomics

Phylum	Class	Order	Family	Genus Species
Actinobacteria	Actinomycetia	Bifidobacteriales	*Bifidobacteriaceae*	*Bifidobacterium pseudocatenulatum*
	Coriobacterriia	Coriobacteriales	*Atopobiaceae*	*Olsenella sp.*
			Coriobacteriaceae	*Collinsella aerofaciens*
Bacteroidetes	Bacteroidia	Bacteroidales	*Bacteroidaceae*	*Bacteroides fragilis, Bacteroides thetaiotaomicron, Bacteroides xylanisolvens, Bacteroides zhangwenhongi, Phocaeicola dorei, Phocaeicola vulgatus*
			Muribaculaceae	*Sodaliphilus pleomorphus*
			Prevotellaceae	*Prevotella sp.*
Euryarchaeota	Methanobacteria	Methanobacteriales	*Methanobacteriaceae*	*Methanobrevibacter smithii*
Firmicutes	Bacilli	Lactobacillales	*Enteroccocaceae*	*Enterococcus faecalis, Erysipelotichaceae* bacterium
			Lactobacillaceae	*Lactobacillus amylovorous*
			Streptococcaceae	*Streptococcus suis*
	Clostridia	Clostridiales	*Eubacteriaceae*	*Eubacterium callanderi, Eubacterium maltosivorans, Eubacterium maltosivorans, Eubacterium sp.*
			Lachnospiraceae	*Anaerobutyricum hallii, Blautia argi, Blautia producta, Blautia sp.,* unidentified sp.
		Eubacteriales	*Christensenellaceae*	*Christensenella sp.*
			Clostridiaceae	*Clostridium bornimense, Clostridium sp., Mordacella sp.*
			Lachnospiraceae	*Cellulosilyticum sp., Coprococcus comes, Enterocloster bolteae, Lachnoclostridium scidens, Lachnoclostridium sp., Lachnospiraceae eligans, Roseburia intestinalis*
			Oscillospiraceae	*Dysomobacter welbionis, Faecalibacterium prauznitzii, Flavonifractor plautii, Oscillibacter sp., Ruthenibacterium lactatiformans*

Phylum	Class	Order	Family	Genus Species
			Peptostreptococcaceae	*Clostridioides difficile*
			Unclassified	*Flintibacter sp., Intestinimonas butyriciproducens*
	Erysipelotrichia	Erysipelotrichales	Coprobacillaceae	*Catenibacterium sp.*
			Turicibacteraceae	*Turicibacter sp.*
Spirochaetes	Spirochaetia	*Spirochaetales*	*Treponemataceae*	*Treponema sp.*
Unclassified				Uncultured human bacteria

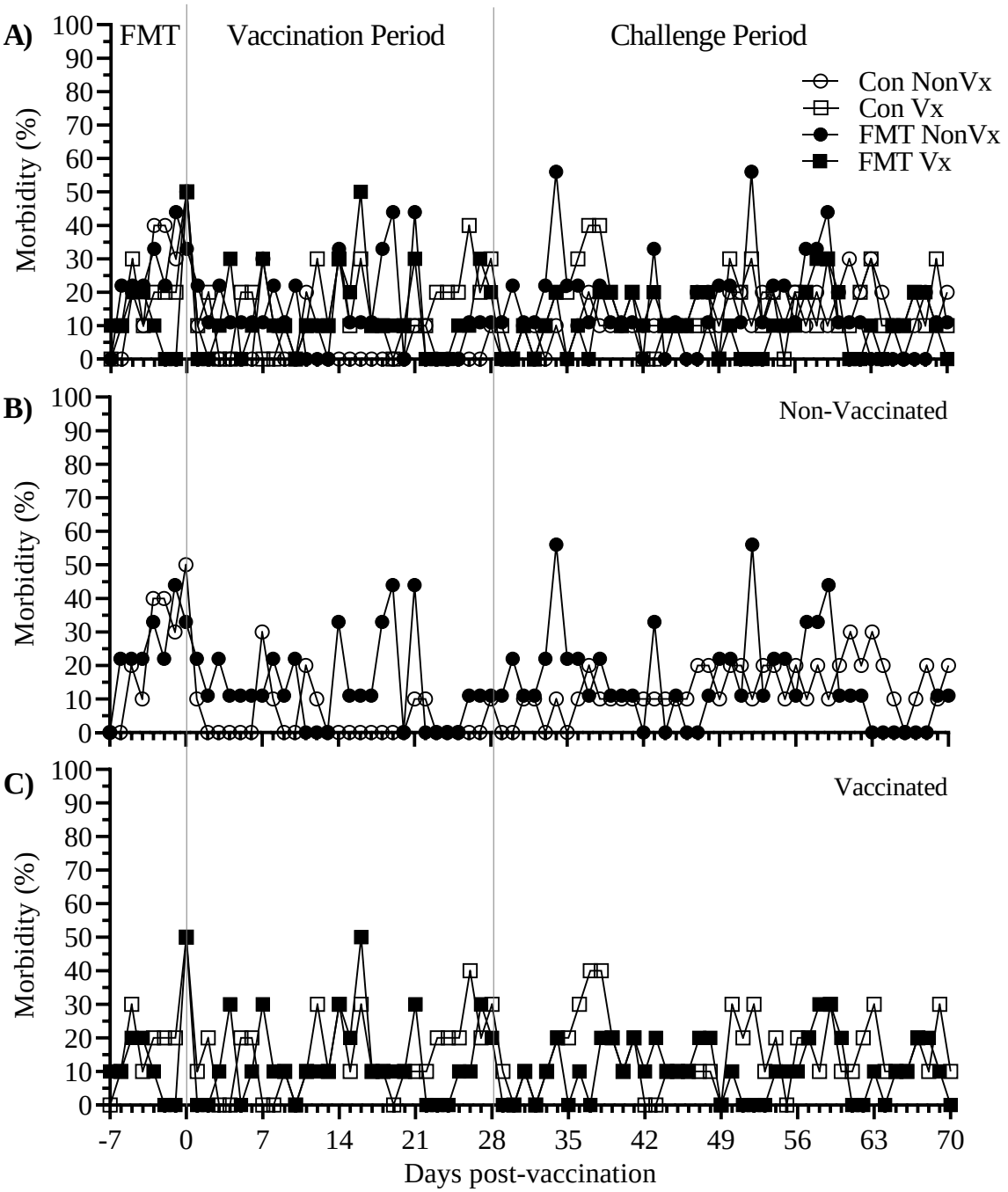

Figure 5.1.Morbidity of pigs with and without fecal microbiota transplantation in addition to with and without PRRSV MLV vaccination prior to PRRSV challenge.

A) Percent morbidity during the FMT (-7 to 0 dpv), vaccination period (0 to 28 dpv) and the challenge period (28 to 70 dpv); data is shown as the percent of pigs in each group with clinical signs associated with PRRSV infection. **B and C)** show non-vaccinated and vaccinated groups, respectively.

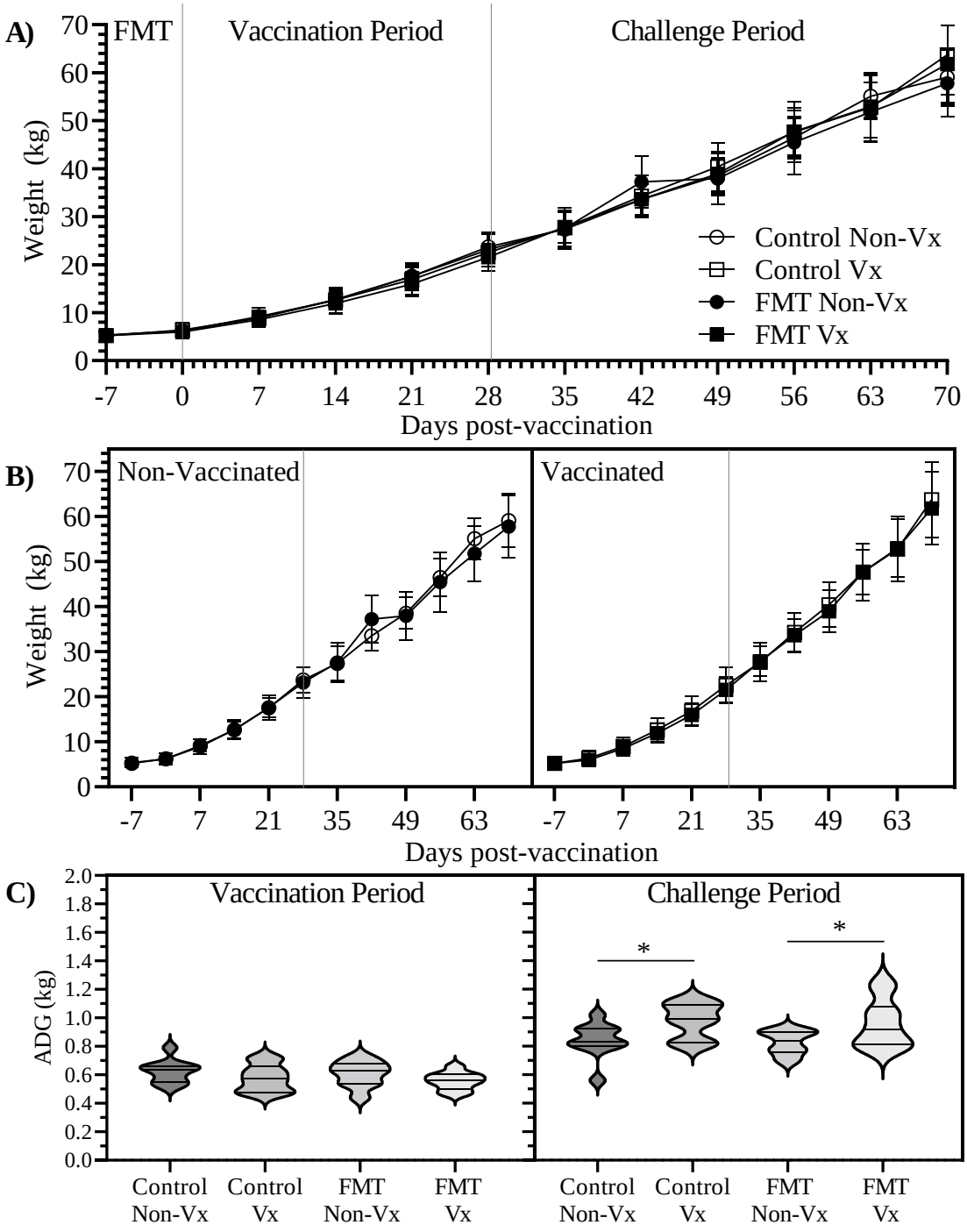

Figure 5.2 Weight gain and ADG during transplantation (FMT), PRRS MLV vaccination and after PRRSV challenge.

A and B) Data is shown as the mean weight in kg ± one standard deviation over the course of the 70-day study. **A)** Weight gain is shown together for all groups and well as, **B)** separated for pairwise comparison. There was significant variation associated time and time by vaccination

status ($p < 0.0001$ and $p = 0.04$; three-way ANOVA). Weights were found to be similar throughout the vaccination and challenge period ($p > 0.1$; Tukey's multiple comparison test). **C)** Data is shown as total ADG as violin plots, showing the frequency distribution of the data; lines are the median and quartiles. Mean ADG after PRRSV challenge was significantly different based on treatment ($p = 0.002$; two-way ANOVA) and inter-individual variation ($p = 0.009$; two-way ANOVA). Within the control groups, non-vaccinated pigs had significantly lower ADG compared to vaccinated pigs ($p = 0.03$; Tukey's multiple comparison test). Within the transplanted groups, non-vaccinated pigs also had significantly lower ADG in contrast to vaccinated pigs ($p = 0.02$; Tukey's multiple comparison tests).

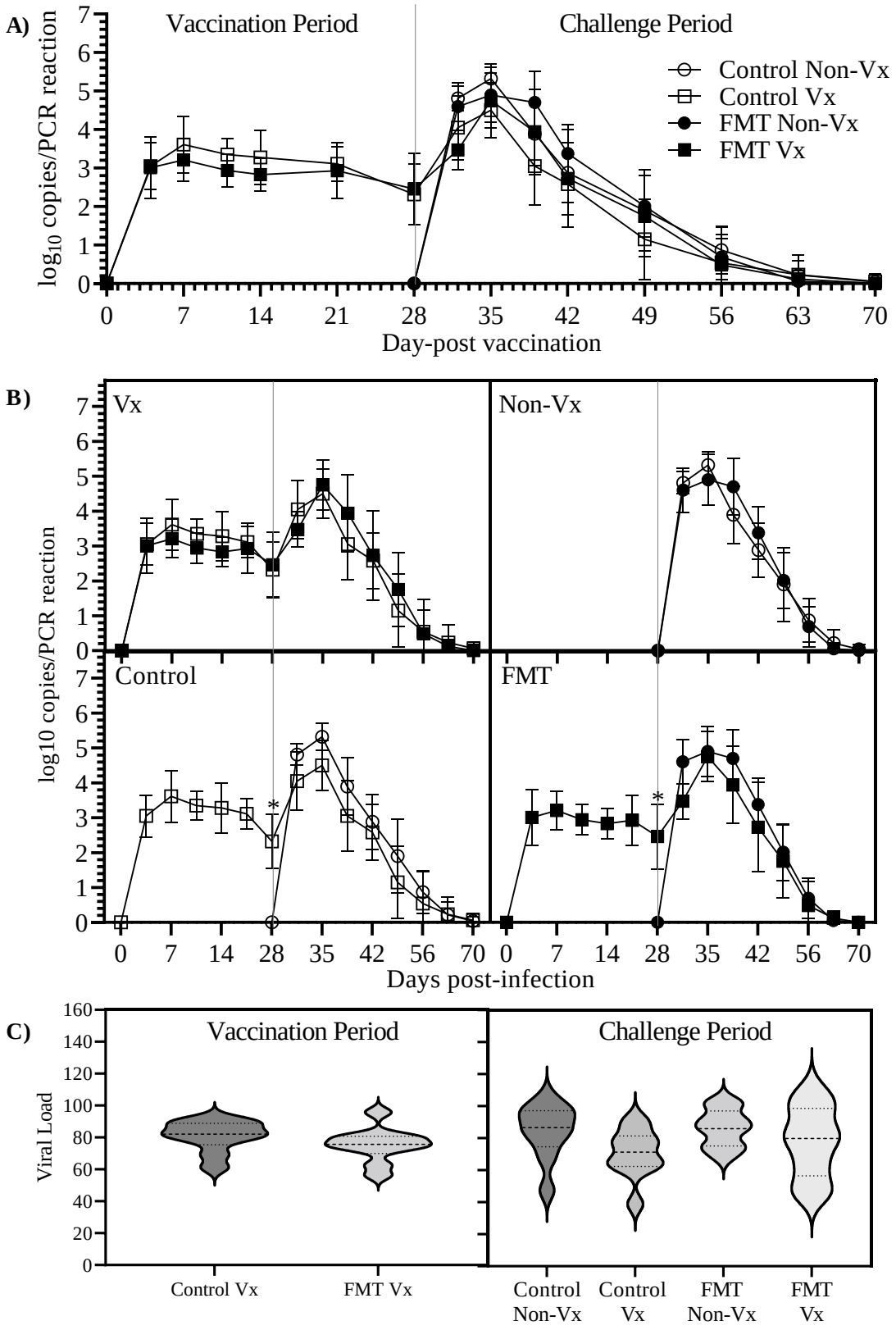
A)
Vaccination Period
Challenge Period
Control Non-Vx
Control Vx
FMT Non-Vx
FMT Vx
log10 copies/PCR reaction
Day-post vaccination
B)
Vx
Non-Vx
Control
FMT
log10 copies/PCR reaction
Days post-infection
C)
Vaccination Period
Challenge Period
Viral Load
Control Vx
FMT Vx
Control Non-Vx
Control Vx
FMT Non-Vx
FMT Vx

Figure 5.3 Time course of PRRSV viremia and viral load during vaccination and challenge periods.

A and B) Data is shown as the $\log_{10}$ copy number per PCR reaction ± 1 standard deviation. **A)** Viremia is shown for all groups and well as, **B)** separated for pairwise comparison. While viremia was lower in transplanted pigs than control pigs during the vaccination period, this difference that was not significant ($p > 0.1$; Šidák's multiple comparisons). On the day of challenge (28 dpv), both non-vaccinated groups had lower viremia compared to their vaccinated counterparts (*$p < 0.0001$; Tukey's multiple comparisons test). **C)** Data is shown as total viral load as violin plots, showing the frequency distribution of the data; lines are the median and quartiles. There was no significant difference in viral load both during the vaccination period as well as the challenge period ($p = 0.24$; unpaired *t*-test, and $p > 0.1$; two-way ANOVA with Tukey's multiple comparisons test).

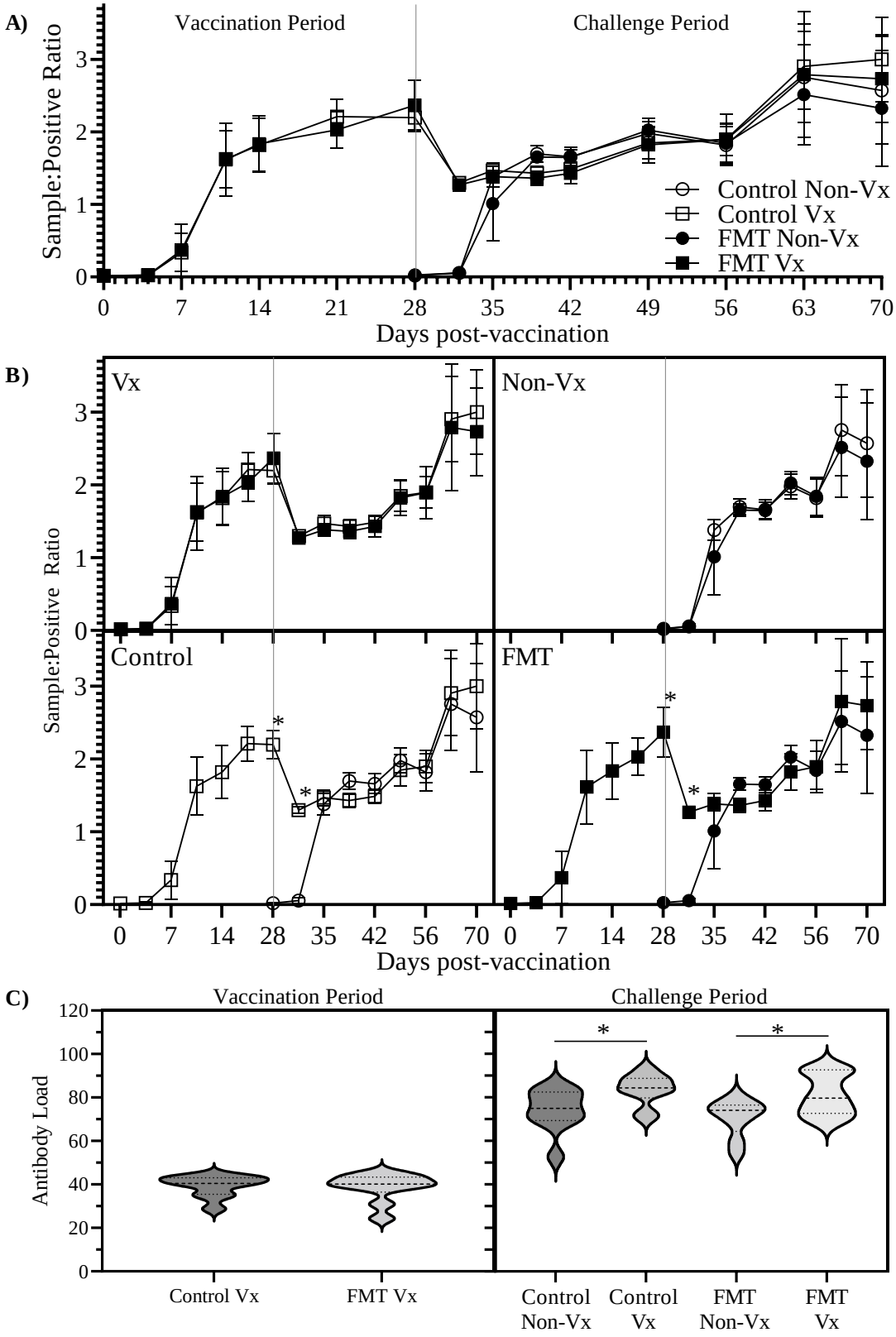
A)
Vaccination Period
Challenge Period
Sample:Positive Ratio
Control Non-Vx
Control Vx
FMT Non-Vx
FMT Vx
Days post-vaccination
B)
Vx
Non-Vx
Control
FMT
Sample:Positive Ratio
Days post-vaccination
C)
Vaccination Period
Challenge Period
Antibody Load
Control Vx
FMT Vx
Control Non-Vx
Control Vx
FMT Non-Vx
FMT Vx

Figure 5.4 Detection of antibody in transplanted and control pigs.

A and B) Data is shown as the mean sample:positive ratio ± 1 standard deviation for PRRSV antibodies. **A)** During the vaccination period, there was significant variation attributed with time and individual pigs ($p < 0.0001$; two-way ANOVA). After PRRSV challenge there was significant variation due time, vaccination status, and time by vaccination status ($p < 0.0001$; three-way ANOVA); additionally there was a trend towards significance for variation due to transplantation status ($p = 0.07$; three-way ANOVA). **B)** On challenge day (28 dpv) together with 32 dpv, antibody levels were significantly higher in vaccinated compared to the non-vaccinated groups (*$p < 0.0001$; unpaired *t*-tests). **C)** Data is shown as total antibody load as violin plots, showing the frequency distribution of the data; lines are the median and quartiles. There was no significant difference in antibody load during the vaccination period ($p = 0.85$; unpaired *t*-test). After challenge, there was significant variation associated with treatment and individual pigs ($p = 0.02$ and $p = 0.0015$, respectively; two-way ANOVA). Within control and transplanted, non-vaccinated pigs had a lower antibody load compared to vaccinated pigs (*$p = 0.03$ and $p = 0.02$; Tukey's multiple comparisons test).

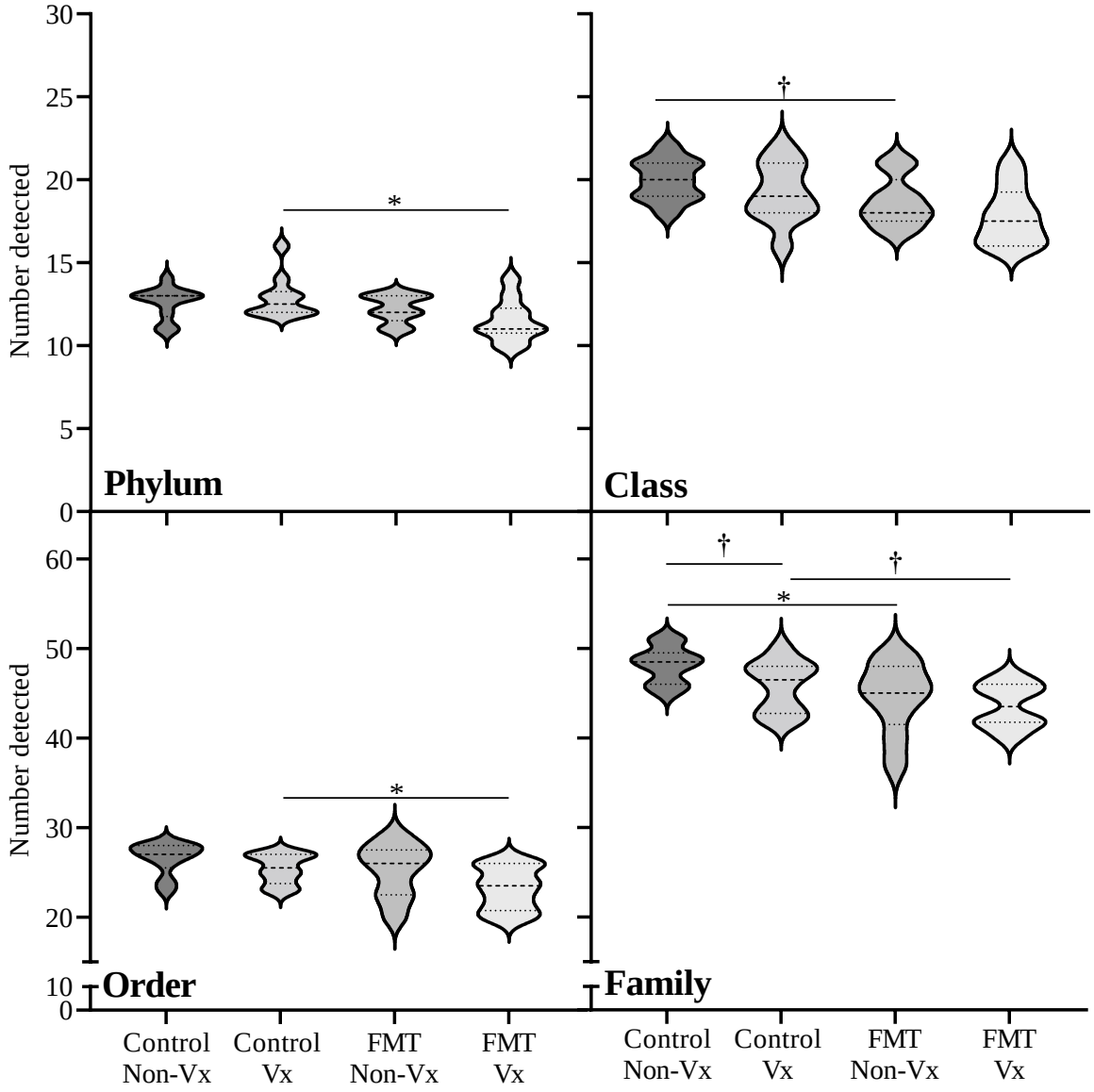

Figure 5.5 Fecal microbiome diversity in pigs after 7 days of transplantation and prior to PRRRS MLV vaccination as detected by metagenomics.

Data is shown as violin plots, showing the frequency distribution of the data; lines are the median and quartiles. Total number of microbial phyla (top left panel), classes (top right panel), order (bottom left panel) and microbial species (bottom left panel) detected by the metagenomics with the four groups. There was a decrease in the number of phyla, orders and families within the vaccinated groups, between control and transplanted pigs (*p = 0.02, *p = 0.04, and †p = 0.06, respectively; Mann-Whitney tests). There was also a decrease in the number of classes and families within non-vaccinated groups, between control and transplanted pigs (†p = 0.05 and *p = 0.03, respectively; Mann-Whitney tests).

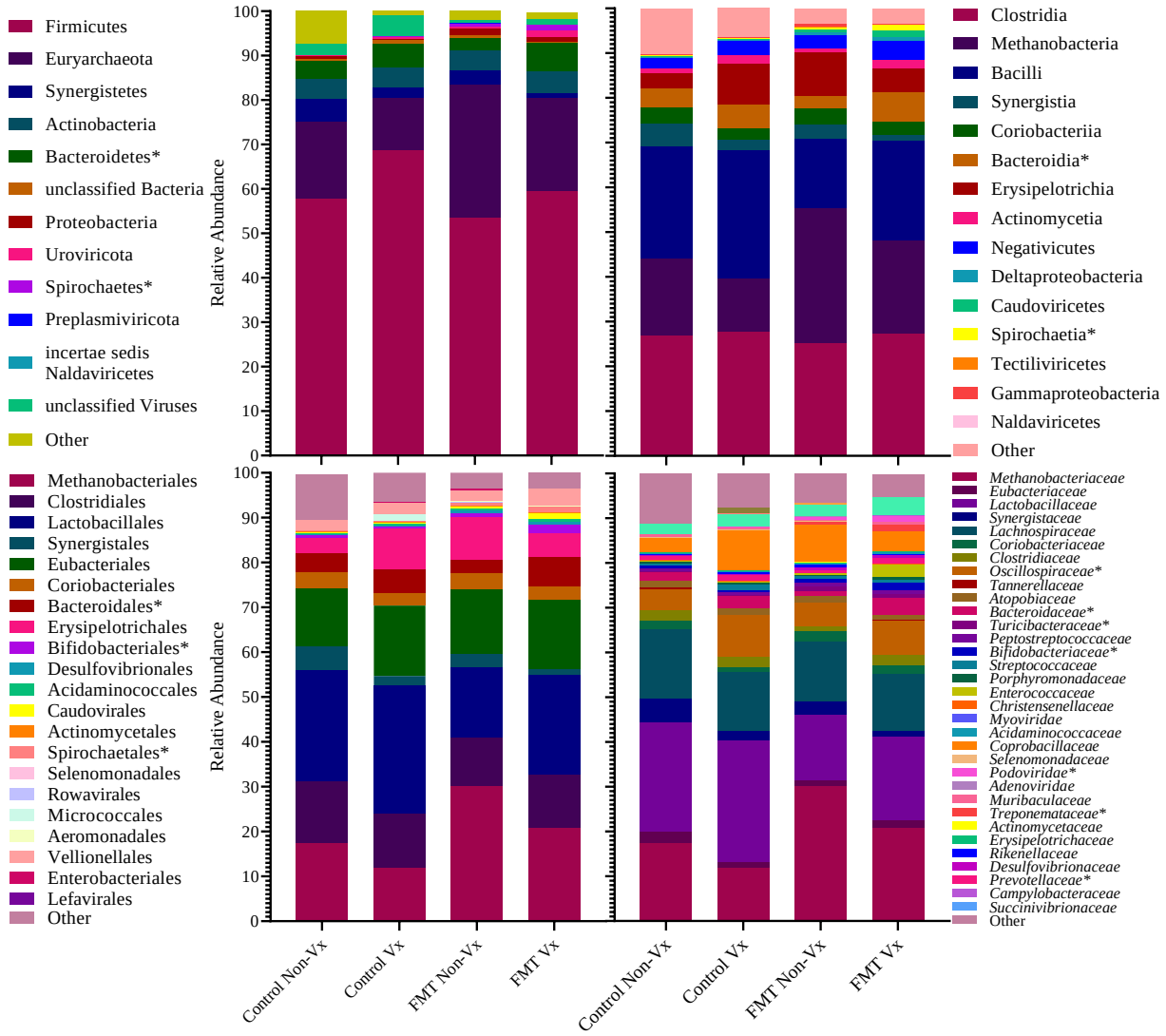

Figure 5.6. Fecal microbiome composition as detected by metagenomics after 7 days of transplantation and prior to PRRRS MLV vaccination.

Data shown as the mean relative abundance of bacterial families for high and low growth groups. Families making up 0.1% or less of all sequences detected plus unclassified microbes were grouped together and classified as "Other". **Top left panel)** Mean relative abundance of microbial phyla. There was a significant difference between the four groups within the phyla Bacteroidia and Spirochaetia (*$p < 0.05$; Kruskal-Wallis test). **Top right panel**) Mean relative abundance of microbial classes. There was a significant difference between the four groups within the classes Bacteroides and Spirochaetes (*$p < 0.05$; Kruskal-Wallis test). **Bottom left panel)** Mean relative abundance of microbial orders. There was a significant difference between the four groups within the orders Bacteroidiales, Bifidobacteriales and Spirochaetales (*$p < 0.05$; Kruskal-Wallis test). **Bottom right panel)** Mean relative abundance of microbial families. There was a significant difference between the four groups within the families *Oscillospiraceae*, *Bacteroidaceae*, *Turicibacteraceae*, *Bifidobacteriaceae*, *Prevotellaceae*, *Treponemataceae*, and *Podoviridae* (*$p < 0.05$; Kruskal-Wallis test).

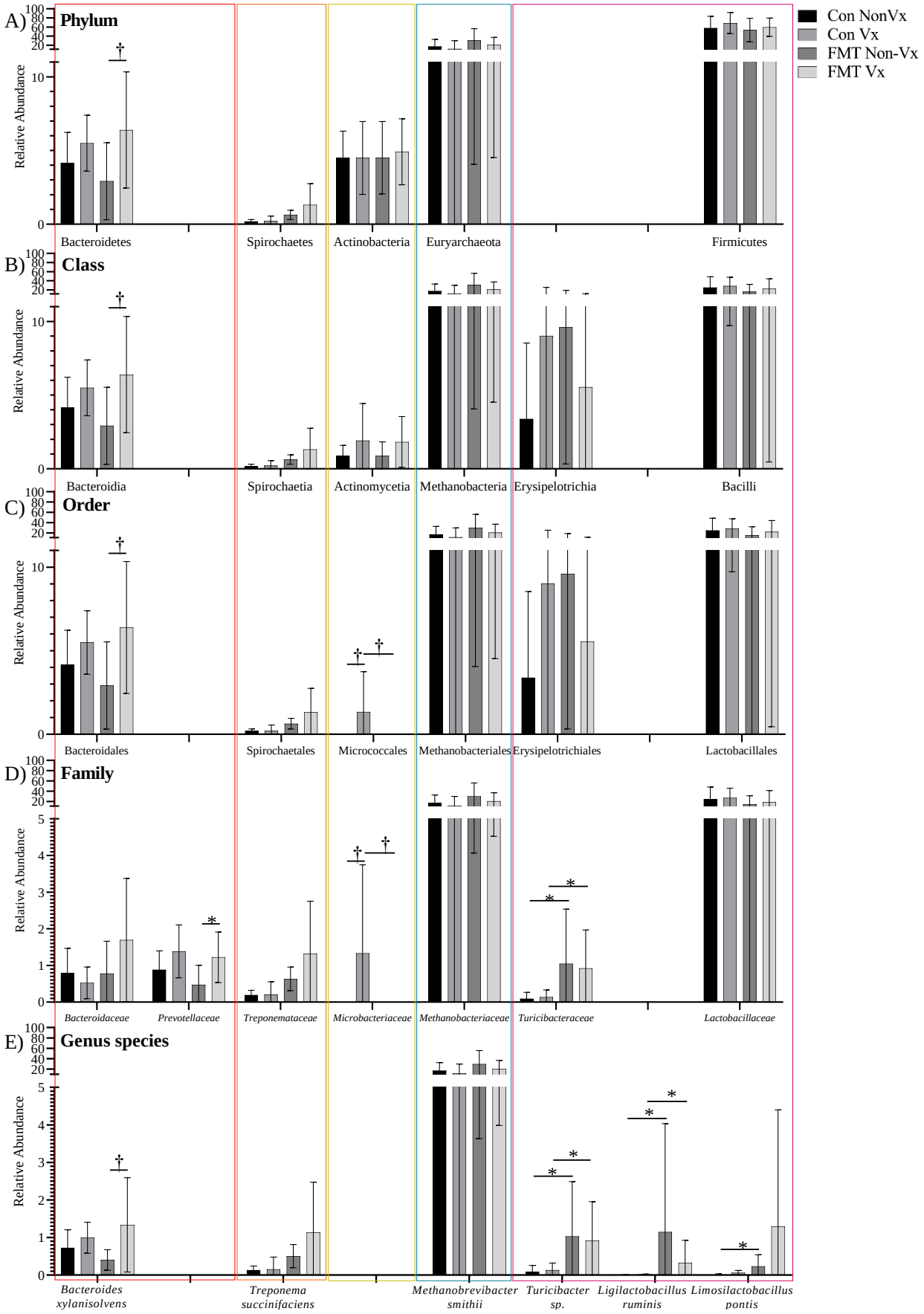
Con NonVx
Con Vx
FMT Non-Vx
FMT Vx
A) Phylum
Relative Abundance
Bacteroidetes
Spirochaetes
Actinobacteria
Euryarchaeota
Firmicutes
B) Class
Bacteroidia
Spirochaetia
Actinomycetia
Methanobacteria
Erysipelotrichia
Bacilli
C) Order
Bacteroidales
Spirochaetales
Micrococcales
Methanobacteriales
Erysipelotrichiales
Lactobacillales
D) Family
Bacteroidaceae
Prevotellaceae
Treponemataceae
Microbacteriaceae
Methanobacteriaceae
Turicibacteraceae
Lactobacillaceae
E) Genus species
Bacteroides xylanisolvens
Treponema succinifaciens
Methanobrevibacter smithii
Turicibacter sp.
Ligilactobacillus ruminis
Limosilactobacillus pontis

Figure 5.7. Differences found by metagenomics at different taxonomic levels 7 days after transplantation and prior to PRRSV MLV vaccination.

Data is shown as relative abundance between the four groups. Colored boxes represent microbial groups within the same taxonomic lineage (e.g. The phylum Bacteroides contains the class Bacteroidia, the family *Bacteroidaceae* in which the species *Bacteroides xylanisolvens* is contained. Also within the phylum Bacteroides, the family *Prevotellaceae* is contained). **A)** Within the phyla Bacteroidetes, there was a trend towards significant changes between the transplanted groups, with non-vaccinated pigs having a lower relative abundance compared to vaccinated pigs (†p = 0.09; Dunn's multiple comparisons test). **B)** Within the class Bacteroidia, there was a trend towards significant changes between the transplanted groups, with non-vaccinated pigs having a lower relative abundance compared to vaccinated pigs (†p = 0.09; Dunn's multiple comparisons test). **C)** Within the order Bacteroidiales, there was a trend towards significant changes between the transplanted groups, with non-vaccinated pigs having a lower relative abundance compared to vaccinated pigs (†p = 0.09; Dunn's multiple comparisons test). Within the order Micrococcales, there was a trend towards significant differences between control, non-vaccinated and vaccinated pigs (†p = 0.08; Dunn's multiple comparisons test) in addition to vaccinated, control and transplanted pigs (†p = 0.08; Dunn's multiple comparisons test). **D)** The family *Prevotellaceae* showed significant differences between the transplanted groups; non-vaccinated pigs had significantly lower *Prevotellaceae* relative abundance compared to vaccinated pigs (*p = 0.04; Dunn's multiple comparison test). Within the family *Microbacteraceae* there were trends towards significant differences between control, non-vaccinated and vaccinated (†p = 0.08; Dunn's multiple comparisons test) as well as vaccinated, control and transplanted groups (†p = 0.08; Dunn's multiple comparisons test). Between the non-vaccinated groups, control pigs had lower *Turicibacteraceae* relative abundance than their transplanted counterparts (*p = 0.04; Dunn's multiple comparisons test). **E)** Within transplanted pigs, non-vaccinated pigs had lower relative abundance of *Bacteroides xylanisolvens* than vaccinated pigs (†p = 0.055; Dunn's multiple comparisons test). Control pigs had a lower relative abundance of *Turicibacter sp.* compared to transplanted pigs, both non-vaccinated and vaccinated (p = 0.04 and 0.009; respectively; Dunn's multiple comparisons test). Control pigs also had a lower relative abundance of *Ligilactobacillus ruminis* compared to transplanted pigs, both non-vaccinated and vaccinated (*p = 0.01 and *p = 0.0499; respectively; Dunn's multiple comparisons test). Additionally, control pigs were found to have a lower relative abundance of *Limosilactobacillus pontis* compared to transplanted pigs, within non-vaccinated pig (*p = 0.03; Dunn's multiple comparisons test).

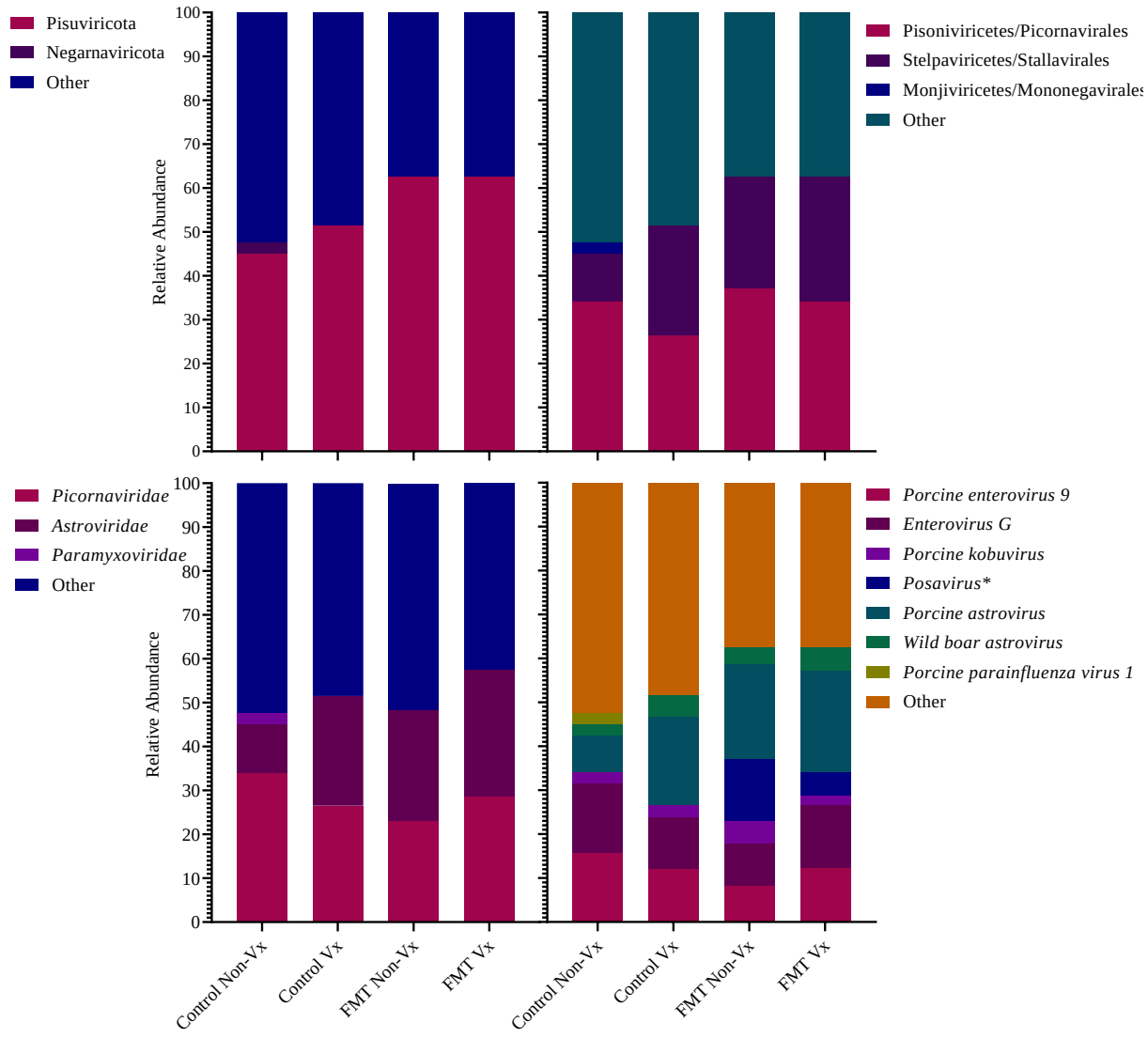

Figure 5.8. Microbial diversity as detected by RNA-Seq after 7 days of transplantation and prior to PRRRS MLV vaccination.

Data shown as the mean relative abundance of bacterial families for high and low growth groups. Families making up 0.1% or less of all sequences detected in 1 or more sample subsets in addition to unclassified microbes are grouped together and classified as "Other". Top left panel: Mean relative abundance of microbial phyla. Top right panel: Mean relative abundance of microbial classes/orders. Bottom left panel: Mean relative abundance of microbial families. Bottom right panel: Mean relative abundance of microbial species. Posavirus was found in higher quantities in transplanted pigs than control pigs, a significant difference (*p = 0.02; Kruskal-Wallis test with Dunn's multiple comparisons test)

Chapter 6 - Conclusions and Future Directions

PRRS has historically been and continues to be a difficult disease to control; genetic mutations lead to phenotypic variations and make control through vaccines difficult. We demonstrated that even between two isolates that disease progression can be almost non-overlapping. Since the current PRRS MLV vaccines are inefficient, we identified the need for alternative or adjunct control methods. While many methods are being explored, the gut microbiome offers several benefits. It can be used to not only to modify microbial populations within the gut, but also lead to immunological changes throughout the body without using antimicrobials. Gut microbiome modulated immunological changes could also enhance in the efficacy of the PRRS MLV vaccine.

Within our first study we demonstrated how genetic changes of PRRSV can result in significant variation in clinical disease presentation. This data should be used to compare phenotypic outcomes with host genotypes. Our group has previously shown the pig SNP WUR10000125 (WUR) was associated with weight gain and viremia variation.[87,89,174] In order to correlate previously identified SNPs with outcome we identified the need to create larger objective sets of clinical phenotype data. Since clinical data is inherently subjective, we created a new scoring system that transformed qualitative data into semi-quantitative data, which we designated the mean clinical score. Future work should focus on correlating not only this phenotypic data to genotypic data with genome-wide association studies, but also expand our knowledge of other PRRSV isolates. This system could also be used to identify resistance to other high impact swine diseases such as African and classical swine fever.

Within two of our studies we used PCV with PRRSV as a co-infection model. However, in current production settings, PCV2 is less commonly found due to the widespread use of

vaccines. Other co-infection organisms should be explored that create more common conditions, specifically bacteria such as *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, and *Bordatella bronchiseptica*.

The remaining studies were dedicated to understanding how gut microbiome differences could aid in PRRSV control. Within these studies we utilized various methods of microbiome analysis; the Lawrence Livermore Detection Microbial Detection Array (LLMDA), 16 rDNA sequencing as well as next generation sequencing (NGS) on an Illumina Platform. These studies highlight the advantage and disadvantages of the different methods. LLMDA is a relatively simple method to detect all species that have been annotated with NCBI Genbank; however, quantitative data cannot be gained from this method. 16S rDNA results in semi-quantitative data of relative abundance; however, it can only detect bacterial species. NGS has the ability to provide both qualitative and quantitative data; however, it requires more expertise to analyze. We are the first study, to our knowledge to use RNA-seq to identify RNA viruses within the gut microbiome. Future studies should consider the use of this technology to describe viruses, as we found one virus, posavirus, significantly different between experimental treatments.

We understand that limitations may have affected our studies the associated results. Our first study had sufficient numbers for genome-wide association studies; however, our initial gut microbiome studies lack sufficient sample size to identify minor effects. This likely means that, in terms of significant associations, we are likely at the tip of the iceberg. For example, small effects seen by relatively low abundance microbes would not been detected. Therefore, larger samples sizes are needed to fully demonstrate associations and determine molecular and metabolic pathways that can be manipulated through the gut microbiome.

In our studies we focused on the gut microbiome; there are advances to this model. It is a non-invasive sample to collect that can be tested serially. However, sampling within different locations could result in different results. This includes not only different locations within the feces itself, but different locations in the gastrointestinal tract, in addition to other body systems such as the respiratory tract. Even within the fecal samples itself, microbes may not be homogenously distributed. In future studies it might be better to homogenize the samples with a blender before analyzing the microbiome. Sampling in different gastrointestinal locations will also give different results. Previous studies have shown that microbes differ along the swine gastrointestinal tract,[498] as well as between the mucosal layer and contents within the gut lumen.[499] However, collecting these samples may be invasive or result in terminal data due to the methods required to collect them. In terms of PRRS, it would also be good to look at the respiratory microbiome and the immune response in the respiratory tract. Our previous data has shown that the respiratory tract is not a sterile environment.[328] It is likely that respiratory microbiome differences are correlated to differences in PRRSV-associated outcomes and immunological responses. Therefore, it is necessary to study this environment to determine if microbial modulation would aid in PRRSV vaccine efficacy as well as decrease PRRSV-associated disease. Respiratory immune response is also important to understand; future studies should consider bronchioalveolar lavage to analyze secretory IgA and other cytokines.

The gut microbiome is incredibly complex and minute genetic and environmental alterations can have substantial effects. While the pigs used in these study represent commercial pigs, they are still genetic crosses. Genetics has been shown to affect the gut microbiome.[278] While these pigs were sibling pairs, we couldn't rule out differences due to genetics. It will be important to rule out differences due to host genetics when understanding gut microbiome

variations. Additionally, previous studies have shown the effect of antimicrobials on the swine gut microbiome; some of the earliest microbiome studies were conducted to study antimicrobial effects on the gut microbiome.[248,249,252] While the sows used for FMT had not received antibiotics in 15 months, antibiotics are known to have long-term effects[50,500-502] and could lead to changes for which we cannot account. Additionally, with many gut microbes contributing to outcome, it is hard to parse out the effect of single organisms. In an ideal situation, pigs would only have one microbe within their gut at a time to study its effects. However, synergistic effects would be missed using this method. One method of depleting the microbiome involves the use of multiple antimicrobials. With a growing concern for antimicrobial resistance, and with the known effects of antimicrobials, this may no longer be the best way to look at specific microbes. While consideration can be given to cesarean section pigs lacking a gut microbiome, this is time intensive and requires expertise in this area. For that reason, other options should be considered. Realistically and fully understanding the complexity of gut microbiome is, thus, complicated at best.

Within our first FMT study, we used fecal material from two high-health sows as our FMT to study the effects on PRRSV- and PCV2-associated outcome. Because we are using feces to transplant into the pigs, we can't define all of the components being given. Feces is known to contain colonocytes, secretory IgA (sIgA), plant material, sugars, oligosaccharides, live and dead microorganisms, in addition to microbial metabolites such as short-chain fatty acids.[503] In addition to the microbial composition, future studies should identify prebiotics and postbiotics that might be contained in the fecal transplant material. Synergistic effects occurring between pre-, pro-, post-, and synbiotics (pre- and probiotic combination) have been previously described and likely complicate our understanding of the gut microbiome.[365,366,504] It would be relevant to

isolate parts of the transplant material and give them individually as well as together. Immunological studies should be done to look at lymphocyte populations within the fecal transplant material as well as sIgA.

Within the last two studies we looked at the relationship of the gut microbiome and PRRSV MLV vaccine. In one study we looked at gut microbiome differences in high and low growth, PRRSV MLV vaccinated, PRRSV/PCV2 challenged pigs. To fully understand the effect of the vaccine, we still need to compare non-vaccinated to the vaccinated pigs. In this study we also used the PRRSV/PCV2 co-infection model. This could complicate vaccine effects, as we know that the PRRSV vaccine potentiates PCV2 replication.[116] Pigs used in this study were subclinical; this had both advantages and disadvantages. While these pigs had no received antimicrobials, differences in clinical disease were more subtle, likely making gut microbiome differences more difficult to detect.

Within the next study, we gave the FMT from the first study to look at its effects on the PRRS MLV vaccine. We found that FMT lessened vaccine-associated viremia. Previous studies have shown that viremia is correlated with virus shedding;[482] therefore, decreased viremia could potentially lead to decreased virus shedding. Oronasal and fecal secretions should be analyzed for virus shedding after FMT administration. RNA-seq data allowed us to determine the population of RNA viruses within the gut microbiome. However, this technology has the capability to provide additional information. It can be used to determine transcriptionally active microbes within the gut, as well as the host fecal transcriptome. This could provide information about microbes that are active within the gut and metabolic and immunological pathways that are up or down regulated.

For future studies involving the gut microbiome and the PRRS MLV vaccine it would be good to look at high and low antibody producers to compare gut microbiome differences. Differential antibody production might better predict what bacteria are associated with improved outcome than growth and better to discriminate between groups. It would also be beneficial to administer single microbial isolates isolated in the previous studies to determine their effects on the PRRSV vaccine.

PRRSV is a genetically diverse virus that leads to changes in phenotypic outcome, making it a difficult disease to diagnose, control and prevent. The gut microbiome is a promising alternative tool for PRRS control, due to its important role in both immunity and weight gain. Our research provides initial evidence for the potential application of the microbiome as an alterative tool for improving response during PRRSV vaccination and infection.

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