Novel control and detection strategies for porcine viruses

by

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B.V.Sc. & A.H., Tribhuvan University, 2014
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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

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Abstract

Pork is the most widely consumed animal protein globally. Viral diseases affecting pork production have a major socio-economic impact on pork producers around the world. Novel techniques to prevent and control the spread of viral swine diseases are necessary to guard swine health and ensure global food security.

African swine fever virus (ASFV) is a current threat to global pork production due to its high case fatality rate, lack of commercial vaccine, recent transboundary spread into new regions of the world, and challenges of virus control. ASFV causes a severe hemorrhagic disease characterized by high fever, loss of appetite, hemorrhage, and depression. Pigs infected with highly virulent ASFV strains frequently die within 2 weeks with up to 100% mortality rates. Economic losses due to the introduction of ASFV into the United States (US) are estimated to be $15 billion if disease control allows the US to reenter export markets within two years. Over $50 billion in losses could occur if the disease spreads to the feral swine population and the US is unable to eliminate the disease over a ten-year period. Thus, preventing introduction and further spread of ASFV is critical for the US and any country currently negative for the virus. Feed ingredients used in US commercial swine diets are sourced in large volumes from countries currently positive for the virus. ASFV has been shown to be stable in feed ingredients subjected to transoceanic conditions and transmission can occur through the natural consumption of contaminated feed. Despite evidence supporting the role of feed in ASFV introduction risk and transboundary spread, diagnostic tools for surveillance and detection of ASFV in feed remains an industry challenge.

Porcine reproductive and respiratory syndrome (PRRS) has the highest economic impact on swine production in the US, with estimated annual losses to the US pork industry at $663.91

million. It is caused by PRRS virus (PRRSV), a single-stranded RNA virus belonging to the family *Arteriviridae*. The major economic impact of PRRS on the global swine industry is due to diminished weight gain and respiratory disease in growing pigs (Holtkamp et al., 2013). PRRSV is frequently isolated along with porcine circovirus type 2 (PCV-2) in US field cases. PCV-2 is a single-stranded DNA virus belonging to the family *Circoviridae*. It is associated with a group of disease syndromes termed porcine circovirus-associated disease (PCVAD), characterized by muscle wasting, respiratory disease, jaundice, or pallor, and reduced weight gain in growing pigs. PRRSV and PCV-2 both cause systemic infections primarily targeting pulmonary and lymphoid tissue and can be used in co-infection models to reproduce PCVAD experimentally. Co-infection with these two viruses modulates host immunity resulting in increased viral replication and enhanced polymicrobial disease compared to single infections alone. Recent research has shown that the gut microbiome, or collection of microorganisms living in the gastrointestinal tract, is associated with vaccine efficacy and health outcomes following PRRSV infection in pigs. Moreover, the gut microbiome is known to play an essential role in local and systemic immunity and that gut microbiome-based therapeutics such as fecal microbiota transplant (FMT) have therapeutic and prophylactic potential for diseases outside the gastrointestinal system.

This dissertation includes four research studies to investigate novel control and detection strategies for porcine viruses.

The objective of the first study was to evaluate feed dust as a novel diagnostic sample type for the detection of ASFV nucleic acid and infectious titer in experimentally contaminated feed. Moist swabs were used to collect dust from creep feeders after natural consumption of feed inoculated with 3.1–5.4 $\log_{10}$ $TCID_{50}$/g ASFV Georgia 2007 in the presence and absence of

antimicrobial feed additives. ASFV DNA was detectable by qPCR in feed dust for at least 8 h after feed inoculation and in the presence of antimicrobial feed additives. Virus isolation could detect infectious ASFV in feed dust when swabbed 30-min post-inoculation. Overall, this study provides valuable proof of concept data on feed dust as a potential surveillance tool for ASFV in contaminated feed and ingredients.

The objective of the second study was to investigate the effects of PRRS MLV vaccination on the gut microbiome composition and diversity of nursery pigs. At twenty-eight days post-vaccination, fecal samples were collected from both vaccinated (n = 12) and nonvaccinated (n = 12) pigs. Gut microbiomes were compared between the two groups using the Lawrence Livermore Microbial Detection Array (LLMDA). Vaccinated pigs had increased microbial species diversity and increased Firmicutes to Bacteroidetes ratio. Significant differences were also noted in microbiome composition at different taxonomic levels. Specifically, the *Enterobacteriaceae* family was detected at a significantly higher rate in vaccinated pigs compared to nonvaccinated pigs. Overall, these results suggest that infection with PRRS MLV may modulate the gut microbiome and that certain microbiome characteristics may contribute to vaccine efficacy.

The objective of the third study was to characterize the FMT material through several approaches, including the identification of antibiotic (ABx) resistance genes, evaluate the bacterial species cultivability, and determine concentration of culturable microorganisms. A total of 26 bacterial isolates were identified by mass spectrometry out of which 24 isolates were culturable. Overall, the study shows that FMT material can be characterized and pure cultures of cultivable fecal bacteria can be obtained using culture-based techniques, and the cultivable portion of the FMT material is dominated by anaerobes.

The objective of the fourth study was to develop a microbiota therapeutic with a pre-defined consortium of cultivable microbes isolated from the FMT material shown to be associated with improved health outcomes in growing pigs with PCVAD. This study aimed to investigate the efficacy of the cultivable microbiota therapeutic as a means to improve disease outcomes following PRRSV and PCV-2d co-infection. Pure cultures of 24 bacterial species were pooled together in sterile normal saline with 10% glycerol mimicking their composition in the fecal transplant material. One group of pigs (n = 50) was administered the microbiota therapeutic while a control group (n = 50) was administered a sterile mock transplant. All pigs were co-infected with PRRSV and PCV-2 after 7 days of the therapeutic administration. Microbiota therapeutic appeared safe as no adverse effects were noted in transplanted pigs, increased average daily gain of transplanted pigs prior to co-infection (-8 to 0 dpi) and in the late stage of co-infection (38 to 42 dpi). However, microbiota therapeutic largely failed to replicate the overall benefits of FMT in PCVAD affected pigs.

Taken together, the dissertation provides insights into a novel diagnostic sample for ASFV detection in swine feed and proposes novel methods to reduce PRRS associated losses including modulating pig gut microbiome to improve PRRS MLV vaccine efficacy and developing and utilizing a pre-defined microbial consortium to improve or aid current therapies for PCVAD.

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Abstract

Pork is the most consumed animal protein worldwide. Viral diseases affecting pork production have a huge socio-economic impact on global pork producers. Novel techniques to prevent and control the spread of viral swine diseases are necessary to guard swine health and ensure global food security.

African swine fever virus (ASFV) is a significant threat to pig production and protein availability worldwide. African swine fever (ASF) is a severe hemorrhagic disease characterized by high fever, loss of appetite, ataxia, and depression. Pigs infected with highly virulent ASFV strains frequently die within 2 weeks with up to 100% mortality rates. ASF is the most consequential porcine disease today due to its high case fatality rate, recent incursions into historically negative countries and regions, and, most importantly, extreme viral control challenges. Current control strategies rely on early disease detection through rapid field recognition, strict biosecurity, and large-scale culling of infected pigs. Moreover, ASF causes serious socio-economic consequences on national and international trade of animal and pork products including export restrictions, control of animal (and product) movement, and animal quarantine. Subsequently, there is a significant impact on both the global meat economy and the global market for agricultural commodities including feed grain. Economic losses due to ASFV's introduction into the United States are estimated, on the low end, to be $15 billion if a country can control disease and reenter export markets within two years. On the upper end, over $50 billion in losses could occur if the disease spreads to the feral swine population and a country is unable to eliminate the disease over a ten-year period.

Porcine reproductive and respiratory syndrome (PRRS) has the highest economic impact on swine production worldwide with estimated annual losses to the United States industry at

$663.91 million. It is caused by PRRS virus (PRRSV), a single-stranded RNA virus belonging to the family *Arteriviridae*. The major economic impact of PRRS on the global swine industry is due to diminished weight gain and respiratory disease in growing pigs (Holtkamp et al., 2013). PRRSV is frequently isolated along with porcine circovirus type 2 (PCV-2) in the US field cases. PCV-2 is a single-stranded DNA virus belonging to the family *Circoviridae*. It is associated with a group of disease syndromes termed porcine circovirus-associated disease (PCVAD), characterized by muscle wasting, respiratory disease, jaundice, or pallor, and reduced weight gain in growing pigs. PRRSV and PCV-2 both cause systemic infections primarily targeting pulmonary and lymphoid tissue and are used in co-infection models for reproducing PCVAD. Co-infection with the two viruses modulates host immunity resulting in increased viral replication and enhanced polymicrobial disease compared to single infections alone.

The objective of the first study was to evaluate feed dust as a novel diagnostic sample type for the detection of ASFV nucleic acid and infectious titer in experimentally contaminated feed. Moist swabs were used to collect dust from creep feeders after natural consumption of feed inoculated with 3.1–5.4 $\log_{10}$ $TCID_{50}$/g ASFV Georgia 2007 in the presence and absence of antimicrobial feed additives. ASFV DNA was detectable by qPCR in feed dust for at least 8h after feed inoculation and in the presence of antimicrobial feed additives. VI could detect infectious ASFV in feed dust when swabbed 30-min post-inoculation. Overall, this study provides valuable proof of concept data on feed dust as a potential surveillance tool for ASFV in contaminated feed and ingredients.

The objective of the second study was to investigate the effects of PRRS MLV vaccination on the gut microbiome composition and diversity of nursery pigs. At twenty-eight days post-vaccination, fecal samples were collected from both vaccinated (n = 12) and

nonvaccinated (n = 12) pigs. Gut microbiomes were compared between the two groups using the Lawrence Livermore Microbial Detection Array (LLMDA). Vaccinated pigs had increased microbial species diversity and increased Firmicutes to Bacteroidetes ratio. Significant differences were also noted in microbiome composition at different taxonomic levels. Specifically, the *Enterobacteriaceae* family was detected at a significantly higher rate in vaccinated pigs compared to nonvaccinated pigs. Overall, these results suggest that infection with PRRS MLV may modulate the gut microbiome and that certain microbiome characteristics may contribute to vaccine efficacy.

The objective of the third study was to develop a microbiota therapeutic with a pre-defined consortium of viable microbes isolated from the original fecal microbiome transplant material shown to be associated with improved health outcomes in growing pigs under a porcine respiratory disease complex model. Aerobic and anaerobic cultures isolated a total of 24 culturable bacteria as identified by MALDI-TOF mass spectrometry. Pure cultures of all 24 species were pooled together in sterile normal saline with a 10% glycerol solution mimicking their composition in the original fecal transplant material.

The objective of the fourth study was to investigate the efficacy of microbiota therapeutic developed in the third study as a means to improve disease outcomes following PRRSV and PCV-2d co-infection. One group of pigs (n = 50) was administered the microbiome therapeutic while a control group (n = 10) was administered a sterile mock transplant. Pigs from both groups were co-infected with PRRSV and PCV-2 after 7 days of the therapeutic administration. Microbiome therapeutic was safe on pigs as evidenced by weight gain in the therapeutic and control pigs prior to co-infection. Overall, this study highlights the beneficial effect of microbiota therapeutic as evidenced by the improved average daily gain in the therapeutic

administered pigs following PRRSV and PCV-2 co-infection. Additionally, it identifies potential limitations.

Taken together, the data presented in this dissertation provided insights into a novel diagnostic sample for ASFV detection and prospects of modulating gut microbiome to improve vaccine efficacy and utilizing pre-defined microbiome consortia to improve or aid current therapies for porcine epidemic diseases.

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Dedication

I would like to dedicate my dissertation work to my family. A special feeling of gratitude to my loving parents, Hari Prasad and Pramila Devi Khanal for always encouraging and pushing me to conquer my dream. My Husband, Santosh, who has shown up every day for me, encouraged me to keep going and be my constant support in every challenge throughout graduate school and life in general. You never gave up on me, I love you. Last but not least, my darling daughter, Ruby who helped me realize my strengths and took off my stress with her sweet gestures and warm hug while things were difficult and I was losing hope. I love you my baby.

Chapter 1 - Detection of African swine fever virus in feed dust collected from experimentally inoculated complete feed using quantitative PCR and virus titration assays

Abstract

African swine fever virus (ASFV) is a current threat to global pork production due to its high case fatality rate, lack of efficacious vaccine, and recent transboundary spread into new regions of the world. Preventing introduction and further spread of ASFV is critical for countries currently negative for the virus. ASFV is stable in feed ingredients subjected to transoceanic conditions and transmission occurs through the natural consumption of contaminated feed. In this study, we investigated the use of feed dust collected from experimentally inoculated feed as a novel diagnostic sample type for ASFV detection. Moist swabs were used to collect dust from creep feeders after natural consumption of feed inoculated with 3.1 – 5.4 $\log_{10}$ $TCID_{50}$/g ASFV Georgia 2007 in the presence and absence of antimicrobial feed additives. Results validate the potential use of feed dust swabs as a novel diagnostic surveillance tool for detection and quantification of viral nucleic acid and infectious virus titer in ASFV-contaminated feed.

Introduction

African swine fever virus (ASFV) is a significant threat to worldwide pig production and protein availability. First described in 1921 in Africa (Eustace Montgomery, 1921), ASFV infection results in high case fatality of domestic swine and wild boar due to widespread hemorrhage (Blome et al., 2013). The only member of the family *Asfarviridae* (Alonso et al., 2018), ASFV is a large and complex virus with high environmental stability and largely unknown correlates of immunological protection. Risk of ASFV transboundary spread has

escalated due to recent incursions of the virus into new countries and regions that have historically maintained a negative status. Namely, new ASFV introductions into major pork producing countries include China in August 2018 (Zhou et al., 2018), Belgium in September 2018 (Forth et al., 2019), Vietnam in January 2019 (Le et al., 2019), and Germany in September 2020 (Sauter-Louis et al., 2020). Without an efficacious vaccine or treatment available for protection or amelioration of disease (Sang et al., 2020), the overwhelming objective of negative countries is to prevent ASFV introduction into domestic and wild pig populations.

Contaminated feed and feed ingredients are one potential route for introduction and transmission of ASFV into new herds and regions (Niederwerder, 2021). Feed ingredients intended for use in commercial swine diets are a global commodity and significant volumes are traded between countries with opposing ASFV epidemiological situations (Patterson et al., 2020). Further, ASFV has demonstrated broad stability in a wide-range of commonly imported feed ingredients exposed to transoceanic environmental conditions (Dee et al., 2018; Stoian et al., 2019) and ASFV is transmissible through the ingestion of contaminated plant-based feed (Niederwerder et al., 2019). Contamination of feed and feed ingredients may be due to unsafe manufacturing practices or environmental exposure, such as during harvest or processing. For example, feed additives from contaminated dried porcine blood in China (Wen et al., 2019) and feed from contaminated grasses and grains in Latvia and Estonia (Olsevskis et al., 2016; Nurmoja et al., 2020) have been implicated as likely contributors to ASFV introduction and spread in the field.

Despite evidence supporting the role of feed in ASFV introduction risk and transboundary spread, diagnostic tools for surveillance and detection of ASFV in feed remains an industry challenge. Feed dust has shown to be a useful and sensitive diagnostic surveillance tool

for detection of pathogens such as *Salmonella enteritidis* and porcine epidemic diarrhea virus in livestock feeds and environments (Davies & Wray, 1996; Dee et al., 2014; Jones & Richardson, 2004; Gebhardt et al., 2018). Further, utilizing feed dust for pathogen testing may help overcome the diagnostic challenges associated with representative sampling and heterogeneous contamination in direct testing of bulk feed (Davies & Wray, 1997; Jones et al., 2020; Binter et al., 2011). Thus, the objective of the study described herein was to evaluate feed dust as a novel diagnostic sample type for detection of ASFV nucleic acid and infectious titer in experimentally contaminated feed.

Materials and Methods

Virus. ASFV Georgia 2007 isolate was derived from splenic homogenate as previously described in detail (Niederwerder et al., 2019). For virus titration, porcine alveolar macrophages (PAMs) were collected from 3 – 4-week-old pigs. PAMs were cultured for 24 hours in RPMI (Roswell Park Memorial Institute; HyCloneTM 1640, Fisher Scientific) media supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in 5% CO2 prior to use in titration assays. Ten-fold serial dilutions of the splenic homogenate were prepared in triplicate and dilutions were added to PAMs in a 96-well plate. After 3 days of incubation at 37°C, PAMs were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X. Monoclonal antibody directed at ASFV p30 (kindly provided by Dr. Ying Fang) was added at 1:6,000 followed by 1-hour incubation at 37°C. After washing the plate 2 times with phosphate buffered saline (PBS), goat-antimouse antibody (Alexa Fluor 488, Invitrogen, Thermofisher) was added at 1:400 and the plate was incubated for 1 hour at 37°C. Infection of cells was assessed by observing fluorescence under the inverted microscope and the 50% tissue culture infectious dose per ml (TCID50/ml) was calculated using the method of Spearman and Karber (Finney, 1964).

Inoculation of feed and dust swab collection. All work with ASFV was performed at the Biosecurity Research Institute under Biosafety Level 3 (BSL-3) containment conditions. Feed dust samples were collected opportunistically during an in vivo study investigating the efficacy of feed additives for reducing ASFV infectivity to pigs during natural consumption. Swabs were collected during four independent studies (A, B, C, D; Table 1.1), which included 24 treatments (6 treatments/study). Treatments included complete swine feed in meal form inoculated with sterile RPMI media (negative control), ASFV Georgia 2007 (positive control), and ASFV Georgia 2007 with antimicrobial feed additives (mitigation samples). Treatments were prepared by adding 10 mL of sterile RPMI media or ASFV Georgia 2007 (4.1 – 6.4 log10 TCID50/ml) to each 100 g volume of complete swine feed. Liquid inoculum was allowed to absorb onto feed for 1 min at room temperature prior to homogenization by swirling for 5 sec and mixing for 15-20 sec in wide-mouth high-density polyethylene round bottles (0.5 – 1.5 L bottles; NalgeneTM, ThermoScientific). Mitigation samples included either 1% inclusion of a medium chain fatty acid blend (MCFA; Purina Animal Nutrition, LLC) based additive or 0.33% inclusion of an aqueous formaldehyde and propionic acid (Sal CURB®, Kemin Industries, Inc.) based additive. Liquid mitigants were allowed to absorb onto ASFV-inoculated feed at room temperature for 1 min prior to homogenizing as described above.

Feed treatments were provided for natural consumption in 23-cm stainless-steel creep feeders (Vittetoe Inc.). Once pigs had consumed the feed treatments, dust swabs were collected from each creep feeder using a sterile polyester-tipped swab pre-moistened with buffered peptone water (PuritanTM Environmental Sampling Kit, FisherScientific). Approximately 25% of the feed contact surface area (~103.9 cm2) on the creep feeder was swabbed in a horizontal side-to-side motion while rotating the swab (Figure 1.1). The time between ASFV inoculation of

feed and collection of dust swabs was dependent on consumption rate and recorded as the length of environmental exposure to room temperature. After collection, swabs were aseptically stored in 4 ml of buffered peptone water at 4°C for 14 – 16 hours. Swabs were processed by vortexing for 10 sec followed by centrifugation at 4000×g for 10 min at 4°C. Swab supernatants were stored at −80°C until diagnostic testing was performed.

qPCR. Nucleic acid was extracted from each swab supernatant using the MagMAXTM 96 Viral RNA Isolation Kit (ThermoFisher). Briefly, paramagnetic beads were mixed with a bead enhancer solution and 20µL of the bead mix was added to 1.5mL snap-cap microcentrifuge tubes (FisherScientific). Supernatant samples (50µL) were added and tubes were vortexed for 1 min prior to adding 130µL lysis/binding solution (containing lysis/binding concentrate, carrier RNA, and 100% isopropanol). Tubes were vortexed for 5 min before placing on a magnetic stand to capture the beads. Beads were washed twice with 150µL wash solution 1 and twice with 150µL wash solution 2. After air drying, DNA was eluted from the beads with 50µL elution buffer.

PCR was designed to amplify a conserved region of ASFV p72 (King et al., 2003) as previously described (Niederwerder et al., 2019). Briefly, the PCR master mix included 10µL SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories), 4µL nuclease-free water, and 1µL primer/probe mix (Integrated DNA Technologies). Extracted DNA samples (5µL) were added to 15µL of master mix in a Hard-Shell® 96-well PCR plate (Bio-Rad Laboratories). The plate was sealed, centrifuged briefly to remove air bubbles, and placed into a CFX96 Real-Time System (Bio-Rad Laboratories). The PCR conditions consisted of 95°C for 2 min, followed by 45 cycles of 94°C for 30 sec, 58°C for 1 min, and 72°C for 45 sec. Data was analyzed using CFX

Manager software version 3.1 (Bio-Rad Laboratories) and results are reported as the cycle threshold (Ct) values. Ct values ≥ 40 were considered negative.

Virus isolation and titration. Swab supernatant samples were initially screened for the presence of infectious ASFV by testing 2-fold serial dilutions in single columns of PAMs. Dilutions (1:2 – 1:256) were created in RPMI media, added to confluent monolayers of PAMs in 96-well plates, and incubated for 1 h at 37°C. PAMs were washed and RPMI media replaced prior to a 3-day incubation at 37°C. Following incubation, cells were fixed and IFA performed. Swab supernatants demonstrating evidence of ASFV infection in single columns were subsequently tested in triplicate to estimate virus titer. Two-fold serial dilutions were prepared in triplicate for titration on confluent monolayers of PAMs following the protocol described above. ASFV infection of cells was observed under an inverted fluorescence microscope and the log10 TCID50/ml titer was calculated according to method of Spearman and Karber (Finney, 1964).

Results

Across the four studies, ASFV inoculation dose ranged between 3.1 – 5.4 $\log_{10}$ $TCID_{50}$/g of feed and the time interval between inoculation of feed and dust swab collection ranged between 30 – 660 min (**Table 1.1**). Dust swabs collected from RPMI-inoculated feed (n = 4; negative controls) had no detectable ASFV on PCR or virus isolation (VI) across all studies. Detection of ASFV on PCR and VI in dust swabs collected from ASFV-inoculated feed varied across the four studies dependent on inoculation dose, environmental exposure, and feed additive inclusion. In study A, all dust swabs collected from ASFV-inoculated feed (n = 5) were negative for ASFV across both diagnostic assays; study A contained the lowest inoculation dose (3.1 $\log_{10}$ $TCID_{50}$/g) and the longest time (11 hr) of environmental exposure. In contrast, dust swabs collected from ASFV-inoculated feed in the remaining three studies (B, C, D; n = 15) all had

detectable ASFV nucleic acid (Ct values 26.33 – 34.64), including those dust swabs collected from feed treated with MCFA- or formaldehyde-based additives. Four dust swabs from studies C and D (positive controls; 2/study) had detectable ASFV on VI; specifically, infectious ASFV was detected in dust swabs collected 30 min after ASFV inoculation of feed (5.4 $\log_{10}$ $TCID_{50}$/g) in the absence of antimicrobial feed additives. ASFV titers of the positive dust swab supernatants ranged between 2.6 – 2.9 $\log_{10}$ $TCID_{50}$/ml.

Discussion

Herein, we evaluated the use of feed dust as a novel diagnostic sample type for detection of nucleic acid and infectious virus in ASFV-contaminated feed. This study provides valuable proof of concept data on feed dust as a potential surveillance tool for ASFV in contaminated feed and ingredients. To generate this proof of concept data, we took advantage of an *in vivo* swine trial to collect feed dust. This provided the opportunity to collect dust from creep feeders post-consumption, which likely contained porcine saliva and nasal secretions consistent with real-world application, and allowed us to circumvent the challenges of creating *in vitro* feed dust in a BSL-3 biosafety cabinet with laminar airflow. However, the *in vivo* trial limited the number of feed doses from which we were able to collect dust and created variability in environmental exposure times between ASFV inoculation and swab collection.

Across the two diagnostic assays investigated in the current study, qPCR was more sensitive for detection of ASFV in feed dust than VI on cell culture. ASFV DNA was detectable by qPCR in feed dust for at least 8 hours after feed inoculation and in the presence of antimicrobial feed additives. VI could detect infectious ASFV in feed dust when swabbed 30 minutes post-inoculation, albeit at concentrations approximately 2.7 $\log_{10}$ $TCID_{50}$ lower than the inoculation dose. Dust swabs collected from feed inoculated with the lowest ASFV dose (3.1

log_{10} $TCID_{50}$/g) were negative for ASFV on both PCR and VI. This is noteworthy as the minimum infectious dose of ASFV Georgia 2007 in feed through natural consumption has been defined as 4.0 log_{10} $TCID_{50}$ (Niederwerder et al., 2019), which equates to approximately 10 g of the aforementioned feed. Further, feed dust was positive for nucleic acid but negative for infectious virus when collected after 8 hours at a dose of 5.1 log_{10} $TCID_{50}$ ASFV per gram. Taken together, qPCR is a sensitive diagnostic assay for detecting ASFV contamination of feed; however, the presence of nucleic acid does not equate to ASFV infectivity and determining biological activity is essential to defining transmission risk.

Environmental exposure time between ASFV inoculation of feed and collection of feed dust likely played a role in ASFV detection sensitivity between studies. Decay of ASFV in inoculated feed exposed to environmental conditions of room temperature has been previously described. For example, unprocessed field crops inoculated with ASFV Armenia 2008 (6.0 log_{10} HAD_{50}/20g) and exposed to 2 hours of drying at room temperature were positive for ASFV DNA but negative for infectious virus (Fischer et al., 2020). Further, compound feed inoculated with ASFV Volgograd-Kalach 2012 (7.0 log_{10} HAU_{50}/10g) and stored at room temperature had infectious virus titers detected after 1 day but were VI negative on cell culture after 5 days (Sindryakova et al., 2016). However, complete feed inoculated with ASFV Georgia 2007 (5.0 log_{10} $TCID_{50}$/5g) and exposed to lower mean environmental temperatures (12.3°C) maintained infectious virus titers for at least 30 days (Stoian et al., 2019). Overall, variation in environmental exposure associated with consumption time likely impacted ASFV detection in feed dust and temperature-dependent decay of ASFV in feed requires further characterization.

Interestingly, this study also provided evidence consistent with previous work reporting antiviral efficacy of MCFA- and formaldehyde-based feed additives against ASFV in cell culture

and transboundary shipment models. Specifically, inclusion of 1% MCFA or 0.33% formaldehyde-based additives decreased ASFV infectivity while maintaining detectable ASFV DNA in nine feed ingredients exposed to 30 days of environmental conditions simulating transoceanic shipping (Niederwerder et al., 2020). Consistent with these findings, mitigated feed dust was negative for infectious virus within 30 minutes of treatment in studies C and D, whereas non-mitigated feed dust had quantifiable ASFV titers. Although mitigated feed dust generally had higher qPCR Ct values, neither MCFA nor formaldehyde-based additives eliminated ASFV nucleic acid. Characteristic of most antimicrobial feed additives, inactivation of swine viruses does not typically translate to destruction of viral nucleic acid (Niederwerder, 2021) and should be considered an important factor in feed diagnostics.

To our knowledge, this is the first study providing proof of concept that feed dust from ASFV-contaminated feed can be used to detect ASFV DNA on qPCR and infectious ASFV on PAMs. Data supports the use of feed dust as a diagnostic tool for ASFV detection in feed and identifies potential limitations. Further investigations are warranted to define the sensitivity of feed dust as a diagnostic sample for ASFV-contaminated feed at lower inoculation doses, longer incubation periods, and varied environmental conditions.

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Industrial Research Organization's Australian Animal Health Laboratory and Bob Rowland of the University of Illinois.

Table 1.1 Detection of African swine fever virus nucleic acid and infectious titer in feed dust swabs collected from creep feeders after natural consumption of experimentally inoculated complete swine feed†

	Feed Treatment	ASFV Inoculation Dose ($TCID_{50}$/g)	Inoculation to Swab Collection (min)	Detection of ASFV in Feed Dust	
				qPCR (Ct)	Titer ($TCID_{50}$/ml)
A	ASFV	3.1 log_{10}	660	–	–
	ASFV			–	–
	ASFV + Formaldehyde			–	–
	ASFV + Formaldehyde			–	–
	ASFV + Formaldehyde			–	–
	RPMI media			–	–
B	ASFV	5.1 log_{10}	480	30.29	–
	ASFV			30.96	–
	ASFV + MCFA			34.64	–
	ASFV + MCFA			34.44	–
	ASFV + MCFA			31.87	–
	RPMI media			–	–
C	ASFV	5.4 log_{10}	30	28.46	2.9 log_{10}
	ASFV			26.33	2.7 log_{10}
	ASFV + Formaldehyde			30.31	–
	ASFV + Formaldehyde			30.60	–
	ASFV + Formaldehyde			29.28	–
	RPMI media			–	–
D	ASFV	5.4 log_{10}	30	26.88	2.6 log_{10}
	ASFV			27.71	2.6 log_{10}
	ASFV + MCFA			27.59	–
	ASFV + MCFA			28.38	–
	ASFV + MCFA			27.30	–
	RPMI media			–	–

†Data from 4 independent studies shown (A, B, C, D). Treatments include feed inoculated with ASFV (positive control), feed inoculated with ASFV and 0.33% formaldehyde (A and C), feed inoculated with ASFV and 1.0% MCFA (B and D), and feed inoculated with sterile RPMI media (negative control). Inoculation dose shown as the ASFV Georgia 2007 virus titer ($TCID_{50}$) per gram of complete feed. $TCID_{50}$ estimates were calculated from triplicate dilutions on porcine alveolar macrophages and determined using the method of Spearman and Karber (Finney, 1964). Approximate time between ASFV inoculation of feed and collection of feed dust dependent on rate of natural feed consumption by pigs and is shown in minutes. Feed dust was collected using moist swabs applied to stainless-steel creep

feeders covering an area of 103.9 cm^2. qPCR Ct values of ≥ 40 were considered negative.
Key: ASFV, African swine fever virus; Formaldehyde, aqueous formaldehyde-based feed additive; MCFA, medium chain fatty acid-based feed additive; RPMI, Roswell Park Memorial Institute; qPCR Ct, quantitative polymerase chain reaction cycle threshold; $TCID_{50}$, 50% tissue culture infectious dose; –, negative

Figure 1.1 Area swabbed for collection of feed dust from stainless-steel creep feeder.

Shaded region (dark grey; 103.9 cm^2) showing approximate area covered by side-to-side path (red line) of swab collection. Feed dust was collected using sterile polyester-tipped moist swabs following natural consumption of feed treatment by nursery pigs.

Chapter 2 - Vaccination with a porcine reproductive and respiratory syndrome-modified live virus vaccine is associated with increased gut microbiome diversity in nursery pigs

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is the most costly disease of swine production in the United States. Although PRRS modified live virus (MLV) vaccines are widely utilized to reduce PRRS-associated loses, the currently available vaccines are considered inadequate for disease control. Recently, the gut microbiome has been associated with vaccine efficacy and health outcomes following PRRSV infection in pigs. The objective of the current study was to investigate the effects of PRRS MLV vaccination on the gut microbiome composition and diversity of nursery pigs. Weaned pigs (average 23.4 ± 2.1 days) were obtained from a single commercial source and vaccinated with a commercial PRRS MLV vaccine (Ingelvac PRRS MLV; Boehringer Ingelheim Animal Health). Twenty-eight days post-vaccination, fecal samples were collected from both vaccinated (n = 12) and nonvaccinated (n = 12) pigs. Gut microbiomes were compared between groups using the Lawrence Livermore Microbial Detection Array (LLMDA). Increased microbial species diversity and increased Firmicutes to Bacteroidetes ratio was found in the vaccinated pigs when compared to the nonvaccinated pigs. Significant differences were also noted in microbiome composition at the level of phylum, family and species. Specifically, Enterobacteriaceae family was detected at a significantly higher rate in vaccinated pigs compared to nonvaccinated pigs (p = 0.027, Fisher's exact test). PRRS MLV vaccination was associated with changes in the gut microbiome

composition and diversity in nursery pigs. This suggests that infection with PRRS MLV may modulate the gut microbiome and that certain microbiome characteristics may contribute to vaccine efficacy.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) has highest economic impacts to swine production worldwide with estimated annual losses to the US industry at $663.91 million (Holtkamp et al., 2013). The etiologic agent of PRRS is the PRRS virus (PRRSV), which is a single stranded RNA virus belonging to the family *Arteriviridae* (Benfield et al., 1992). The major economic impact of PRRS on the global swine industry is due to diminished weight gain and respiratory disease in growing pigs (Holtkamp et al., 2013). PRRSV is frequently isolated along with porcine circovirus type 2 (PCV-2) in US field cases (Pallarés et al., 2002). PCV-2 is a single stranded DNA virus belonging to the family *Circoviridae*. It is associated with a group of disease syndromes termed porcine circovirus associated disease (PCVAD), characterized by muscle wasting, respiratory disease, jaundice, or pallor, and reduced weight gain in growing pigs (Segales, 2012). PRRSV and PCV-2 both cause systemic infections primarily targeting pulmonary and lymphoid tissue and are used in co-infection models for reproducing PCVAD. Co-infection with the two viruses modulates host immunity resulting in increased viral replication and enhanced polymicrobial disease compared to single infections alone (Niederwerder et al., 2016; Megan C Niederwerder et al., 2015; B. R. Trible et al., 2012).

There are two types of PRRS vaccines available commercially; killed virus vaccines and modified live virus vaccines (MLV). PRRS MLV vaccines are predominately used in the endemic herds to reduce PRRS-associated losses by reducing wildtype viremia, virus shedding, and respiratory disease while improving growth performance (Cano et al., 2007; Kritas et al.,

2007). However, several challenges remain regarding PRRS MLV vaccine safety and efficacy including lack of complete protection to heterologous strains, persistent MLV infections in vaccinated hosts, shedding and spread of vaccine virus to naïve animals (Charerntantanakul, 2012), reversion to virulence (Botner et al., 1997), recombination between MLVs and wild-type strains (Wenhui et al., 2012), and potentiation of PCV-2 infection (Megan C Niederwerder et al., 2015). Hence, new strategies are needed to improve the efficacy of PRRSV vaccines.

The term gut microbiota refers to the population of microorganisms including bacteria, viruses, fungi, protozoa, and archaea that reside in the gastrointestinal tract. Gut microbiota and their genomes are collectively known as gut microbiome. The gut microbiome is shown to play an essential role in local and systemic immunity along with nutrient availability (Ichinohe et al., 2011; Jamieson, 2015). Understanding the role of gut microbiome for improving the response of swine to infectious viral diseases including PRRS is an emerging area of study and the focus of our work. Previous work investigating associations between the gut microbiome and clinical outcome in PRRSV-PCV-2 coinfected pigs has demonstrated several microbiome characteristics such as increased gut microbiome diversity, increased *Ruminococcaceae* species, increased *Streptococcaceae* species, reduced *Methanobacteriaceae* species and fecal microbiota transplantation to be associated with improved disease outcome including reduced virus replication, improved weight gain and decreased morbidity (Niederwerder et al., 2016; Ober et al., 2017; Niederwerder et al., 2018a). The mechanisms through which the gut microbiome impacts outcome of porcine respiratory infection remain largely unknown. However, it is believed to be through systemic dissemination of gut microbial-derived components and metabolic degradation products such as SCFAs and their immunomodulatory activity (Dang & Marsland, 2019).

Despite growing evidence in support of the influence of gut microbiome on immune responses to vaccination against infectious diseases, only a few studies have focused on the effect of vaccination on gut microbiome in turn. A study by Eloe-Fadrosh et al. (2013) demonstrated no disruption in the microbiome composition in adults after vaccination with either a single dose or four-dose of oral live-attenuated typhoid vaccine Ty21a when compared to unvaccinated controls. In contrast, influence of vaccination on gut microbiome was reported for vaccines against Rotavirus (Harris et al., 2016), Poliovirus (Huda et al., 2014) in infants and PRRSV (Yuan et al., 2022; Zhang et al., 2021) in pigs. Increased abundance of *Streptococcus bovis* and a decreased abundance of species in the Bacteroidetes phylum of fecal microbiota was correlated with rotavirus vaccine response in infants (Harris et al., 2016). A high relative abundance of actinobacteria was correlated to low T cell responses and virus-specific IgG levels in serum after the oral immunization of infants with a poliovirus vaccine (Huda et al., 2014). Higher abundance of *Lactobacillus* was reported to be associated with higher antibody titer in serum after intramuscular immunization of pigs with a PRRSV vaccine and PRRSV challenge (Zhang et al., 2021). Similarly, a previous study investigating effect of killed PRRSV vaccine on gut microbiome in pigs demonstrated enhanced taxonomic richness and greater community diversity of gut microbiome in vaccinated/challenged pigs compared to unvaccinated/ unchallenged controls indicating incomplete protection conferred by vaccine against viral impacts on the microbiome (Yuan et al., 2022). Further studies are required to determine whether there is measurable difference in the microbiome composition in response to administration of PRRS MLV vaccine and identify microbial species associated with vaccination.

In this study, we presented identification of fecal microbiome from nursey pigs which were either parenterally vaccinated with the PRRS MLV vaccine or served as

unvaccinated controls at 28 days post vaccination by utilizing The Lawrence Livermore Microbial Detection Array (LLMDA). Subsequent efficacy of vaccination was demonstrated after PRRSV PCV-2 co infection. We found that infection with PRRS MLV vaccine resulted in greater microbiome diversity, higher Firmicutes: Bacteroides and increased prevalence of several microbial families including but not limited to *Enterobacteriaceae*. This result suggests that the gut microbiome characteristics associated with vaccination may serve as a tool for improving PRRS MLV vaccine efficacy in pigs.

Materials and Methods

Animals and housing. All experiments involving use of 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. This study was conducted as part of the PRRS Host Genetics Consortium (PHGC) as previously described (Lunney et al., 2011). A subset of pigs (n = 100; mean age 23.4 ± 2.1 days) were obtained at weaning from a PRRSV negative high health commercial herd. The piglets were not vaccinated for PCV-2 and were utilized without regards to maternal antibody as the sow herd they were sourced from was previously vaccinated with a PCV-2 capsid subunit vaccine. All piglets were housed in a single environmentally controlled room at the Kansas State University Large Animal Research Center and maintained under BSL-2 conditions. The piglets were randomly distributed and housed in groups of 7-10 in a 13.4 square meter pen. Pigs were given access to food and water ad libitum.

Viruses. The challenge inoculum prepared for this study included the PRRSV and PCV-2b viral isolates originated from the lymph node of a pig with severe postweaning multisystemic wasting syndrome (PMWS) as previously described (B. R. Trible et al., 2012; Trible et al., 2011).

PRRSV (isolate KS62, GenBank Accession #KM035803) was isolated by propagation on MARC-145 cells and titrated as described previously by Megan C Niederwerder et al. (2015) and Ober et al. (2017). Briefly, PRRSV isolate was serially diluted 1:10 in minimal essential medium (MEM; Corning) supplemented with 7% FBS (Sigma-Aldrich), penicillin-streptomycin (Pen Strep; 80 Units/mL and 80 µg/mL, respectively; Gibco), 3 µg/mL amphotericin B (Fungizone; Gibco), and 25 mM HEPES (Life Technologies). The dilutions were then added in quadruplicate to confluent MARC-145 cells in a 96-well tissue culture plate (BD Falcon). Following a 4-day incubation at 37° C in 5% CO2, cells were examined for PRRSV-induced cytopathic effects. The 50% tissue culture infectious dose (TCID50/mL) was calculated using the method of Reed and Muench (Reed & Muench, 1938).

PCV-2b (GenBank accession no. JQ692110) was isolated by utilizing the heat stability of the virus to prepare a lymph node suspension enriched for PCV-2 as previously described (Niederwerder et al., 2016; Megan C Niederwerder et al., 2015; Ober et al., 2017). Analysis of the heat-treated PCV-2 tissue homogenate used to prepare challenge inoculum yielded negative results for most heat-stable agents, but was positive for two ubiquitous swine viruses, including Torque teno sus virus (TTSuV) and porcine endogenous retrovirus (PERVs) (Jaing et al., 2015).

PCV-2b infectivity was titrated on swine testicle (ST) cells. Briefly, serial 10-fold dilutions of PCV-2b 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 of gentamicin (Lonza). Following a 3-day incubation at 37° C in 5% CO_2, cells were fixed and permeabilized with 80% acetone. Cells were then stained with fluorescein isothiocyanate (FITC)-labeled porcine anti-PCV (Veterinary Medical Research and Development, Inc.). Infected cells were visualized using an inverted fluorescent microscope and the $TCID_{50}$/mL was calculated using the method of Reed and Muench (Reed & Muench, 1938).

The inocula for pigs were prepared by combining the challenge viruses to yield a 2-mL dose consisting of $10^{3.6}$ $TCID_{50}$ PCV-2b and 10^5 $TCID_{50}$ PRRSV in MEM. The 2-ml dose was split; 1 ml was administered intranasally and 1 ml was administered intramuscularly.

Experimental design and sample collection. After 4 days of acclimation, 50 of the total 100 pigs were randomly 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 100 pigs were infected with PRRSV and PCV-2b. Individual body weights were determined on 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 dpv. 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 100 pigs during the week prior to co-infection for microbiome analysis.

At 42 days post-infection (dpi) or 70 dpv, 24 pigs were selected to represent vaccinated (n = 12) and non-vaccinated (n = 12) pigs. The two groups were balanced according to initial weight on 0 dpi. Any pig displaying clinical signs which required veterinary medical treatment was excluded from the study. At 42 dpi, all 24 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. All pigs were observed daily for the presence of clinical signs associated with PRRSV/PCV-2 co-infection 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. Pigs were visually examined by an attending veterinarian or veterinary assistant on each day of the study period. Appropriate treatment was administered to any pig showing moderate to severe clinical disease under the direction of a veterinarian. Treatment was administered for clinical presentations, such as 1) dyspnea, 2) mucopurulent nasal discharge, 3) lameness with joint effusion, 4) pallor or jaundice with muscle wasting, and 5) lethargy or depression with pyrexia. Clinically affected pigs were administered parenteral antibiotics, including ceftiofur hydrochloride, oxytetracycline, or enrofloxacin. Any pig with overt clinical disease and a rectal temperature of ≥104°F was administered a nonsteroidal anti-inflammatory drug, such as flunixin meglumine or meloxicam. Pigs with documented clinical disease requiring veterinary medical treatment was excluded from selection for fecal microbial analysis.

At 42 dpi, all pigs were humanely euthanized by intravenous injection of pentobarbital sodium. A board-certified veterinary pathologist, blinded to the selection of pig groups, performed complete necropsies and histopathologic evaluations. One section from each lung lobe was collected 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 on a scale of 0-4 as previously described (Niederwerder et al., 2016; Megan C Niederwerder et al., 2015; Ober et al., 2017). Briefly,

scores were given 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.

Measurement of PRRSV and PCV-2 viremia. Nucleic acid was extracted from 50 μL of serum using the Ambion's MagMAX 96 Viral Isolation Kit (Applied Biosystems) in accordance with the manufacturer's instructions as previously described (Niederwerder et al., 2016; Megan C Niederwerder et al., 2015; Ober et al., 2017). 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. 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-2 DNA was quantified using SsoAdvanced Universal SYBR green supermix (Bio-Rad) as previously described (Niederwerder et al., 2016; Megan C Niederwerder et al., 2015). Briefly, 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 PCV-2 PCR was carried out on a CFX96 Touch Real-Time PCR Detection System using the following settings: activation at 98 °C for 2 min, followed by 40 cycles of denaturing at 98 °C for 5 s and annealing/extension at 60 °C for 10 s. The PCR assay results were reported as $\log_{10}$ PCV-2 DNA starting quantity (copy number) per 20 μL reaction volume.

Microarray analysis of fecal samples. The Lawrence Livermore Microbial Detection Array (LLMDA) developed at the Lawrence Livermore National Laboratory was used to analyze microbiome composition and diversity of fecal samples collected prior to co-infection. This array detects annotated sequences of microbes within GenBank®, the National Institute of Health genetic sequence database. The version 7 of the LLMDA in the 4plex180 K probe format was used in this study, which targets a total of 10,612 microorganisms including 5457 bacteria, 4377 viruses, 327 archaebacteria, 319 fungi, and 132 protozoa. The LLMDA oligonucleotide probes are around 60 nucleotides in length and have roughly equivalent affinities for their complementary target DNA molecules (McLoughlin, 2011). 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 (Niederwerder et al., 2016; Ober et al., 2017; Niederwerder et al., 2018a; Jaing et al., 2015; Constance et al., 2021; Gardner et al., 2010; Rosenstierne et al., 2014).

The PowerViral™ Environmental RNA/DNA Isolation Kit (MO BIO, San Diego, CA) was used to extract nucleic acid from the fecal samples. Briefly, 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 min 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). For each sample, 10 µL of the extracted DNA and RNA was amplified using the

random amplification procedure as previously described (Rosenstierne et al., 2014). The amplified cDNA and DNA was purified with the Qiaquick PCR purification columns (Qiagen) and quantified using 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 h. 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 h 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). All arrays were scanned to a resolution of 3 µm using the SureScan Microarray Scanner (Agilent Technologies Inc., Santa Clara, CA). Microarray data was generated from the microbe sequences using the CLiMax method developed at Lawrence Livermore National Laboratory (Gardner et al., 2010), 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 vaccinated and non-vaccinated 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 Firmicutes bacterial species by the total number of Bacteroidetes bacterial species detected for each pig.

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

Results

Vaccinated pigs had overall lower weight gain. Mean weights at the beginning of the study (0 dpv) were 5.7 ± 0.6 kg and 5.7 ± 0.8 kg for the vaccinated and non-vaccinated groups, respectively (p = 0.82, unpaired t-test; **Figure 2.1**). A significant divergence in the mean absolute weekly weights of the two groups occurred at 21, 28, and 42 dpv. At 21 dpv, mean weights of 14.1 ± 1.3 and 15.5 ± 1.8 kg, were measured in vaccinated and non-vaccinated groups,

respectively ($p = 0.03$, unpaired t-test). Mean weights on 28 dpv or on the day of co-infection were 18.0 ± 1.6 and 20.0 ± 1.8 kg for the vaccinated and non-vaccinated groups, respectively ($p = 0.01$, unpaired t-test). It was only on 14 dpi (42 dpv) that mean weights between the vaccinated and non-vaccinated groups were significantly different after co infection. Mean weight for the vaccinated and non-vaccinated pigs were 28.5 ± 2.6 and 31.0 ± 3.0 kg, respectively ($p = 0.04$, unpaired t-test). Overall, mean weekly weights continued to be similar during the post coinfection period.

Vaccinated pigs had reduced PRRSV replication. The result showed a biphasic distribution of PRRS viremia with a vaccine virus replication peak followed by a challenge virus replication peak (**Figure 2.2A**). Detectable level of the vaccine virus was seen in the serum samples collected throughout the 28 days vaccine period with peak viremia occurring at 11 dpv and subsequent decline of virus replication. No PRRS virus was detected in the serum of the non-vaccinated pigs during the vaccine period. After infection with wildtype PRRSV, vaccinated pigs had a more rapid incline in virus replication followed by a more rapid decline compared to non-vaccinated pigs. However, non-vaccinated pigs had increased mean PRRS viremia compared to vaccinated pigs for majority of the serum sampling days after infection with challenge virus. Vaccinated pigs peaked PRRS challenge virus replication at 35 dpv whereas non-vaccinated pigs peaked 4 days later at 39 dpv. Mean PRRS viremia was 3.13 $\log_{10}$ copies/PCR in vaccinated pigs on 35 dpv and 5.29 $\log_{10}$ copies/PCR in non-vaccinated pigs on 39 dpv. At 39 dpv, there was a trend towards a significant reduction in the PRRS viremia of vaccinated pigs, with a mean of 2.7 $\log_{10}$ copies/PCR reaction being detected compared to 5.3 log10 copies/PCR reaction in the non-vaccinated group ($p<0.001$, unpaired *t*-test). This trend continued at 42 dpv, where vaccinated pigs (mean 2.4 $\log_{10}$ copies/PCR reaction) had reduced

PRRSV detected in serum compared to the non-vaccinated group (3.8 $\log_{10}$ copies/PCR reaction; p = 0.027, unpaired *t*-test). Serum virus declined to undetectable levels in vaccinated pigs after 42 days of infection whereas PRRSV reappeared in the serum of non-vaccinated pigs at 70 dpv after it dropped down to almost undetectable level at 63 dpv. On 70 dpv, mean PRRSV viremia was 0.5 and 0.0 $\log_{10}$ copies/PCR reaction for the non-vaccinated and vaccinated groups, respectively (p = 0.037; unpaired *t*-test). Overall, vaccinated pigs had reduced PRRS viremia compared to non-vaccinated pigs during the co-infection period.

For PCV-2, vaccinated pigs had a more rapid incline in virus replication followed by a more gradual decline demonstrated a plateau of continued virus detection in the serum until 70 dpv (**Figure 2.2B**). Vaccinated pigs peaked PCV-2 challenge virus replication at 49 dpv whereas non-vaccinated pigs peaked a week later at 56 dpv. On 35 dpv, there was a trend towards vaccinated pigs having significantly higher PCV-2 viremia compared to non-vaccinated pigs (p = 0.016; unpaired *t*-test); PCV-2 detection was approximately 0.5 $\log_{10}$ copies/PCR reaction greater in vaccinated pigs. On 56 dpv, abrupt decline was seen in PCV-2 viremia in vaccinated pigs however, it was not a significant drop. Additionally, it did not follow the declination trend hence forth, instead maintained consistent replication. Overall, vaccinated pigs peaked PCV-2 replication sooner and initiated a decay earlier compared to non-vaccinated pigs. However, mean viral loads were similar between the two groups at 70 dpv; 1.22 $\log_{10}$ copies/PCR reaction for vaccinated pigs and 1.17 $\log_{10}$ copies/PCR reaction for non-vaccinated pigs (p = 0.932; unpaired *t*-test).

Lesions associated with PCVAD in Vaccinated and non-vaccinated pigs.

Tracheobronchial lymph node was examined for lymphoid depletion associated with porcine circovirus associated disease (PCVAD) at 70 dpv by a board-certified pathologist blinded to the

selection of pig groups. Representative pictures of microscopic lymphoid lesions are shown in **Figure 2.3A**. Every non-vaccinated pig had some degree of lymphoid depletion. The mean depletion scores were ## and 2.1 for vaccinated and non-vaccinated group of pigs, respectively (p = ##, Mann-Whitney U test). Lung tissues from each lung lobe were examined for interstitial pneumonia associated with PCVAD and compared between vaccinated and non-vaccinated pigs. Representative gross and microscopic images of lung lesions in a non-vaccinated pig are shown in **Figures 2.3B-C**. Almost all non-vaccinated pigs had some degree of interstitial pneumonia, with only a single pig having normal microscopic lung appearance. One third of non-vaccinated pigs had moderate to severe multifocal interstitial pneumonia with 50-75% lung lobe involvement. Compare mean lung lesion scores between two groups ###

Vaccinated pigs had increased fecal microbiome diversity and shifts in microbial composition. Microbial Detection Array was used to determine the presence of microbial families and species in fecal samples obtained from all pigs. Overall, a total of 16 phyla, 61 microbial families and 225 microbial species were detected by the microarray. Majority of the identified microbial species fell within the phyla Firmicutes (95/225), Proteobacteria (47/225) or Bacteroidetes (26/225). From the sixty-one unique classifications of microbial families identified, most of the families were bacterial (n = 54), but other represented groups included archaea (n = 2), eukaryotes (n = 1) and viruses (n = 4). Both DNA and RNA viruses belonging to families *Astroviridae, Circoviridae, Parvoviridae* and *Picornaviridae* were detected. Few unclassified bacteria were also detected.

Microbiome diversity was determined using the LLMDA data as the number of families and species detected in the feces of each pig across both groups (**Figures 2.4A and B**). The mean number of families detected were similar in both groups; 29.0 ± 5.0 and 30.3 ± 3.8 in

vaccinated and non-vaccinated groups of pigs, respectively ($p = 0.540$; Mann-Whitney U test). Interestingly, significant difference was detected in the mean number of species between the two groups ($p = 0.005$, Mann-Whitney U test). The mean number of species detected in vaccinated and non-vaccinated pigs were 60.5 ± 9.6 and 74.3 ± 10.4, respectively. This data suggests that PRRS MLV vaccination may contribute to increased replication of diverse microbial species in the gut.

The Firmicutes/Bacteroidetes ratio was determined by dividing the number of Firmicutes species by the number of Bacteroidetes species. Vaccinated pigs had a significantly higher Firmicutes to Bacteroidetes species ratio ($p = 0.002$, Mann-Whitney U test). Specifically, vaccinated pigs had increased numbers of Firmicutes species detected; 40 ± 5.9 species compared to 25.8 ± 6.4 species in non-vaccinated pigs ($p < 0.0001$, Mann-Whitney U test). Both vaccinated and non-vaccinated pigs had decreased but similar number of species detected in Bacteroidetes phylum; 7.5 ± 2.0 and 7.8 ± 2.0, respectively ($p = 0.612$, Mann-Whitney U test).

Majority of the microbial families detected by the LLMDA had similar prevalence rates in both vaccinated and non-vaccinated group of pigs. Ten microbial families were detected at significantly different prevalence rates between the two groups when individual families were compared (**Figure 2.4C**). Families including *Acidaminococcaceae, Carnobacteriaceae, Enterobacteriaceae,* and *Erysipelotrichaceae* were detected in significantly higher proportion ($p = 0.005$, $p = 0.003$, $p = 0.027$, $p = 0.003$, respectively, Fisher's exact test) of vaccinated pigs. Families including *Desulfovibrionaceae, Rikenellaceae, Xanthomondaceae* were detected in significantly lower proportion of vaccinated pigs ($p = 0.04$, $p = 0.01$, $p = 0.03$, and $p = 0.04$, respectively, Fisher's exact test). Interestingly, family *Circoviridae* was exclusively detected in vaccinated pigs ($p = 0.037$; Fisher's exact test) whereas families *Ceratobasidiaceae* and

Comamodaceae were only detected in non-vaccinated pigs ($p = 0.001$, $p = 0.037$, respectively; Fisher's exact test).

Species diversity within each family was analyzed for differences associated with effects of vaccination, fifteen significant differences were detected (**Figure 2.5B**). Vaccinated pigs had increased species diversity within the group of unclassified bacteria ($p = 0.002$; Mann-Whitney U test). Similarly, there was a trend towards increased species diversity in vaccinated pigs with families including *Acidaminococcaceae, Bradyrhizobiaceae, Carnobacteriaceae, Enterobacteriaceae, Erysipelotrichaceae, Eubacteriaceae, Lachnospiraceae, lactobacillaeae, Streptococcaceae,* and *Veillonellaceae* ($p = 0.008$, $p < 0.001$, $p = 0.002$, $p = 0.014$, $p = 0.002$, $p = 0.005$, $p < 0.001$, $p = 0.049$, $p = 0.002$, and $p = 0.003$, respectively; Mann-Whitney U test). Within the *Rikenellaceae* family, there was a trend towards less species diversity in vaccinated pigs; 0.3 ± 0.5 species in vaccinated pigs and 0.8 ± 0.4 species in low growth pigs ($p = 0.01$; Mann-Whitney U test). Furthermore, a trend towards increased species diversity was observed in family *Circoviridae* which was only detected in vaccinated pigs ($p = 0.037$; Mann-Whitney U test). Similarly, families *Ceratobasidiaceae* and *Comamodaceae* which were only detected in non-vaccinated pigs had increased species diversity within these families ($p = 0.001$ and $p = 0.037$, respectively; Mann-Whitney U test). Overall, the greatest number of species were detected in the *Lactobacillaceae* family in both groups, with a mean of 7.8 ± 2.3 and 5.7 ± 1.8 species detected in vaccinated and non-vaccinated group of pigs, respectively. Moreover, the difference was statistically different ($p = 0.049$; Mann-Whitney U test).

Twenty-four microbial species detected on the LLMDA, were identified at significantly different prevalence rates between the two groups when individual species were compared (**Figure 2.5A**). Most of these microbial species ($n = 20$) were detected at significantly

greater rates in vaccinated pigs. *Lactobacillus vagilis* was detected in five of vaccinated pigs (5/12) while none of non-vaccinated pigs had this species present ($p = 0.005$; Fisher's exact test). Furthermore, five bacterial species in the family *Lachnospiraceae* had higher prevalence in vaccinated pigs which included *Ruminococcus torques, Blautia wexlerae, Dorea longicate, Lachnospiraceae* bacterium C6A11, and *Oribacterium* sp. NK2B42 ($p = 0.037$, $p = 0.003$, $p = 0.009$, $p = 0.036$, and $p = 0.037$, respectively; Fisher's exact test). Similarly, two bacterial species in the family *Streptococcaceae* were identified at greater rates in vaccinated pigs. *Streptococcus gallolyticus* and *Streptococcus salivarius* were found in 12 and 8 of the vaccinated pigs, respectively, compared to 6 and 1 of the non-vaccinated pigs, respectively ($p = 0.013$ and $p = 0.009$, respectively; Fisher's exact test). A trend towards having higher prevalence in vaccinated pigs was also identified in two bacterial species *Megasphaera elsdenii* ($p = 0.009$) and *Megasphaera* sp. BV3C16-1 ($p = 0.009$ and $p = 0.036$, respectively; Fisher's exact test) in the family *Veillonellaceae*. Other bacterial species detected at a greater rate in vaccinated pigs includes *Phascolarctobacterium succitutens, Bradyrhizobium* sp. JGI 0001019-M21, *Alkalibacterium* sp. AK22, Clostridium sp. ATCC 29733, *Clostridiales* bacterium VE202-27, *Eubacterium biforme, Eubacterium ramulus*, *Mycoplasma hyopneumoniae*, and *Ruminococcus* sp. 5 1 39B FAA ($p = 0.005$, $p = 0.0001$, $p = 0.003$, $p = 0.037$, $p = 0.037$, $p = 0.027$, $p = 0.012$, $p = 0.027$, and $p = 0.003$, respectively; Fisher's exact test). Interestingly, Fur seal faeces associated circular D virus in the family *Circoviridae* had a greater detection rate in vaccinated pigs. In contrast, microbial species including *Rhizoctonia solani, Comamodaceae* bacterium JGI 0001003-E14, *Desulfovibrio alkalitolerans*, and *Methanobacterium paludism* trended towards having lower prevalence in vaccinated pigs ($p = 0.001$, $p = 0.037$, $p = 0.037$, and $p = 0.027$, respectively; Fisher's exact test).

Discussion

In recent years, it has been shown that the gut microbiome can influence immune response to vaccinations for infectious diseases (Lynn et al., 2022). However, the mechanisms by which the microbiome affects response to vaccination and how vaccination may in turn influence the microbiome remain largely undetermined. PRRS MLV vaccines are widely used to decrease PRRS-associated losses in endemic herds, but the currently available commercial vaccines are inadequate for disease control (Montaner-Tarbes et al., 2019). Hence, additional strategies are imperative for reducing the economic cost of PRRSV on pig production (Constance et al., 2021).

The concept of gut-lung axis, a cross-talk between gut microbiota and the lungs (Keely et al., 2012) allows us to conceive that utilization of beneficial gut microbes may provide an opportunity to improve immune response to PRRS MLV vaccines.

Before vaccination, there was no significant difference in the mean weight of pigs in vaccinated and non-vaccinated groups suggesting that the pigs in the different groups started with the similar weights. Overall, vaccinated pigs maintained lighter bodyweight compared to non-vaccinated pigs with a significant reduction in ADG during the third and fourth weeks after vaccination (M. C. Niederwerder et al., 2015). The negative impact of vaccines during the 28-days vaccine period is consistent with our previous finding . Similarly, a study by Pretzer et al. (1996) and (Opriessnig et al., 2005) reported negative effect of PRRS-MLV vaccine on growth performance of vaccinated pigs compared to non-vaccinated controls. Further, Linhares et al. (2012) showed no significant difference in ADG between vaccinated and non-vaccinated groups when they investigated a novel concept of using PRRS-MLV vaccine as therapeutic vaccine against PRRSV infection. However, continued negative effect on ADG for second-week post-

challenge remarkably differed from our previous finding which showed a positive effect on ADG during the first two weeks post-challenge (Megan C. Niederwerder et al., 2015).

Viremia levels for PRRSV and PCV-2 indicated that all non-vaccinated controls were free of PRRSV during 28 days vaccine period. This observation confirms that there was no external and cross-contamination among the vaccine and non-vaccine control groups which henceforth validates subsequent findings from this study. Additionally, detectable level of vaccine virus observed during the vaccine period indicates that vaccinated pigs were successfully infected with PRRS MLV vaccine. In consistence to previous study, non-vaccinated pigs attained peak PRRSV replication four days after vaccinated pigs attained its peak at 35 dpv (Megan C. Niederwerder et al., 2015). In the present study, total PRRS viral load in vaccinated pigs was significantly reduced when compared to unvaccinated pigs after challenge with wild-type PRRSV ($p = 0.014$, unpaired *t-test*) which suggests that vaccinated pigs were able to respond to the challenge better than unvaccinated pigs. This effect of the PRRS MLV on PRRSV infection is similar to that seen in a previous study, which showed a significant reduction in the total PRRS viremia level in a heterologous challenge model (Megan C. Niederwerder et al., 2015). In contrast, total viremia levels for PCV-2 did not differ significantly between the two groups during 42 days post-challenge period ($p = 0.860$, unpaired t-test) in consistence to the finding from our previous study as mentioned earlier (Megan C. Niederwerder et al., 2015).

Microbial analysis utilizing LLMDA identified a total of 16 phyla, 61 microbial families and 225 microbial species in fecal samples from all twenty-four pigs. Notable differences in the microbiome profile between the two groups were seen. Similar to our previous findings, phylum Firmicutes was the predominant phyla detected in the microarray analysis including 95 out of total 225 microbial species (Niederwerder et al., 2016; Ober et al., 2017). The majority of the

microbial species detected in the increased number of vaccinated pigs are Firmicutes except two of them which are Proteobacteria and Tenericutes. Vaccination was associated with a greater prevalence of phylum Firmicutes and a lower prevalence of phylum Tenericutes along with a significant and complete absence of phyla Fusobacteria and Basidiomycota. Previous studies on the influence of the gut microbiome on vaccine responses have shown a positive association between the relative abundance of phylum Firmicutes and humoral & cellular immune response to oral rotavirus vaccines in infants (Harris et al., 2016). Although we are unable to calculate the relative abundance of bacteria in different phyla using LLMDA, we can utilize the proportional representation of these bacteria in the overall gut microbial community to predict their functional capacities considering this limitation. These characteristics may predispose improved immune response in the presence of wild-type PRRSV after vaccination. However, other vaccine studies have demonstrated contradicting results showing positive association of relative abundance of phylum Actinobacteria (particularly Bifidobacterium) (Huda et al., 2014; Mullié et al., 2004) and negative association of relative abundance of phyla Proteobacteria (Huda et al., 2014) & Bacteroidetes (Harris et al., 2016) with humoral and cellular vaccine responses to oral and parenteral vaccines in infants. Since microarray cannot detect the relative abundance of microbial species, possibilities exist that there may have been a significant difference in the abundance of microbial species detected in these phyla despite smaller number of species detected in them.

Additionally, increased Firmicutes to Bacteroidetes ratio was observed in vaccinated pigs. Early gut microbiome studies comparing lean versus obese humans demonstrated that obese humans had increased Firmicutes and decreased Bacteroidetes abundance than their lean counterparts (Ley et al., 2006; Guo et al., 2008; Turnbaugh et al., 2009). Additionally, previous

work reported increased Firmicutes to Bacteroidetes ratio in high growth nonvaccinated pigs after PRRSV/PCV-2 co-infection (Ober et al., 2017). However, similar type of positive correlation between Firmicutes to Bacteroidetes ratio and weight gain was not evident in the current study. There were no significant differences in the ADG between vaccinated and non-vaccinated pigs. Consistent with our finding, there is also evidence of no significant differences in the relative abundance of phyla Firmicutes and Bacteroidetes between high and low body weight growing pigs (Oh et al., 2020). There are also some arguments been made that phylum-wide changes in the gut microbiota composition may not be absolutely considered a biomarker for obesity. Instead changes in microbiota composition at the lower taxonomic level or say an abundance of specific bacterial species within a phylum might be responsible for increased weight gain in an individual (Gérard, 2016). For instance, if increased abundance of firmicutes has been found associated to increased weight gain in humans (Ley et al., 2006), gut microbiome modulation by some species belonging to Lactobacillus (specifically, *L. acidophilus L. ingluviei* or *L. fermentum*) were found associated with weight gain whereas specific strains of *L. gasseri* and *L. plantarum* used as food supplements presented an anti-obesity effect in humans and animals (Million et al., 2012). Also, since microarray cannot detect the relative abundance of microbial species, possibilities exist that there may not have been a significant difference in the abundance of microbial species detected in the phylum Firmicutes despite a larger number of species detected in this phylum.

In the current study, increased gut microbiome diversity was noted at the species level in vaccinated pigs. Greatest species diversity was seen in family *Lactobacillace.* All the species detected under this family belong to genus *Lactobacillus*. In a previous study by Zhang et al. (2021), *Lactobacillus* was reported significantly abundant in high growth vaccinated pigs and

its abundance was associated with a better host immune response against PRRSV challenge in the PRRSV immunized pigs. This indicates that *Lactobacillus* may be used to improve PRRS MLV vaccine efficacy in pigs. Apart from *Lactobacillace*, bacterial families *including Acidaminococcaceae, Bradyrhizobiaceae, Carnobacteriaceae, Enterobacteriaceae, Erysipelotrichaceae, Eubacteriaceae, Lachnospiraceae, Streptococcaceae,* and *Veillonellaceae* showed greater species diversity whereas families including *Rikenellaceae, Ceratobasidiaceae,* and *Comamodaceae* showed lower species diversity in the vaccine group. While not much is known about the effect of the vaccine on these microbial families previously, a relatively high abundance of family *Lachnospiraceae* was shown associated with higher cellular and humoral immune response to oral typhoid (Eloe-Fadrosh et al., 2013) and rotavirus (Harris et al., 2016) vaccines. Members of this family hydrolyze complex polysaccharide substrates like xylanase, α-amylase, α- and β-glucosidase and α- and β-galactosidase and produce short-chain fatty acids including acetate, butyrate, and propionate (Stackebrandt, 2014; Sagheddu et al., 2016). Additionally, the abundance of *Lachnospiraceae* was correlated with high antibody titer in mice immunized with rabies vaccine (Zhang et al., 2020). Hence, *Lachnospiraceae* may have potential to regulate host immune response during PRRS MLV infection. Family *Enterobacteriaceae* belongs to phylum Proteobacteria which was correlated to best clinical outcome pigs post PRRSV-PCV-2 infection. This indicates that increased prevalence of Proteobacteria may regulate host immune response and result in improved clinal outcome in vaccinated/challenged pigs. In a previous study, an increased abundance of family *Bradyrhizobiaceae* (particularly genera *Bradyrhizobium*) was found in control (uninfected) chickens compared to low pathogenic influenza virus (subtype H9N2) infected chickens (Yitbarek et al., 2018). This suggests that increased prevalence of bacterial species of this genus are potentially associated with improved

respiratory viral disease outcome. In another study, family *Carnobacteriaceae* was shown inversely correlated to Toll like receptor 2 (TLR 2) expression in Chinese patients with functional gastrointestinal disorders which indicates that it regulates gut immune responses (Dong et al., 2017). TLR2 is one of the best characterized TLRs that detect microbial membrane components and play a key role in the activation of innate and adaptive immune responses (Kramer & Genco, 2017). Interestingly, family *Circoviridae* was only detected in vaccinated pigs. This result demonstrates negative impact of PRRS MLV vaccine as reported in a previous study that PRRS MLV vaccine potentiates PCV-2 replication in vaccinated PRRS/PCV-2 co-infected pigs compared to non-vaccinated co-infected controls (Megan C. Niederwerder et al., 2015)

Overall, greater species diversity was observed in vaccinated pigs compared to their unvaccinated counterparts which indicate that vaccination results significant shift in microbiome composition with an increase in diversity. Increased microbiome diversity has been often associated with improved protection from infectious diseases and vice versa in both humans, non-human primates and animals. For instance, live attenuated *Shigella dysenteriae* vaccination resulted in a higher intestinal microbiome diversity in macaques along with improved protection from *S. dysenteriae* infection (Seekatz et al., 2013). Another study comparing microbiome of healthy individuals with hepatitis C virus (HCV) infected individuals showed reduced microbial diversity in HCV infected individuals (Inoue et al., 2018). Similar results have been shown for infectious diseases of the respiratory tract. In our previous study in pigs, improved clinical outcome following co-infection with PRRSV and PCV-2 was associated with increased gut microbiome diversity (Niederwerder et al., 2016; Ober et al., 2017). Moreover, experimental increment of microbial diversity through fecal microbiota transplantation showed early antibody

production, a significant reduction in coughing frequency and lower lung lesion scores following *Mycoplasma hyopneumoniae* infection in pigs (Schachtschneider et al., 2013). Similarly, in a mouse model of a human respiratory pathogen (*Streptococcus pneumoniae*), antibiotic-treated mice (reduced microbiome diversity) showed accelerated mortality rate, increased *S. pneumoniae* load in lungs, increased interstitial pneumonia and diminished primary alveolar macrophage function following *S. pneumoniae* infection (Schuijt et al., 2016). Hence, accumulating evidences have shown associations between microbiome diversity and improved outcome following challenge with bacterial and viral respiratory pathogens in swine including *M. hyopneumoniae,* PRRSV, and PCV-2.

Altogether, infection with PRRS MLV modulates the gut microbiome composition and diversity in nursery pigs and certain microbiome characteristics may contribute to vaccine efficacy. Further, well-designed studies to characterize specific properties of individual families or species are needed to make insightful predictions about their role in the development and regulation of host immune responses. This will help identify bacterial species that can provide better protection to different infectious diseases and can also help improve vaccine efficacy and duration of protection.

Acknowledgements

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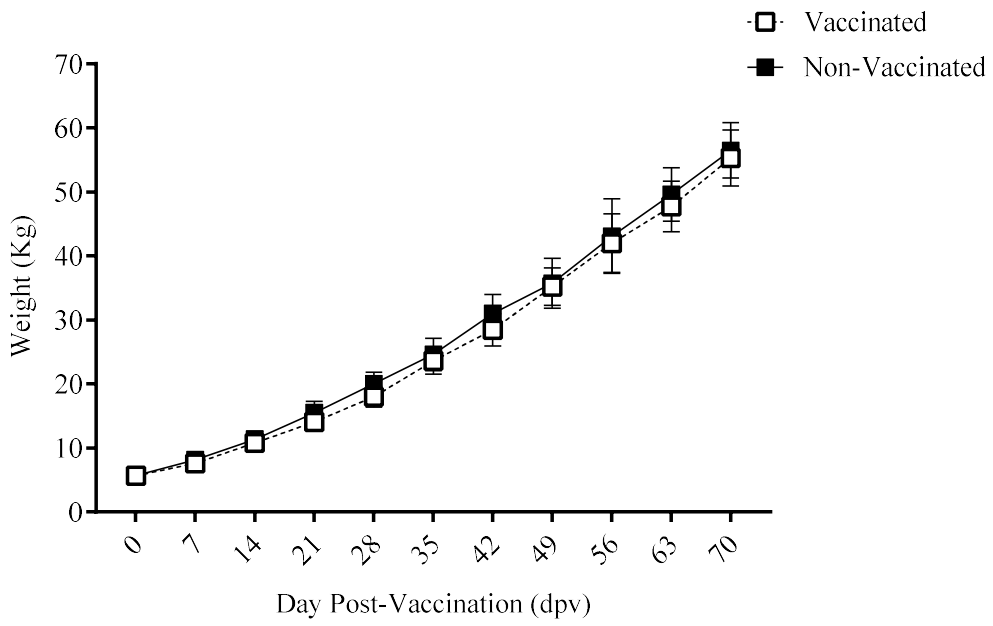
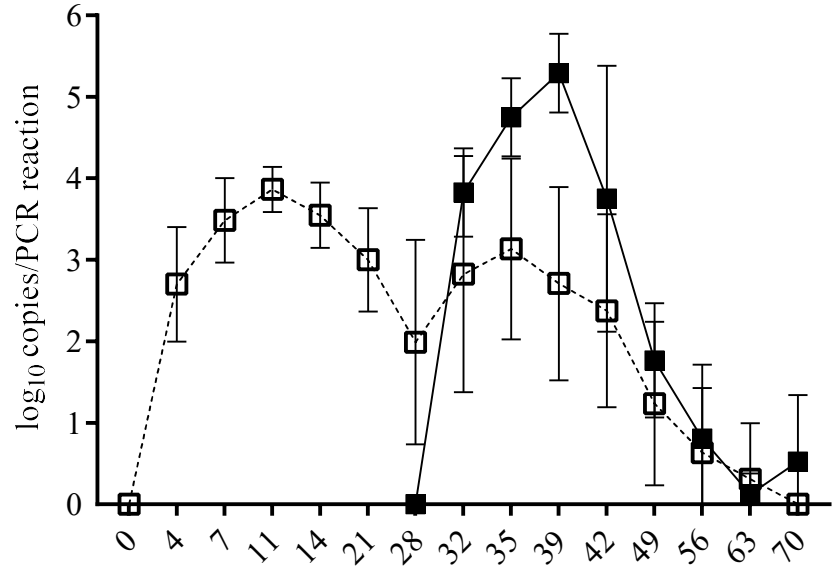


Figure 2.1 Weight gain in vaccinated and non-vaccinated pigs after PRRSV MLV vaccination and co-infection with PRRSV and PCV-2b

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 (*$p \leq 0.05$ and **$p \leq 0.01$; unpaired t-test) at 21, 28, and 42 dpv.

A. PRRSV viremia



B. PCV-2 viremia

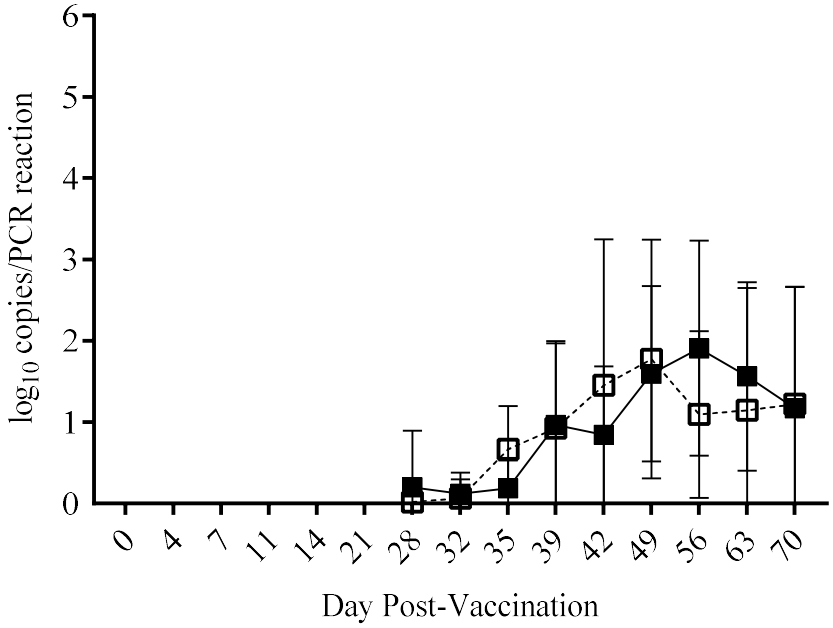


Figure 2.2 PRRSV and PCV-2 virus replication in vaccinated and non-vaccinated pigs after PRRSV MLV vaccination and PRRSV/PCV-2 co-infection

Data shown as the mean log10 copies/PCR reaction ± standard deviation for each virus and growth rate group (A and B). Pigs were coinfected with PRRSV and PCV-2b at 28 dpv. Asterisks identify statistically significant differences in mean serum virus load between the two groups ($p \leq 0.05$). (A) PRRSV viremia. On 28 dpv, PRRS virus replication was detected only in the vaccinated group and mean viremia was significantly greater in vaccinated pigs (***$p \leq 0.001$, unpaired t-test). Beginning on 32 dpv and until 42 dpv the non-vaccinated group had a trend towards significantly higher PRRSV replication. On 70 dpv, the non-vaccinated group had a trend towards viremia rebound (*$p = 0.05$, unpaired t-test). (B) PCV-2 viremia. On 35 dpv, the vaccinated group had a trend towards significantly higher PCV-2 viremia (*$p \leq 0.05$, unpaired t-test).

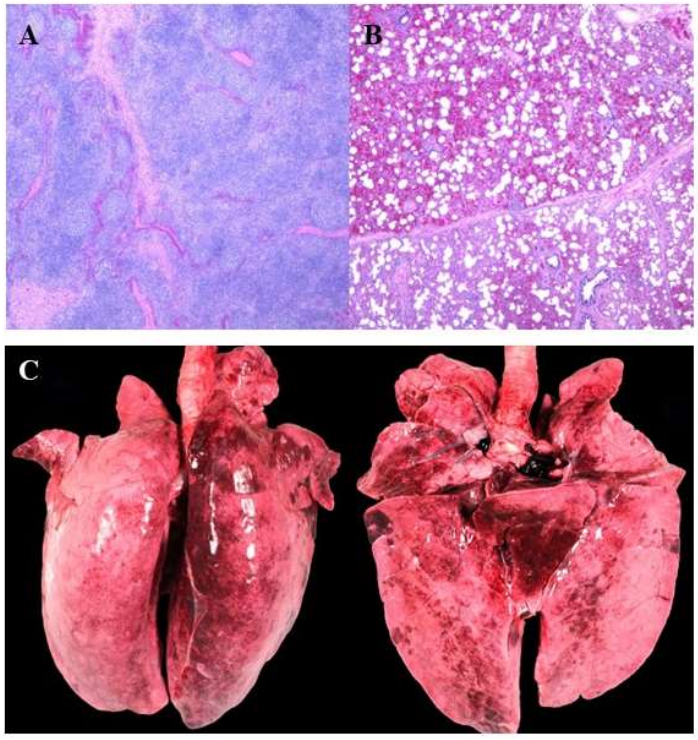


Figure 2.3 Representative gross and microscopic lesions associated with porcine circovirus associated disease (PCVAD)

Images shown are from a representative non-vaccinated pig. (A) H&E-stained tracheobronchial lymph node with severe lymphoid depletion (2×). (B) H&E-stained lung showing severe diffuse interstitial pneumonia affecting greater than 75% of lung (2×). (C) Dorsal and ventral gross lung showing extensive congestion, hemorrhage, and pneumonia.

A. Family Diversity

B. Species Diversity

C. Firmicutes:Bacteroidetes Ratio

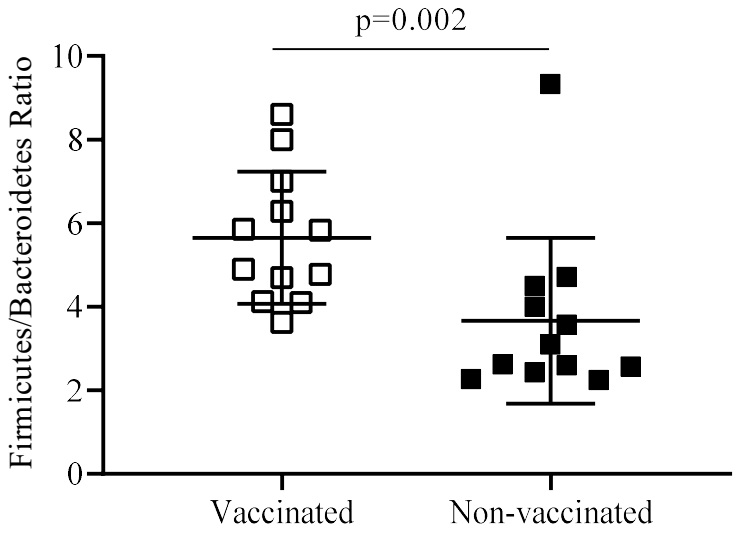


Figure 2.4 Fecal microbiome diversity in vaccinated and non-vaccinated pigs after PRRS MLV vaccination and prior to PRRSV and PCV-2 co infection

Data shown as the total number of microbial families (A) and microbial species (B) detected by the LLMDA prior to co-infection for individual pigs. Group means and standard deviations are represented by horizontal lines. No significant difference in microbiome diversity was detected on a family level ($p = 0.54$; Mann-Whitney U test) whereas microbiome diversity was significantly greater on a species level in vaccinated pigs ($p = 0.005$, Mann-Whitney U test). C) Data shown as Firmicutes:Bacteroidetes ratio for each pig in the two groups. Horizontal lines represent mean ± one standard deviation. Vaccinated pigs had a trend towards significantly higher ratios when compared non-vaccinated pigs ($p = 0.002$, Mann-Whitney U test).

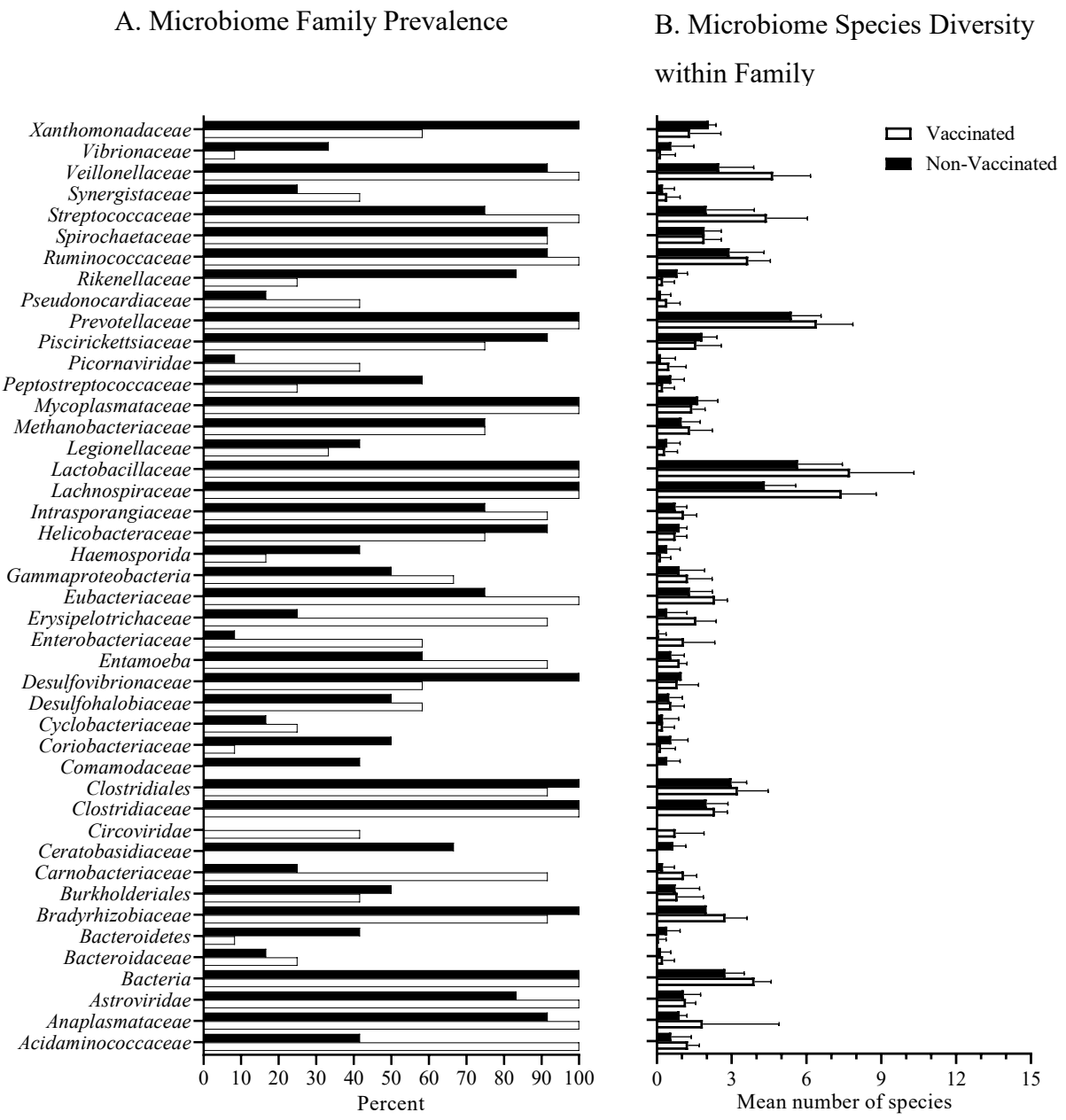


Figure 2.5 Fecal microbiome composition in vaccinated and non-vaccinated pigs PRRS MLV vaccination and prior to PRRSV and PCV-2 co infection

(A) Microbiome family prevalence is shown as percent of vaccinated (n = 12) and non-vaccinated (n = 12) pigs with each family detected on the microarray. Asterisks highlight statistically significant differences in family prevalence detected between the two groups ($p \leq 0.05$). (B) Number of species detected within each microbial family. Data is shown as the mean number of species detected ± one standard deviation in each family identified in vaccinated and non-vaccinated group of pigs. Asterisks highlight statistical significance ($p \leq 0.05$).

Chapter 3 - Characterization of a fecal microbiota transplant shown to improve clinical outcomes in growing pigs with porcine circovirus associated disease

Abstract

Fecal microbiota transplant (FMT) involves introducing the fecal microbial community from a healthy donor into a recipient's gastrointestinal tract to modulate their gut microbiome. FMT has been previously shown to improve health outcomes in growing pigs with porcine circovirus-associated disease (PCVAD). The objective of the current study was to characterize the transplant material shown to be beneficial to the clinical outcome of pigs challenged with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus-2 (PCV-2). Further, the study aimed to characterize the FMT material, determine its cultivability, and quantify the culturable microbes. Aerobic and anaerobic culture methods were used to isolate, identify and quantify bacteria from the transplant material. The cultivable fecal microbiota was further cultured using selective and enriched media and/or broth to obtain individual pure cultures, enumerated and mixed together in sterile normal saline with glycerol to prepare a predefined consortium referred to in this paper as microbiome therapeutic. A total of 26 bacterial isolates were identified by mass spectrometry out of which 24 isolates were culturable. The total microbial load of the original FMT material was 1.58 x107 CFU/mL including total aerobic and anaerobic counts of 1.11 x106 (7.0%) and 1.47 x107 (93.0%) CFU/mL, respectively. The majority of the bacterial load (53.9%) was comprised of *Bifidobacterium boum* as identified by mass spectrometry. Overall, the study shows that FMT material can be characterized and pure cultures of cultivable fecal bacteria can be obtained using culture-based techniques, and the

cultivable portion of the FMT material is dominated by anaerobes. Further, this knowledge could help regulate the composition of FMT materials and understand the mechanisms behind the benefits of the transplant material.

Introduction

Fecal microbiota transplant (FMT) is a microbiome-based intervention that aims to restore or modify the gut microbiota of a diseased individual by introducing the entire fecal microbial community from a healthy donor into the recipient's intestinal tract. The goal of the FMT is to improve the composition and function of the recipient's gut microbiome. It has mainly shown remarkable efficacy in the treatment of recurrent Clostridium difficile infections that is unresponsive to standard antibiotic therapy (Gupta et al., 2022). Additional FMT-treatable gastrointestinal diseases in humans include irritable bowel syndrome and intestinal inflammation. More recently, there is increasing evidence of correlative effects on organ systems outside of the gut, including liver diseases, metabolic syndrome, neurological disorders, malignant tumors, and infectious diseases (Biazzo & Deidda, 2022; Qu et al., 2022; Chunxi et al., 2020). In animals, oral transfaunation of mature cattle with ruminated ingesta or rumen contents is a more common historic practice to restore ruminal microbes in cases of digestive or metabolic disorders other than feces (DePeters & George, 2014; Mandal et al., 2017). However, various recent studies have explored the therapeutic, prophylactic, and immunogenic potential of FMT in animals. Some of the examples include canine parvovirus infections (Pereira et al., 2018), Inflammatory bowel disease (Niina et al., 2019), acute diarrhea (Chaitman et al., 2020), and *Clostridium difficile* associated diarrhea (Sugita et al., 2019; Diniz et al., 2021) in dogs, colitis in horses (Mullen et al., 2018) and *Mycoplasma hyopneumoniae* (Schachtschneider et al., 2013), porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus

type 2 (PCV-2) (Niederwerder et al., 2018a)and porcine epidemic diarrhea virus (Clement et al., 2016) in pigs.

Previous work has demonstrated prophylactic administration of FMT for consecutive seven days decreased the severity of clinical signs following PRRSV and PCV-2 co-infection. Specifically, reduced mortality, improved weight gain, decreased macroscopic lung lesions, reduced PRRSV and PCV-2 viremia, and increased levels and sustained production of antibodies in FMT-administered nursery pigs were observed when compared to saline mock transplanted pigs (Niederwerder et al., 2018a). Although the FMT material provided benefits to clinical outcomes, the mechanism by which this occurred and the specific components of the FMT which played a role in reducing PCVAD remained unclear.

Thus, the objective of the study described herein was to characterize the FMT material through several approaches, including identification of antibiotics (ABx) resistance genes to further understand their safety and to determine their cultivability, and concentration of culturable microorganisms.

Materials and methods

Isolation of bacteria from the original fecal microbiota transplant material. An aliquot of the fecal microbiota transplant (FMT) material, previously described in Niederwerder et al. (2018) was serially diluted in sterile water using ten-fold dilutions from 10-1 through 10-5. The 10-1 dilution was inoculated onto blood agar (tryptic soy agar with sheep blood). The 10-2 through 10-4 dilutions were inoculated onto blood agar, Columbia colistin nalidixic acid agar with sheep blood (CNA agar), MacConkey agar, and Brucella agar with 5% sheep blood with hemin and vitamin K (Brucella agar). The 10-5 dilution was inoculated onto Brucella agar. Selective and differential media were used to aid in the generation of pure cultures and

differentiation among species based on morphologic differences. Inoculated plates of blood agar, CNA agar, and MacConkey agar were incubated overnight under aerobic conditions at 37°C with 5% CO_2. Following the incubation period, all plates were examined for bacterial growth and morphologic differences among individual colonies. Colonies of each distinct morphology observed on the plates were then re-inoculated onto fresh blood agar plates for isolation. These plates were incubated as described previously.

Inoculated plates of Brucella agar were incubated overnight at 37°C under anaerobic conditions utilizing an anaerobe container with a Mitsubishi anaerobic environment-generating sachet and anaerobic indicator. Following incubation, all plates were examined for bacterial growth and morphologic differences among colonies. Individual colonies of each unique morphology were re-inoculated onto fresh plates of Brucella agar for isolation and incubated under the same anaerobic conditions. Following overnight incubation, individual colonies from each plate were re-inoculated onto fresh plates of blood agar and incubated under aerobic conditions to determine if the isolates were facultative or obligate anaerobes.

All plates inoculated for isolation were inspected to ensure each contained a pure culture isolate. Bacterial growth from each of the pure cultures was harvested from the plates and suspended in sterile normal saline containing 10% glycerol. Two 500-µL aliquots were prepared from each pure culture for quantification. The remaining 9 mL of each culture was stored in a 15-mL conical tube. All tubes and vials containing individual bacterial suspension and their aliquots respectively were stored at -80°C until their use.

Quantification of bacterial isolates and original FMT material. Each isolate was quantified by performing ten-fold serial dilutions of individual isolates in sterile normal saline. Dilutions from 10-1 through 10-10 were prepared. The 10-3 through 10-10 dilutions were

inoculated onto blood agar for aerobic organisms (n = 19) and Brucella agar for anaerobic organisms (n = 7). Plates were then incubated as previously described for both aerobic and anaerobic growth conditions. All plates were examined for growth after 24 hours of incubation. Plates with insufficient growth were returned to the incubator for additional 24-hour periods up to 72 hours of total incubation. Two of the isolates failed to grow during the quantification process (X and Y). Concentration for the remaining 24 isolates was calculated in colony forming unit per milliliter (CFU/mL). Quantification calculations were based on plates containing between 30-300 colonies.

The plates inoculated with 10-1 through 10-5 dilutions of the original FMT material were used to estimate the CFU/mL of each of the isolates present in the original FMT material. This was completed by counting the number of colonies present for each distinct colony morphology present on each type of agar (blood agar, CNA agar, MacConkey agar, and Brucella agar).

Identification of isolates was completed using two diagnostic assays, including 1) MALDI-TOF mass spectrometry and 2) the Lawrence Livermore Microbial Detection Array (LLMDA).

Culturing methods utilized to grow additional cultures of bacterial isolates. Concentrations of aerobic isolates O (*Streptococcus alactolyticus*) and Q (*Lactobacillus mucosae*) were inadequate from the initial growth on blood agar and additional cultures were required. Isolates O and Q were cultured directly into BHI broth and incubated at 37°C with 5% CO2 for 48 hours. Following incubation, the liquid cultures were preserved with the addition of 10% sterile glycerol before storing at -80°C. Quantification was performed as previously described. Similarly, Concentrations of anaerobic isolates T (*Clostridium perfringens*), V (*Clostridium perfringens*), and Z (*Bifidobacterium boum*) were inadequate from the initial

growth, and additional cultures were required. First, T and V were inoculated onto Brucella agar and incubated at 37°C with 5% CO_2 for 24 to 48 hours under the anaerobic conditions described previously. Following incubation, bacterial growth was transferred to thioglycolate (FTG) broth and incubated for 24 hours. Liquid cultures were preserved with 10% sterile glycerol and stored at -80°C. Second, isolate Z was cultured in De Man, Rogosa, and Sharpe (MRS) broth in an anaerobic chamber/glove box and bench-top nitrogen gas system for 24 hours at 37°C. Culture material containing isolate Z was preserved with 10% glycerol and anaerobically stored at -80°C in glass vials. Quantification of the isolates was performed as previously described until all isolates reached the desired concentrations.

Fecal resistome and microbiome characterization. Shotgun metagenomics was used to characterize differences in the microbiome and antimicrobial resistome composition in the fecal samples obtained from each donor sow (n = 2) and fecal transplant material (n = 1) developed utilizing these fecal samples.

Total DNA was extracted from each sample using the DNeasy Power Soil Pro Kit (Qiagen, Cat No. 47014, Hilden, Germany) according to the manufacturer's instructions. Briefly, 250 mg of the fecal sample was placed in power bead tubes containing 800 µL CD1 buffer. After vortexing, bead tubes containing samples were processed on a Mini Bead-beater (Biospecproducts Cat. No. 1001, Bartlesville, OK, U.S.) at 2200 rpm for 20 seconds, repeated three times with an interval of 30 seconds in between bead beating cycle. Following centrifugation (16,000 x g for 2 minutes), 600 µL of supernatant was transferred into the tube, and all subsequent extraction steps were followed according to the manufacturer's instructions including the inhibitor removal step. DNA concentration was determined by Qubit 4 Fluorometer (ThermoFisher Scientific, Cat No. Q33226, CA, U.S.). After DNA extraction, libraries were

prepared using the Qiaseq FX DNA Library Kit (Qiagen, Germany, Catalog number 180475), starting with 100 ng individual DNA samples. The quality and quantity of libraries were evaluated using TapeStation (Agilent Technologies 4200) and Qubit® 4.0, respectively. Libraries were sequenced on NovaSeq 6000 with SP flow cell chemistry (Illumina Inc., San Diego, CA, USA).

To characterize the resistome composition, the sequencing reads were aligned to the AMR gene database MEGAREs v1.0.0 using AMRPlusPlus pipeline version 1.0 (Lakin et al., 2016). Briefly, raw sequence reads were processed, and low-quality reads and adapter contamination were removed using Trimmomatic (Bolger et al., 2014). The host-associated sequences were removed by aligning the trimmed sequences to the reference Sus scrofa genome using Burrows-Wheeler-Alignment (BWA) (Li & Durbin, 2009). Non-host reads were then aligned to the AMR gene database MEGAREs v1.0.0 using BWA and then converted to a SAM-formatted file using Samtools (Li et al., 2009); later, this was analyzed through ResistomeAnalyzer with the default settings for at least 80% gene fraction (i.e., percentage of the nucleotides within the gene to which at least one read aligns). The counts of aligned sequence reads were recorded at the gene (accession), group, mechanism, class, and type, and were normalized using cumulative sum (CSS) using a default percentile of 0.5 (Paulson et al., 2013) to account for potential differences in sequence depth.

Similarly, microbiome taxonomic composition was determined by classifying non-host sequencing using the Kraken 2 Standard Database (Wood et al., 2019) with default settings as implemented in the AMRPlusPlus pipeline (Lakin et al., 2016).

Results

Microbiome composition of original FMT material. Aerobic and anaerobic cultures isolated a total of 26 bacteria. Isolates were designated A through Z (**Table 3.1**). A total of 24 culturable bacteria were identified using MALDI-TOF mass spectrometry, including single or multiple species from genera *Bacillus, Escherichia, Staphylococcus, Enterococcus, Streptococcus, Lactobacillus, Actinomyces, Clostridium*, and *Bifidobacterium*. Two anaerobic organisms identified as gram-negative isolate X) and gram-positive (isolate Y) were unable to be cultured following their initial isolation on Brucella agar. Additionally, estimates of each isolate's abundance (CFU/mL) in the original fecal transplant material were determined (**Table 3.1**). The cultivable portion of the fecal microbiota was determined by pooling the total bacterial count estimated for each of the isolates present in the original FMT material. The total microbial load of the original FMT material was 1.58 x107 CFU/mL including total aerobic and anaerobic counts of 1.11 x106 (7.0%) and 1.47 x107 (93.0%) CFU/mL, respectively. The majority of the bacterial load (53.9%) was comprised of *Bifidobacterium boum* as identified by mass spectrometry.

Pure cultures of a total of 24 culturable bacteria identified by mass spectrometry were also determined using LLMDA. The pan-microbial array successfully determined the majority of the isolates (23/24); 3 phyla, 9 microbial families, and 23 microbial species were detected (**Table 3.2**). Interestingly, a gram-negative anaerobic bacterium unable to be identified at the genus or species level using the mass spectrometry was identified as *Shigella sonnei* by the pan microbial array. Conversely, the microbial array could not determine an isolate identified as *Actinomyces hyovaginalis* by spectrometry.

Fecal resistome and microbiome characterization. Shotgun metagenomics analysis showed an overall similar microbiome and resistome composition across all three samples including two donor feces and one fecal transplant material.

Metagenomics sequencing uncovered that the majority of the microbial species fell within the phylum Firmicutes across all three samples. Other phyla detected include Actinobacteria, Euryarchaeota, and Bacteroidetes (**Figure 3.1, top left panel**). At the class level, 7 uniquely classified groups were identified in all three samples, with a majority falling within the classes Bacilli, Erysipelotrichia, and Clostridia (**Figure 3.1, top right panel**). Within orders, there were 7 identified microbial groups, with Lactobacillales, Erysipelotrichales, and Clostridiales being the most commonly identified across all three samples (**Figure 3.1, bottom left panel**). At the family level, 6 unique classification groups were identified (**Figure 3.1, bottom right panel**). The most commonly represented families were *Lactobacillaceae* and *Erysipelotrichaceae* across all three samples. Metagenomic sequencing also identified 22 uniquely classified microbes across the 3 samples (**Figure 3.2, left panel**). The most prevalent species in all 3 samples were *Lactobacillus amylovorus*; with 65.5%, 67.9%, and 57.5% prevalence in fecal transplant material, and feces from donor sows A and B, respectively (**Figure 3.2, right panel**). Some of the more prevalent species were *Turicibacter, Megasphaera*, and *Lactobacillus* species.

Each resistance gene type was matched to its corresponding antibiotic and the relative abundance of types resistant to the same antibiotic was summarized. The abundance of various antimicrobial-resistant genes (ARGs) was estimated based on their reads. For instance, tetracycline-associated ARGs (Fecal transplant material = 71.2%, Donor Sow A = 61.4% Donor Sow B=65.1%; tetW, tetQ, tetA, e.t.c.) were found to be the major genes identified across all

three samples. Any class of antibiotic making up 2% or less of all resistant gene types identified was classified as “Other”. The results indicate that the resistant gene types in all three samples conferred resistance to almost all major classes of antibiotics of common veterinary use including tetracycline, Rifampin, Elfamycins, Aminoglycosides, Fluoroquinolones, and MLS. Among these antibiotics, tetracycline contributed to the majority of the percentage of total resistance genes across all three samples (**Figure 3.3**).

Discussion

Despite a long history of FMT as a treatment for various GI diseases, it is more recently that FMT has been recognized as an alternative therapeutic for non-GI diseases including respiratory and neurologic diseases. Lately, FMT has also been investigated as a prophylaxis prior to the development of disease and shown improved disease outcome in pigs. However, it comes with substantial risks to the recipient including transplantation of detrimental pathogens and uncertain donor-recipient compatibility. More recently, bacterial therapeutics with precisely defined consortium of feces-derived viable microbes is gaining consideration as an alternative to FMT as it offers the advantage of being of known, consistent composition and testable safety (Caballero et al., 2017). Additionally, it is believed to provide a better understanding of the roles of each microbial species and, thus improve the identification of microbiome-mediated clinical efficacy (Gerardin et al., 2021).

The current study describes the characterization of the original fecal transplant material and the subsequent formulation of microbiome therapeutic using cultivable microbes from the transplant material. Apart from providing insights on species co-occurrence patterns in the fecal samples, culture-based techniques provide an opportunity to isolate, preserve and exploit the identified microbial diversity in the feces. Aerobic and anaerobic cultures identified several

microbial species, including species from genera *Bacillus, Escherichia, Staphylococcus, Enterococcus, Streptococcus, Lactobacillus, Actinomyces*, *Clostridium*, and *Bifidobacterium.* A previous study by our team has reported similar results. For example, several culturable bacteria, including non-hemolytic *Escherichia coli, Bacillus altitudinis, Streptococcus alactolyticus, Enterococcus hirae, non-hemolytic Staphylococcus sp., Bacteroides vulgatus,* and *Clostridium perfringens were* identified by conventional culture techniques (Niederwerder et al., 2018a). Consistent with a previous study (Wang et al., 2021), fecal transplant material was majorly composed of microbes from phylum Firmicutes and Proteobacteria. Also, bacterial genera found to be most abundantly characterized through culture-dependent techniques were successfully isolated by us. These bacterial genera include *Escherichia, Streptococcus*, and *Lactobacillus* (Wang et al., 2021). Our findings indicate that majority of the microbial load in the original fecal transplant is comprised of anaerobic isolates. It was not surprising as GI tract is an anerobic environment and is more likely to support growth of oxygen-sensitive bacteria.

Characterization of transplant material using culture-dependent techniques shows the presence of several beneficial microbes. For example, *Clostridium* species are excellent inducers of regulatory T cells (T_{regs}) in the colon which was linked to reduced mucosal inflammation and improved systemic antibody responses in mice as shown by oral inoculation of clostridium during the early life of conventionally reared mice (Atarashi et al., 2011). Additionally, *Lactobacillus* species are known to have beneficial roles in swine gut health. For instance, increased relative abundance of *Lactobacillus* was associated with increased antibody titers and milder pathogenic damage in pigs after immunization with PRRSV vaccine (Zhang et al., 2021).

Taken together, robust characterization helps to define the microbiome therapeutic and is key for delivering consistent and efficacious bacterial consortia while addressing safety concerns associated with FMT.

Funding and Acknowledgment

Table 3.1 Characterization of fecal microbiota transplant material shown to be beneficial for prophylactic administration prior to PRRSV/PCV2 co-infection in nursery pigs*

Growth condition	Isolate	Identification MALDI-TOF	CFU/mL
Aerobic	A	*Bacillus* sp.	2.00E+02
	B	*Bacillus* sp.	1.00E+02
	C	*Escherichia coli*	1.00E+02
	D	*Bacillus pumilus*	1.00E+02
	E	*Escherichia coli*	1.00E+03
	F	*Escherichia coli*	1.20E+04
	G	*Escherichia coli*	1.70E+04
	H	*Bacillus megaterium*	1.00E+03
	I	*Staphylococcus simulans*	1.00E+03
	J	*Escherichia coli*	5.90E+04
	K	Gram negative unable to ID	2.00E+03
	L	*Enterococcus mundtii*	5.00E+03
	M	*Enterococcus hirae*	1.20E+04
	N	*Escherichia coli*	3.70E+04
	O	*Streptococcus alactolyticus*	7.30E+05
	P	*Enterococcus faecium*	1.00E+04
	Q	*Lactobacillus mucosae*	1.30E+05
	R	*Actinomyces hyovaginalis*	5.00E+04
	S	*Escherichia coli*	4.00E+04
Anaerobic	T	*Clostridium perfringens*	2.90E+05
	U	*Clostridium perfringens*	6.00E+04
	V	*Clostridium perfringens*	8.00E+05
	W	*Clostridium perfringens*	1.00E+05
	X	Gram negative unable to ID	1.00E+05
	Y	Gram positive unable to ID	4.80E+06
	Z	*Bifidobacterium boum*	8.50E+06
Total quantity of cultivable bacteria in original FMT material			1.58E+07

*MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; ID, identify; ND, not determined

Table 3.2 Cultivable microbiome composition of the original fecal microbiota transplant material as detected by the mass spectrometry and pan microbial array*

Phylum	Family	Genus species -MALDI-TOF	Genus species - LLMDA
Actinobacteria	*Actinomycetaceae*	*Actinomyces hyovaginalis*	ND
	Bifidobacteriaceae	*Bifidobacterium boum*	*Bifidobacterium indicum*
Firmicutes	*Bacillaceae*	*Bacillus* sp.	*Bacillus amyloliquefaciens*
		Bacillus sp.	*Bacillus licheniformis*
		Bacillus megaterium	*Bacillus sp.*
		Bacillus pumilus	*Bacillus safensis*
	Clostridiaceae	*Clostridium perfringens*	*Clostridium* sp.
		Clostridium perfringens	*Clostridium* sp.
		Clostridium perfringens	*Clostridium* sp.
		Clostridium perfringens	*Clostridium* sp.
	Enterococcaceae	*Enterococcus mundtii*	*Enterococcus mundtii*
		Entereococcus hirae	*Enterococcus faecium*
		Enterococcus faecium	*Enterococcus faecium*
	Lactobacillaceae	*Lactobacillus mucosae*	*Lactobacillus mucosae*
	Staphylococcaceae	*Staphylococcus simulans*	*Staphylococcus carnosus*
	Streptococcaceae	*Streptococccus alactolyticus*	*Streptococcus gallolyticus*
Proteobacteria	*Enterobacteriaceae*	*Escherichia coli*	*Escherichia coli*
		Escherichia coli	*Escherichia coli*
		Escherichia coli	*Escherichia coli*
		Escherichia coli	*Escherichia coli*
		Escherichia coli	*Escherichia coli*
		Escherichia coli	*Escherichia coli*
		Escherichia coli	*Escherichia coli*
		Gram negative unable to ID	*Shigella sonnei*

* MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; LLMDA, Lawrence Livermore microbial detection array; ID, identify; ND, not determined
‡Organized alphabetically by phylum

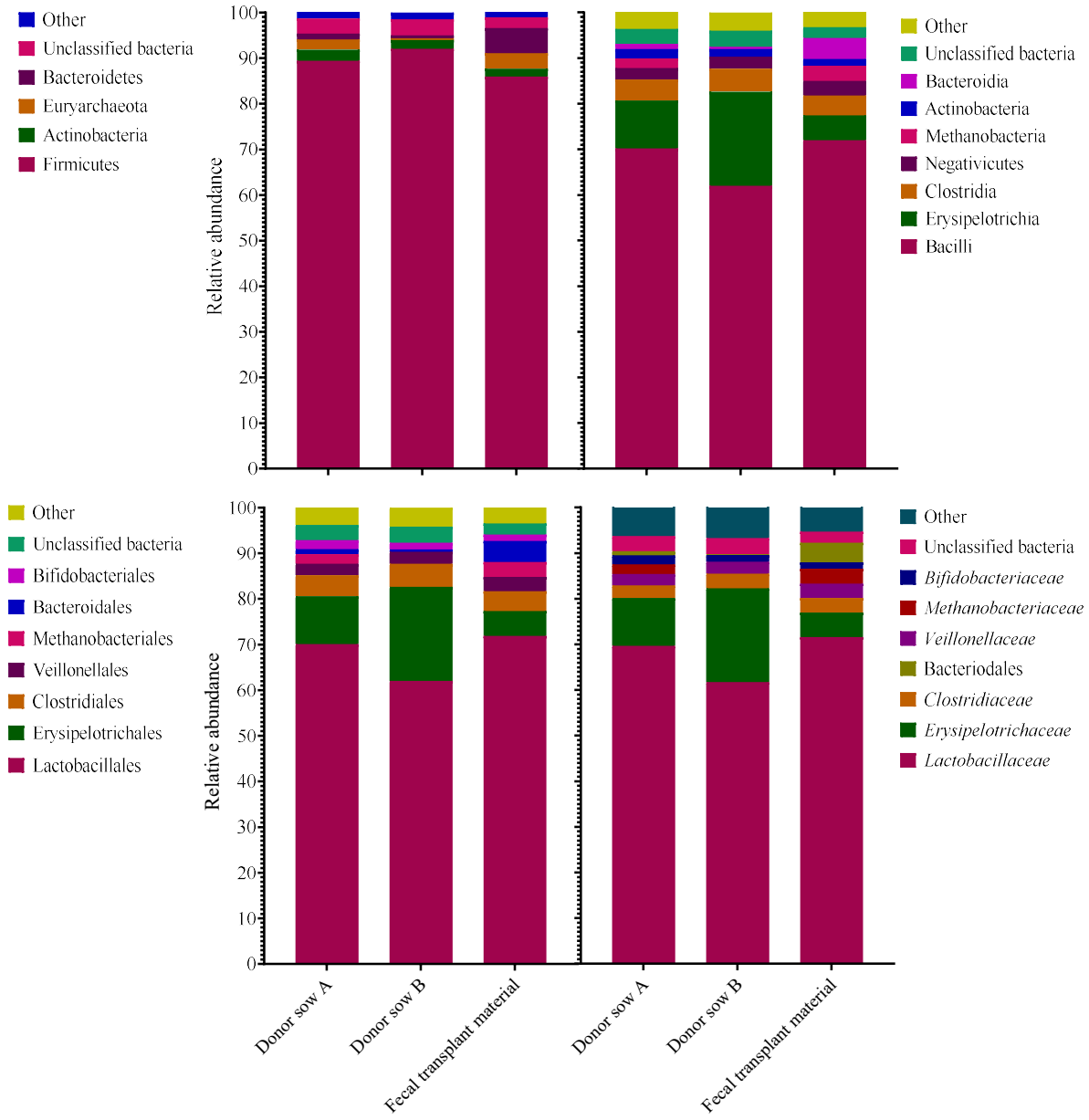


Figure 3.1 Fecal microbiome composition as detected by shotgun metagenomics.

Data shown as relative abundance of different taxonomic levels for fecal samples obtained from two donor sows and laboratory developed fecal transplant material. Each taxonomic level making up 2% or less of all sequences detected were classified as "Other". **Top left panel)** Relative abundance of microbial phyla. **Top right panel)** Relative abundance of microbial classes. **Bottom left panel)** Relative abundance of microbial orders. **Bottom right panel)** Relative abundance of microbial families. Immediate higher taxonomic level identified was listed when microbes were unidentified at the family level.

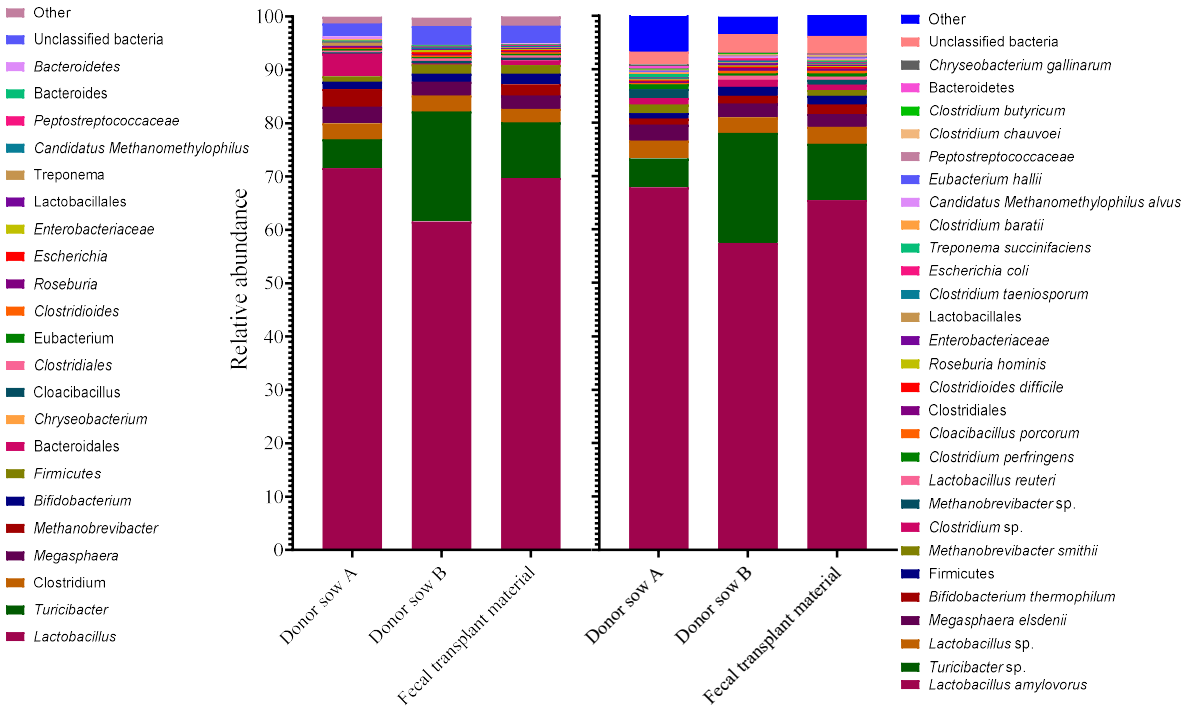


Figure 3.2 Fecal microbial species as detected by shotgun metagenomics.

Data shown as relative abundance of microbial genera (**Left panel**) and species (**Right panel**) for fecal samples obtained from two donor sows and laboratory developed fecal transplant material. Each taxonomic level making up 0.2% or less of all sequences detected were classified as "Other". Immediate higher taxonomic level identified was listed when microbes were unidentified at the genus level.

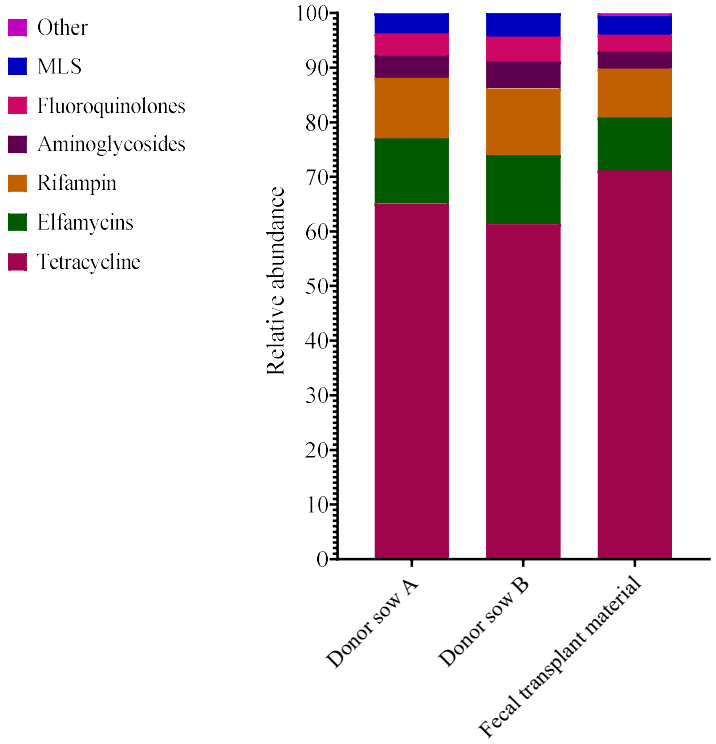


Figure 3.3 Antimicrobial resistome composition as detected by shotgun metagenomics.

Data shown as relative abundance of antibiotic resistance genes (ARG) assigned to each major antibiotic class identified in fecal samples obtained from two donor sows and laboratory developed fecal transplant material. The relative abundance of each ARG type was estimated on the basis of total number of reads. Each antimicrobial making up 2% or less of all resistome sequences detected were classified as “Other”.

Chapter 4 - Microbiota therapeutic had limited benefits in reducing clinical outcomes of porcine circovirus associated disease

Abstract

Co-infection of pigs with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV-2) can cause porcine circovirus-associated disease (PCVAD) in nursery pigs. Fecal microbiota transplantation (FMT) is linked to a reduction in the severity of clinical signs of PCVAD following co-infection with PRRSV and PCV-2. Although FMT is generally considered safe, it is associated with potential risks to the recipient. As such, there is a need to evaluate potential alternatives to FMT such as a live biotherapeutic or microbiota therapeutic in which a defined bacterial consortium can be administered. In the current study, a microbiota therapeutic was created and investigated as a potential alternative to FMT for improving clinical outcomes of pigs following PRRSV-PCV-2 co-infection. The microbiota therapeutic was prepared by mixing a set composition of cultivable microbes from the FMT material previously shown beneficial to clinical outcomes in co-infected pigs. A total of 100 pigs were either administered the microbiota therapeutic (n = 50) or a sterile mock transplant (n = 50) for seven consecutive days prior to co-infection. Overall, the microbiota therapeutic appeared to be safe after administration with no adverse reactions reported, increased average daily gain of transplanted pigs prior to co-infection (-8 to 0 dpi) and in the late stage of co-infection (38 to 42 dpi), and increased the rate of PCV-2 clearance in the serum of transplanted pigs. While some beneficial outcomes could be seen with the microbiota therapeutic, there was not a significant difference in the number of PCVAD affected pigs, serum viral load and the viral specific antibody levels between the groups and the microbiota therapeutic did not replicate the benefits of FMT.

Introduction

Fecal microbiota transplantation is a microbiome-based intervention by which fecal microorganisms obtained from a healthy donor is subsequently transplanted into a diseased individual. Although the use of FMT dates back to the 4th century in China for the treatment of diarrhea in humans (Zhang et al., 2012), its scientific upsurge did not substantially commence until 2013, when it was approved by the United States Food and Drug Administration for the treatment of recurrent and refractory *Clostridium difficile* infection (Food & Administration, 2013). Over the years, FMT has been shown to improve or resolve symptoms of several gastrointestinal (GI) diseases including obesity, inflammatory bowel disease, irritable bowel syndrome, ulcerative colitis, and metabolic syndrome (Almeida et al., 2022). Apart from GI diseases, FMT has been shown as a promising therapy for non-GI disorders like cancer and cardiometabolic, neurological, and respiratory disorders (Almeida et al., 2022). The therapeutic mechanism of FMT is likely associated with the restoration of normal gut flora as dysbiosis of gut flora has been considered to be associated with the development and progression of diverse disease conditions (Li et al., 2016).

More recently, prophylactic interventions of FMT have also been explored for disease prevention. For instance, previous work evaluated FMT as a prophylactic measure to reduce the severity of porcine circovirus-associated disease in nursery pigs (Niederwerder et al., 2018a). In traditional food animal production, antibiotics and growth promoters have been employed as preventive measures. However, there has been a surge of interest in antibiotic alternatives due to concerns regarding antimicrobial resistance. As gut microbiotas are further recognized for their beneficial role in growth and immunity, modulating by FMT may serve as a promising alternative to antibiotics and growth promoters in food animal production.

Porcine reproductive and respiratory syndrome (PRRS) is the most economically important infectious disease for the swine industry worldwide accounting for annual losses of about $664 million in the US alone (Holtkamp et al., 2013). It is caused by the porcine reproductive and respiratory syndrome virus (PRRSV). In clinical conditions, the detection of PRRSV is typically accompanied by the presence of porcine circovirus type 2 (PCV-2) (Pallarés et al., 2002). PCV-2 is a widely studied swine virus as a causative factor for porcine circovirus-associated diseases (PCVAD) and that coinfection with other swine pathogens like PRRSV may enhance the severity of PCVAD (Ouyang et al., 2019). PCVAD is a multisystemic disease syndrome characterized by muscle wasting, respiratory disease, jaundice, and reduced weight gain in growing pigs (Segales, 2012). Many studies have reported that the co-infection of nursery pigs with PRRSV and PCV-2 can reproduce PCVAD in an experimental setting. (Niederwerder et al., 2016; Megan C Niederwerder et al., 2015; Trible et al., 2011).

Previous work demonstrated prophylactic administration of FMT for consecutive seven days decreased the severity of clinical signs following co-infection with porcine reproductive and respiratory syndrome (PRRS) and porcine circovirus-2 (PCV-2) (Niederwerder et al., 2018a). Specifically, reduced mortality, improved weight gain, decreased macroscopic lung lesions, reduced PRRS and PCV-2 viremia, and increased levels and sustained production of antibodies were observed in FMT-administered nursery pigs when compared to saline mock transplanted pigs. Although FMT is generally considered safe, it is associated with risks to the recipient including transplantation of pathogens and uncertain donor-recipient compatibility (Wong & Levy, 2019). Additionally, substantial variability in the gut microbiome composition between individuals and limited long-term establishment of transplanted microbial community highlights the need to develop an alternative to FMT such as live biotherapeutic or microbiota therapeutic.

Microbiota therapeutic is a defined consortium of feces-derived viable microbes (Petrof & Khoruts, 2014). Utilizing therapeutics to reconstitute gut microbiota can allow us to control the set composition of defined consortia. It can provide a consistent ability to replicate the beneficial features of the FMT material. Additionally, the consortia can be screened extensively for the absence of undesired pathogens and can be reproducibly manufactured for large-scale production (Borody, 2019). For example, administration of defined preparation of 33 nonpathogenic stool-derived bacteria has been shown to result in a durable cure of recurrent CDI in 100% (2/2) of patients (Petrof et al., 2013).

The hypothesis tested in the current study was that prophylactic administration of a microbiota therapeutic to 3-week old weaned sibling pairs prior to co-infection with PRRSV and PCV-2 would reduce clinical signs and pathology associated with PCVAD consistent with results from the FMT demonstrated in previous work (Niederwerder et al., 2018a). The microbiota therapeutic was prepared by mixing set composition of cultivable microbes from the fecal microbiota transplant (FMT) material used in Niederwerder et al. (2018a). The study described aimed to investigate if microbiota therapeutic transplantation is comparable to FMT on disease outcome effects following co-infection in pigs.

Materials and methods

Animals and housing. All experiments involving the use of 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. Fifty pairs of barrow siblings (n = 100; 19.1 ± 1.9 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). The sibling pairs were not vaccinated for PCV-2. No prophylactic or therapeutic antibiotics were administered at weaning or within 1 week of arriving at Kansas State. Each sibling pair was divided into either the control or microbiota therapeutic group and the two groups were balanced by the weight of siblings upon arrival. Microbiome therapeutic and control groups were housed in separate but identical environmentally controlled rooms maintained under biosafety level 2 (BSL-2) conditions at the Kansas State University Large Animal Research Center. All pigs were housed in groups of 10 in a 9.1 square meter pen with raised slatted flooring. Pigs were given access to food and water ad libitum.

Viruses. The challenge inoculum prepared for this study included the PRRSV and PCV-2d viral isolates.

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-2d (Benjamin R. Trible et al., 2012). PRRSV was isolated by propagation on MARC-145 cells and titrated as previously described (Megan C Niederwerder et al., 2015; Niederwerder et al., 2018a).

The PCV-2d virus was isolated and titrated as described previously in Niederwerder et al. (2018a). Briefly, the isolation method involved utilizing the heat stability of the virus and preparing liver, lung, and spleen homogenates enriched with PCV-2. In addition, supernatants from these organ homogenates were screened using the Lawrence Livermore Microbial Detection Array (LLMDA) and were confirmed negative for other common pathogens, such as porcine parvovirus, PRRSV, swine influenza virus, and *Mycoplasma hyopneumoniae*, except

porcine endogenous retroviruses (data not shown), which are ubiquitous in swine. PCV-2d infectivity was titrated on swine testicle (ST) cells.

The inocula for pigs were prepared by combining the challenge viruses 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 to administer 1 mL intranasally and 1 mL intramuscularly.

Microbiota therapeutic. The microbiota therapeutic was obtained from the fecal microbiota transplant material utilized in our previous work (Niederwerder et al., 2018a). The FMT material was prepared as described previously by Niederwerder et al. (2018a) from the feces collected from two high-health sows from a commercial farrow-to-wean farm in Kansas

To prepare the microbiota therapeutic, the abundance (CFU/mL) of each isolate in the FMT material was estimated. Cultivable bacteria were grown using various culture methods to obtain quantities sufficient to create a microbiome therapeutic for administration. Once desired titers of all cultivable bacteria were obtained, their pure cultures were pooled together to formulate the microbiota therapeutic. Briefly, the volume of pure cultures was determined by dividing their desired abundance to constitute microbiota therapeutic by their abundance of respective pure cultures. The pre-determined volume of pure cultures of bacteria was pooled together in sterile normal saline with a 10% glycerol solution (**Table 4.1**).

The microbiota therapeutic was submitted to spell out for routine bacterial culture, including aerobic culture and anaerobic culture. Species identification and their abundance estimation was attempted for all bacteria cultured.

Experimental design and sample collection. Approximately 24 h after arriving at Kansas State University, pigs were administered microbiota therapeutic (MT) or a mock transplant (CONTROL) made of 10% glycerol in sterile saline. The microbiota therapeutic was

administered as 5 mL doses delivered once daily for seven consecutive days prior to co-infection. Each dose of microbiota therapeutic was formulated to administer the cultivable bacteria at a dose equivalent to the concentration of each microbial species detected in the FMT material. MT or mock-transplant were delivered through flexible dispensing tips (6.4 mm FlexojectTM 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 s to 1 min.

After 7 days of transplantation, all 100 pigs were co infected with PRRSV and PCV-2d. Individual body weights were collected upon arrival (-8) and on 0, 3, 7, 10, 14, 17, 21, 24, 28, 31, 35, 38, and 42 dpi. Blood samples were collected on -8, 0, 3, 7, 10, 14, 21, 28, 35, and 42 dpi. Fecal samples were collected on -8, 0, 7, 14, 21, 28, and 35 dpi for microbiota analysis. In addition to scheduled sample collection days, blood, feces and weights were collected on the day of death or euthanasia. Ten sibling pairs were serially sacrificed at 0 and 15 dpi each to evaluate the effects of the cultivable bacteria on the outcome of co-infection over time, including measurements of gross and histopathologic lesions. Two pigs from each pen were randomly selected (corresponding to sibling groups in the two rooms) on 0 and 15 dpi for serial sacrifice and subsequent necropsy. While remaining 60 pigs were allowed to develop clinical signs and monitored through the 42 days of the study.

Any pigs displaying following conditions were humanely euthanized under the direction of the attending laboratory animal veterinarian: 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. Throughout the study, all pigs were visually assessed by an attending veterinarian or veterinary assistant every day for clinical signs associated with PRRSV PCV-2 co-infection. These signs included dyspnea, coughing, nasal discharge, ocular discharge and open mouth breathing, lethargy, depression, diarrhea, fever, lameness, joint effusion, reduced body condition, muscle wasting and aural cyanosis. Appropriate treatment was administered under the direction of a veterinarian to any pig showing moderate to severe clinical disease including 1) dyspnea, 2) mucopurulent nasal discharge, 3) lameness with joint effusion, 4) pallor or jaundice with muscle wasting, and 5) lethargy or depression with pyrexia. Parenteral antibiotics, such as ceftiofur hydrochloride, oxytetracycline, or enrofloxacin were administered to clinically affected pigs. Likewise, parenteral flunixin meglumine, a nonsteroidal anti-inflammatory drug was prescribed to any pig with overt clinical disease and a rectal temperature of ≥104°F.

At 42 dpi, all remaining pigs were humanely euthanized by intravenous injection of pentobarbital sodium. Immediately after euthanasia, whole body weights of individual pigs were recorded before a board-certified veterinary pathologist, blinded to the selection of pig groups, performed complete necropsies and histopathologic evaluations on them. The lung was removed and the lung weight was recorded with and without trachea. The lung weight to body weight ratio was calculated as a measure of pulmonary pathology. Both dorsal and ventral views of the entire lung were captured using a digital camera (Canon EOS Rebel T6 DSLR) and the digital images were evaluated using a photo scoring system. Gross anatomical photo scores were expressed as the percentage of lung affected by pneumonia (0-100%) and combined from five

sections of the lung as previously described (Halbur et al., 1995). The photographs were analyzed by a board-certified veterinary pathologist who was blinded to the source of images.

One section from each lung lobe, trachea and tracheobronchial lymph nodes was collected from all pigs at necropsy. Collected 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 on a scale of 0-4 as previously described (Niederwerder et al., 2016; Megan C Niederwerder et al., 2015; Ober et al., 2017). The degree of lymphoid depletion was scored using a 0-3 system as described previously by Niederwerder et al. (2016). Likewise, microscopic tracheitis were estimated using a 0-3 scoring system. Briefly, scores were given as follows: 0, no lymphocyte depletion; 1, mild or small amount of lymphocyte depletion; 2, moderate or intermediate amount of lymphocyte depletion; 3, severe or large extent of lymphocyte depletion.

Microarray analysis of fecal samples. Microbiome composition and diversity of the fecal samples was analyzed using the LLMDA that can detect annotated sequences of microbes associated with infection of vertebrates within GenBank®, the National Institute of Health genetic sequence database. The LLMDA version 7 with the 4plex180 K probe format in this study. This version of the array targets a total of 10,612 microorganisms including 5457 bacteria, 4377 viruses, 327 archaebacteria, 319 fungi, and 132 protozoa. Length of the LLMDA oligonucleotide probes are around 60 nucleotides and have roughly equivalent affinities for their complementary target DNA molecules (McLoughlin, 2011). The probes were designed to detect every sequenced microbial families, with an average of 30 probes per sequence to improve their sensitivity. The LLMDA microarray and statistical analysis method have been extensively tested in several studies for detection of both virus and bacteria in pure or complex environmental and

clinical samples (Niederwerder et al., 2016; Ober et al., 2017; Jaing et al., 2015; Constance et al., 2021; Gardner et al., 2010; Rosenstierne et al., 2014; Niederwerder et al., 2018b).

Nucleic acid was extracted from each fecal sample using the PowerViral™ Environmental RNA/DNA Isolation Kit (MO BIO, San Diego, CA) as previously described in Niederwerder et al. (2018a). Briefly, 250 mg of feces was added to a glass bead tube included in the kit with 600 µL of PV1/β-mercaptoethanol and vortex mixed for 10 min at maximum speed to prepare a homogenate prior to processing them using the PowerViral™ Kit protocol. Nucleic acid was eluted with 100 µL of RNase-Free water and were quantified using the Thermo Scientific™ Nanodrop™ spectrophotometer. For each sample, 10 µL of the extracted nucleic acid (both DNA and RNA)was amplified using the random amplification procedure as previously described by Rosenstierne et al. (2014). The amplified cDNA and DNA was purified and quantified using the Qiaquick PCR purification columns (Qiagen) and Nanodrop™ spectrophotometer, respectively.

Approximately 1 µg of amplified cDNA and DNA were fluorescently labeled using a one-coloring labeling protocol (Roche NimbleGen, Madison, WI) that involves 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 h. The labeled DNA was precipitated in isopropanol and centrifuged for 10 min. The pellet obtained was washed, dried and reconstituted in 50 µL of RNase-Free water and quantified using the Nanodrop™ spectrophotometer.

Next, samples with fluorescently labeled DNA were hybridized to the arrays using the Agilent Technologies Oligo aCGH/ChIP-on-Chip Hybridization kit (Santa Clara, CA). Briefly, 10 µg of fluorescently labeled DNA was mixed with 10x aCGH blocking agent, 2x HiRPM

hybridization buffer and nuclease free water and denatured at 95 °C for 3 min followed by incubation at 65 °C for 3 min.

Each sample was then immediately loaded onto the array and hybridized for approximately 40 h at 65 °C in a microarray rotator oven (Agilent Technologies Inc., Santa Clara, CA) set to a speed of 20. Following hybridization, 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). Afterwards, SureScan Microarray Scanner (Agilent Technologies Inc., Santa Clara, CA) was used to scan all arrays to a resolution of 3 μm. CLiMax method developed at Lawrence Livermore National Laboratory (Gardner et al., 2010) was used to generate the microarray data from the microbe sequences 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 microbiome was measured by calculating the number of families and species detected in each sample. The mean number of families and species were compared between the control and MT pigs. Microbiome composition was compared between the two groups at the level of family and the diversity of species within each family was determined.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 9.5.0 software (La Jolla CA, www.graphpad.com). Mean viremia, antibody levels, and weight measurements were compared between groups using repeated measures analysis with multiple unpaired *t*-tests. 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 families detected were compared using Fisher's exact test.

Results

Characterization of microbiota therapeutic. Microbial characterization of the MT was performed prior to administration. Aerobic and anaerobic culture identified the majority of bacteria used to prepare the MT with few exceptions including *Bacillus pumilus* and *Lactobacillus mucosae*. Overall, the abundance of isolates was reduced by two- to five-fold in the therapeutic compared to their abundance in each pure culture used to prepare the therapeutic (**Table 4.2**).

Microbiota therapeutic improved average daily gain in pigs prior to co-infection. Average daily gain (ADG) was calculated to demonstrate the change in weight over 8 days period prior to co-infection that includes 7 days of consequent therapeutic administration and reported in kg (**Figure 4.1**). Mean ADG for the control group was 0.05 ± 0.06 kg, with a range of -0.07 kg and 0.24 kg. Mean ADG for the MT group was 0.08 ± 0.07 kg, with a range of -0.06 kg and 0.28 kg. The mean ADG was significantly different over the 8-day pre-challenge period between the two groups ($p = 0.045$; unpaired t-test;). Improved ADG in the MT pigs during the pre-challenge or transplantation period indicates no adverse reactions of therapeutic administration on pigs in unchallenged conditions.

MT pigs sacrificed at 0 dpi had similar clinical outcome in both transplanted and control groups. All 20 pigs sacrificed at 0 dpi were negative for PRRSV and PRRSV Ab. Only 1/10 control pigs had a detectable level of PCV-2 viremia (0.05 log10 copies/PCR reaction). Pigs in both groups had almost same level of PCV-2 Ab detected in their serum ($p = 0.95$, unpaired t-

test; data not shown). Mean ADG in MT pigs was 0.07 ± 0.06 was slightly higher than control pigs 0.03 ± 0.06 (p=0.14, unpaired *t*-test; data not shown).

Lung pathology was similar between the MT and mock transplanted pigs. Percent lung affected was not significantly different between the two group (p = 0.13, unpaired t-test; data not shown).

Within 20 pigs sacrificed at 0 dpi, control pigs had a mean of 18.0 ± 23.7% lung affected while MT pigs had a mean of 36.5± 27.8% lung affected. In addition, pigs in both groups had a similar lung weight to body weight ratio at approximately 0.02 (p> 0.1, unpaired t-test; data not shown). Degree of interstitial pneumonia was score in all 20 pigs, with 1/10 control and 2/10 MT pigs having mild to moderate interstitial pneumonia with 50-75% lung lobe involvement (data not shown). MT pigs had slightly higher, but not significant, severity of microscopic lung lesions (p = 0.29, unpaired t-test). Likewise, microscopic tracheitis and tracheobronchial lymph node depletion scores were also slightly higher in MT pigs compared to control pigs, but not significantly different (p = 0.10 and p = 0.21, respectively, unpaired t-test; data not shown).

MT pigs sacrificed at 14 dpi had reduced morbidity. Majority of the 20 pigs sacrificed at 14 dpi did not develop any clinical signs that needed treatment. Only 2% of control pigs (1/10) developed clinical signs that needed treatment at 3-5 and 10-13 dpi and this difference in percent morbidity between the control and MT group was significantly different (p = 0.006, Man Whitney U test; **Figure 4.2**). Among the 20 pigs sacrificed at 14 dpi, MT pigs (*n* = 10) had similar weights compared to control pigs (*n* = 10) during the transplantation and post challenge period (p> 0.5, multiple unpaired t-test; data not shown). Likewise, PRRSV viremia was similar between the groups (p>0.1 multiple unpaired t-test; data not shown). At 3 dpi, slightly higher PRRSV viremia was detected in control pigs, but not statistically different (p = 0.13, multiple

unpaired t-test). The mean viremia at 3 dpi was 5.56 ± 0.36 and 5.15 ± 0.76 in control and MT pigs, respectively. Overall, serum PRRSV viremia determined bi-weekly showed that the virus replication peaked at 7 dpi and started to decay towards 14 dpi. Additionally, PCV-2 viremia was also determined biweekly and appeared similar between the groups. At 7 dpi, control pigs had slightly lower PRRSV viremia compared to MT pigs ($p = 0.44$, multiple unpaired t-test). The mean viremia at 7 dpi was 0.37 ± 0.87 in control pigs and 0.87 ± 1.81 in MT pigs.

Likewise, antibody production against PRRSV nucleocapsid (N) protein and PCV-2 was quantified bi-weekly (**Figure 4.3A**). Antibody levels were comparatively higher in control group at all timepoints, but not significantly higher. However, at 10 dpi, there was trend towards higher level of detectable antibody in control pigs compared to the MT pigs ($p = 0.059$, multiple unpaired t-test). At 10 dpi, mean sample to positive (S:P) ratio in control and FMT pigs was 1.55 ± 0.23 and 1.37± 0.39, respectively. The PCV-2 antibody levels remained similar between the control and MT throughout 14 days post challenge period ($p > 0.1$; multiple unpaired t-test, data not shown).

Gross and microscopic lung pathology appeared slightly severe, but not significant, in MT pigs compared to control pigs. However, there was trend towards higher percent of lung affected in MT pigs in comparison to control pigs ($p = 0.083$, unpaired t-test; **Figure 4.3B**). Control and MT pigs had a mean of 55.5± 18.8% and 69.0 ± 13.7% lung affected, respectively. Lung weight to body weight ratio was approximately same for both groups at 0.021 ($p > 0.1$, unpaired t-test; data not shown). On comparing the degree of interstitial pneumoniae between the groups, 1/10 control and 2/10 MT pigs were found to have severe interstitial pneumonia with over 75% lung lobe involvement (data not shown). MT pigs had slightly higher, but not significant, severity of microscopic lung lesions ($p = 0.36$, unpaired t-test). Likewise,

microscopic tracheitis and tracheobronchial lymph node depletion scores were also slightly higher in MT pigs compared to control pigs, but not significantly different ($p = 0.48$ and $p = 0.28$, respectively, unpaired t-test; data not shown).

Microbiota therapeutic transplanted pigs had improved ADG compared to control pigs prior to and during the PRRSV PCV-2 co-infection period. Weights were measured throughout the study and used to determine the average daily gain (ADG) in both groups. ADG was calculated to demonstrate the change in weight over 8 days pre-challenge and 42 days post-challenge period and reported in kg. Weight steadily increased throughout the study in both groups. Pairwise comparisons were conducted between groups to compare the weights of pigs; however, the weights were found to be similar throughout the study period ($p > 0.05$, unpaired t. test; data not shown).

Since no significant findings were found in weight between groups, ADG was investigated for detailed insights regarding the efficiency of the MT on weight gain in pigs. Pairwise comparisons were conducted between groups to compare the mean ADG between the two groups (**Figure 4.4**). MT transplanted pigs had significantly higher ADG of 0.08 ± 0.07 Kg in contrast to control pigs with ADG of 0.05 ± 0.06 Kg during the therapeutic transplantation period, -8 to 0 dpi (*$p = 0.047$; unpaired t-test). Similarly, MT administered pigs had significantly higher ADG of 1.31 ± 0.21 Kg compared to control pigs, which had ADG of 0.81 ± 0.22 Kg towards the end of the study, between 38 and 42 dpi (*$p < 0.001$, unpaired t-test).

Microbiota therapeutic transplanted pigs had comparable number of PCVAD-affected pigs to that of control pigs after PRRSV-PCV-2 co-infection. The percentage morbidity and survival of the MT and control groups are shown in **Figure 4.5**. Morbidity rates of the control and MT groups were comparable throughout the majority of the study period after co-

infection (**Figure 4.5A**). In the first 21 days after co-infection, 12 control pigs and 10 FMT pigs showed clinical signs, including dyspnea, open-mouth breathing, coughing, tachypnea, mucoid rhinorrhea, conjunctivitis, reduced body condition, lethargy/weakness, and pyrexia. At 22 dpi, the morbidity rates of the control and MT groups diverged, with 12 control pigs (12/21; 57.1%) and 9 FMT pigs (9/24; 37.5%) exhibiting clinical signs sufficient to require veterinary intervention, including depression, dyspnea, tachypnea, coughing, open-mouth breathing, rough hair coat, mucoid oculo-nasal discharge, pyrexia, diarrhea, muscle wasting and loss of body condition, and cyanosis. This difference was larger, but not significant. Clinical disease in both groups did not start to resolve until 35 dpi. It was completely resolved in MT pigs at 38 dpi while 5.9% of control pigs (1/17) required treatment at 42 dpi. Overall, clinical disease in MT and control groups remained consistent with PCVAD.

With regards to mortality, initial death rates were same (3/30) between the two groups with 10% mortality at 20 dpi. However, by the end of the study, the mortality in the control group was 43.3% (13/30) compared to 36.7% (11/30) for the FMT pigs (**Figure 4.5B**). Mortality between 19 and 39 dpi was due to pigs that died or were euthanized due to severity of clinical disease. Overall, the survival rate of the control group (17/30, 56.67%) remained comparable with that of the FMT group (19/30, 63.33%; $p>0.1$, Fisher's exact test). When taken together, pigs that received the MT showed some reduction in PCVAD but the reduction was not significantly different.

Representative gross and microscopic lesions seen in the lungs, and microscopic lesions seen in the tracheobronchial lymph node and trachea in pigs with PCVAD are shown in **Figure 4.6**. Images of none to minimally-affected pigs are included for comparison. Observations of overall gross lesions included bronchopneumonia with consolidation and hemorrhage, interstitial

pneumonia, moderate to diffuse fibrous adhesions between the lungs and thoracic cavity and/or the pericardium, fibrous adhesions in the abdomen, lymphadenopathy with congestion and edema, pericardial effusion, peritoneal effusion, mucopurulent exudate in trachea with hemorrhage, enteritis, and intestinal ulceration, edematous colon and splenomegaly. Gross lung tissue images were captured during necropsy and subsequently scored for severity of lesions (**Figures 4.6A, and 4.6B**).

A lung (with trachea) weight to body weight ratio was calculated for each pig and is presented in **Figure 4.7A**. Control pigs and MT pigs had similar ratios ($p>0.1$, unpaired t-test). Since no significant difference was found in the lung weight to body weight ratio between the two groups when all pigs were included in the analysis, only ratios of pigs that survived the entire study were further explored (**Figure 4.7B**). This showed that MT pigs had significantly higher ratios when compared to control pigs ($p = 0.03$, unpaired t-test). Higher ratios indicate increased cellular infiltrate and edema which is characteristic of interstitial pneumonia. Gross lung lesions were comparable in both the groups ($p>0.1$, unpaired t-test; **Figure 4.7C**). Control pigs had a range of 15 to 100% of lung affected, with a mean of 59.2 ± 22.6%. FMT pigs also had a range of 15 to 100% of lung affected, with a mean of 63.0 ± 22.2%.

Histopathologic assessment of the lesions was also performed through the evaluation of lung, lymph node and trachea. Microscopic lesions in the lungs included lymphoplasmacytic and histiocytic interstitial pneumonia, suppurative interstitial and bronchopneumonia, edema, and hemorrhage. Lymphoid depletion with histiocytic replacement was seen in the tracheobronchial lymph nodes. Lymphoplasmacytic tracheitis with necrosis was observed in the trachea. The severity of interstitial pneumonia was scored in all 60 pigs, with 9/30 pigs in both control and MT group each having severe diffuse interstitial pneumonia with >75% lung lobe involvement

(**Figure 4.7D**). Although MT pigs had higher overall severity of microscopic lung lesions this difference was not statistically significant ($p = 0.38$, Mann-Whitney U test). The severity of tracheitis was also evaluated in all 60 pigs (**Figure 4.7E**). Interestingly, none of the control or MT had severe lymphocyte depletion in trachea. Conversely, control pigs had higher overall severity of tracheitis; however, this difference was not statistically significant ($p = 0.49$, Mann-Whitney U test). Similarly, the overall severity of lymphoid depletion was higher in control pigs with no significant difference in the depletion score between the two groups ($p = 0.77$, Mann-Whitney U test; **Figure 4.7F**).

Overall, MT had a neutral effect in providing protection to the pigs from respiratory and lymphoid diseases. This is due to the comparable number of PCVAD-affected pigs in both control group and MT group.

Microbiota therapeutic increased the rate of serum PCV-2 clearance and promoted antibody production. PRRSV and PCV-2 viremia curves are shown in Figure 8. In both groups, PRRSV viremia followed the typical course, peaking at 7 dpi followed by gradual decay over the next 5 weeks. Mean PRRSV viremia was observed similar between the control and MT groups throughout the study (**Figure 4.8A**). PCV-2 viremia followed the typical course as well. In both groups, PCV-2 replication peaked later in the co-infection period between 14 and 21 dpi, followed by a plateau over the next 3 weeks in the control group (**Figure 4.8B**). The PCV-2-viremia continued to decay after 21 dpi. Although not significantly different, the MT pigs showed a lower tendency of viremia level at 42 dpi ($p=0.12$, multiple unpaired t-test). Overall, MT increased the rate of PCV-2 clearance in the serum of transplanted pigs.

Additionally, antibodies were measured against PRRSV N protein and PCV-2 whole capsid protein (CP 43-233). Detectable PRRSV antibodies were comparable in both groups

(**Figure 4.9A**). PRRSV antibodies level peaked between 28 and 35 dpi. A greater level of PRRSV antibodies was detected in MT pigs on 7, 21, 28 and 42 dpi; however, the differences were not significantly different when compared to control pigs ($p > 0.1$ multiple unpaired t-test).

PCV-2 antibody levels peaked a weak later than PRRSV antibodies between 35 and 42 dpi. PCV-2 antibodies were detected at a greater level in MT pigs on 28, 35, and 42 dpi however, the differences were not significantly different when compared to control pigs ($p > 0.1$ multiple unpaired t-test; **Figure 4.9B**). Overall, it indicates that the MT promoted the production of higher and more sustained levels of antibodies directed at both PRRSV and PCV-2 towards the second half of the study.

Microbiota therapeutic shifted microbiome composition. Fecal microbiotas of the transplanted and control groups were analyzed after MT or mock-transplantation prior to PRRSV PCV-2 co infection by a pan-microbial array (LLMDA). Microbiome diversity was determined using the LLMDA data as the number of families and species detected in the feces of each pig across both groups. On -1 dpi, after 7 days transplantation period, the mean number of families detected was comparable in both groups; 30.5 ± 5.4 and 32.3 ± 4.7 in control and MT groups, respectively ($p = 0.25$; Mann-Whitney *U* test). Interestingly, a significant difference was detected in the mean number of species between the two groups ($p = 0.016$, Mann-Whitney *U* test). The mean number of species detected in control and MT groups were 84.1 ± 18.7 and 72.4 ± 20.5, respectively (**Figure 4.10**). This data suggests that MT may contribute to increased replication of diverse microbial species in the gut.

The majority of the microbial families detected by the LLMDA had similar prevalence rates in both control and MT groups. Three microbial families were detected at significantly different prevalence rates between the two groups when individual families were compared

(**Figure 4.11A**). Families including *Enterobacteriaceae* and *Piscirickettsiaceae* were detected in significantly higher proportions of MT pigs ($p = 0.003$ and $p = 0.03$, respectively, Fisher's exact test). The *Methanobacteriaceae* family was detected in a significantly lower proportion of MT pigs ($p = 0.019$, Fisher's exact test).

Species diversity within each family was analyzed for differences associated with the effects of MT on the gut microbiome of nursery pigs. Significant differences in species diversity were observed within the four families (**Figure 4.11B**). Greater species diversity was observed within families *Methanobacteriaceae* and *Desulfovibrionaceae* in the control pigs ($p = 0.025$ and $p = 0.015$, respectively, Mann-Whitney U test). Within the *Enterobacteriaceae* family, there was a trend towards greater species diversity in MT pigs; 1.9 ± 3.6 species in the MT group and 0.2 ± 0.9 species in the control group ($p = 0.0017$; Mann-Whitney U test). The greatest number of species were detected in the *Reoviridae* family with a mean of 10.3 ± 18.3 and $24.4.7 \pm 26.2$ species detected in MT and control group of pigs, respectively and the difference was statistically different ($p = 0.036$; Mann-Whitney U test; **Figure 4.12B**).

Discussion

Despite a long history of FMT as a treatment for various GI diseases, it is more recently that FMT has been recognized as an alternative therapeutic for non-GI diseases including respiratory and neurologic diseases. Lately, FMT has also been investigated as a prophylaxis prior to the development of disease and shown improved disease outcome in pigs. However, it comes with substantial risks to the recipient including transplantation of detrimental pathogens and uncertain donor-recipient compatibility. More recently, bacterial therapeutics with precisely defined consortium of feces-derived viable microbes is gaining consideration as an alternative to FMT as it offers the advantage of being of known, consistent composition and testable safety

(Caballero et al., 2017; Atarashi et al., 2011). Additionally, it is believed to provide a better understanding of the roles of each microbial species and, thus improve the identification of microbiome-mediated clinical efficacy (Gerardin et al., 2021).

The current study describes efficacy of microbiome therapeutic when used as prophylactic tool to prevent disease outcomes in pigs co-infected with two important swine viruses. This study was conducted in accordance with current swine industry standards where pigs are typically handled at 3 weeks of age post-weaning and without broad-spectrum antibiotic use.

MT was characterized after formulating the therapeutic using bacterial culture to determine the stability of pure cultures of individual microbes together in a concoction. Majority of the microbes used to prepare the therapeutic were isolated; however, their abundance was reduced in the final formulation. Several factors including temperature, pH, and storage at freezing conditions could have affected their abundance. The therapeutic was prepared at room temperature in aerobic conditions which may have limited the viability of obligate anaerobes in the therapeutic. Additionally, most commensal microbial species evolve as members of a complex enteric ecosystem with metabolic interdependencies (Rakoff-Nahoum et al., 2016), indicating that the species that were successfully cultured may be incapable of co-existing in the absence of favorable enteric environment with optimal growth conditions. Interestingly, all microbial species used to formulate the therapeutic had reduced abundance post formulation as shown by microbial enumerations and these changes may be due to reduction in pH caused by lactic acid-producing bacteria like *Lactobacillus* and *Streptococcus.* The reduction in pH has the potential to antagonize many microbial species. Moreover, lactic acid produced by LAB possesses potent bactericidal activity (Chen et al., 2010). Freezing of pure cultures of isolates

prior to formulating the therapeutic could also have reduced the abundance of individual microbes in the therapeutic.

Our findings did not demonstrate adverse effects of MT on pigs prior to co-infection in consistence to lack of deleterious impact of the FMT as shown by our previous study (Niederwerder et al., 2018a). A biphasic clinical disease is observed in pigs co-infected with PRRSV and PCV-2 as previously described by Megan C Niederwerder et al. (2015). Typically, a clinical disease associated with PRRS is seen in the first 21 dpi followed by a clinical disease associated with PCVAD after 21 dpi. PRRSV viremia peaks during the first 21 dpi and PCV-2 replication peaks and plateaus after 21 dpi. In consistent to previous study (Niederwerder et al., 2018a), MT did not have significant impact on the clinical disease associated with PRRS, which is typically seen in the first half of the coinfection period. In spite of that, further research is necessary to understand the impact of MT on PRRSV-only infection model prior to coming into any conclusions.

Overall, MT failed to largely reduce clinical disease due to co-infection in pigs. Since, majority of the microbiota colonizing the GI tract are obligate anaerobes, they are difficult to culture in laboratory settings, suggesting that the bacterial species that contribute to beneficial disease outcomes may be absent in the MT. Additionally, MT was formulated by mixing cultivable fecal microbes in normal saline with 10% glycerol instead of their natural ecosystem with essential metabolites or symbiotic commensals which may have potentially inhibited viability of microbes post-transplantation. Despite its current limitations, a defined microbial consortia-based therapeutic strategy is highly promising. Further investigation is required to design techniques to preserve the defined composition of the bacterial therapeutic to identify

both microbiome-mediated clinical efficacy and possible mechanism of action of individual microbes.

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Table 4.1 Formulation of microbiota therapeutic

Isolate	Abundance in the FMT (CFU/ mL)	Abundance required to administer 5 mL/day for 7 days to 50 pigs (CFU/mL)	Volume of pure culture (mL)	Abundance in pure culture (CFU/mL)	Volume of pure culture used to equal each isolate's abundance in the FMT	
					mL	µL
Bacillus sp.	2.00E+02	3.50E+05	9	1.55E+08		2.3
Bacillus sp.	1.00E+02	1.75E+05	9	4.00E+07		4.4
Escherichia coli	1.00E+02	1.75E+05	9	1.35E+09		0.1
Bacillus pumilus	1.00E+02	1.75E+05	9	1.52E+08		1.2
Escherichia coli	1.00E+03	1.75E+06	9	2.47E+09		0.7
Escherichia coli	1.20E+04	2.10E+07	9	2.18E+09		9.6
Escherichia coli	1.70E+04	2.98E+07	9	7.60E+08		39.1
Bacillus megaterium	1.00E+03	1.75E+06	9	6.60E+07		26.5
Staphylococcus simulans	1.00E+03	1.75E+06	9	1.71E+09		1.0
Escherichia coli	5.90E+04	1.03E+08	9	1.50E+09		68.8
Gram negative unable to identify	2.00E+03	3.50E+06	9	1.05E+09		3.3
Enterococcus mundtii	5.00E+03	8.75E+06	9	7.30E+08		12.0
Entereococcus hirae	1.20E+04	2.10E+07	9	5.10E+08		41.2
Escherichia coli	3.70E+04	6.48E+07	9	8.70E+08		74.4
Streptococccus alactolyticus	7.30E+05	1.28E+09	20	1.49E+08	8.6	8573.8
Enterococcus faecium	1.00E+04	1.75E+07	9	6.90E+08		25.4
Lactobacillus mucosae	1.30E+05	2.28E+08	20	2.52E+07	9.0	9027.8
Actinomyces hyovaginalis	5.00E+04	8.75E+07	9	1.31E+08		667.9
Escherichia coli	4.00E+04	7.00E+07	9	5.50E+08		127.3
Clostridium perfringens	2.90E+05	5.08E+08	18	4.95E+07	10.3	10252.5
Clostridium perfringens	6.00E+04	1.05E+08	9	9.20E+07	1.1	1141.3
Clostridium perfringens	8.00E+05	1.40E+09	18	1.03E+08	13.6	13592.2
Clostridium perfringens	1.00E+05	1.75E+08	9	1.88E+08		930.9
Bifidobacterium boum	8.50E+06	1.49E+10	312	1.73E+09	8.6	8598.3

Table 4.2 Composition of microbiota therapeutic	
Bacteria	**CFU/mL**
Bacillus megaterium	6.00E+03
Escherichia coli	2.60E+05
Staphylococcus simulans	1.00E+04
Enterococcus hirae	>1000000
Enterococcus faecium	1.90E+05
Actinomyces hyovaginalis	>1000000
Enterococcus mundtii	3.00E+04
Streptococcus alactolyticus	>1000000
Escherichia coli	2.00E+05
Escherichia coli	1.00E+04
Escherichia coli	5.00E+04
Escherichia coli	>1000000
Clostridium perfringens	2.20E+06
Clostridium perfringens	3.10E+06
Clostridium perfringens	3.70E+06
Bifidobacterium thermophilum	>10000000

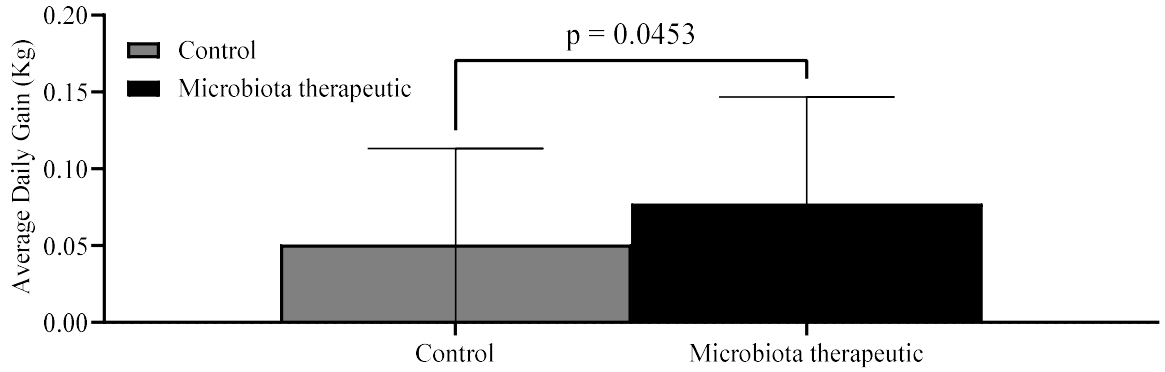


Figure 4.1 Average daily gain (ADG) between -8 and 0 days post-infection (dpi).

Bars represent the mean ADG. Data is shown as the mean ± standard deviation of control and microbiota therapeutic groups.

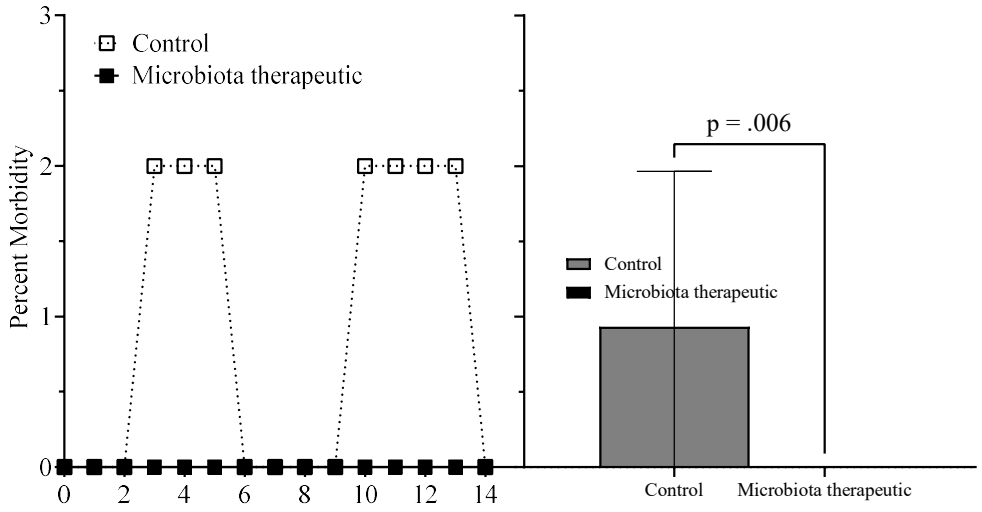


Figure 4.2 Morbidity of pigs sacrificed at 14 dpi with and without microbiome therapeutic transplantation after co-infection with PRRSV and PCV-2d.

Percent morbidity over time; data is shown as the percent of pigs in each group with antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs) prescribed by the veterinarian due to moderate to severe clinical disease.

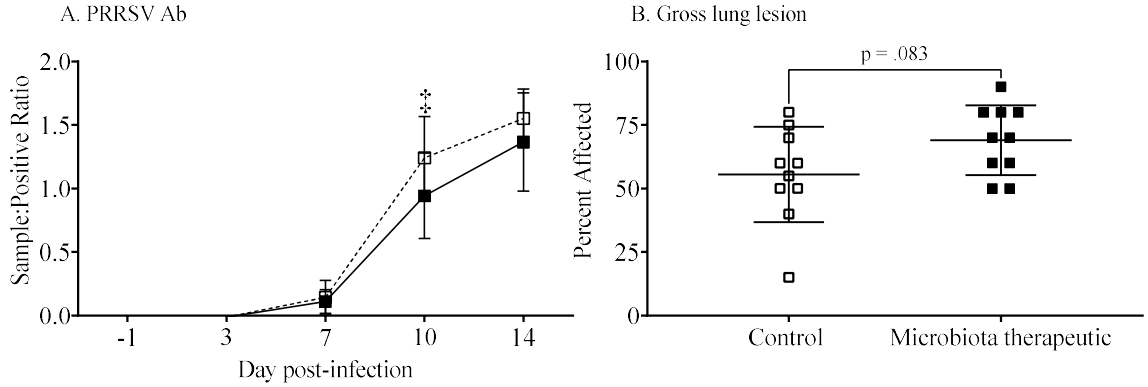
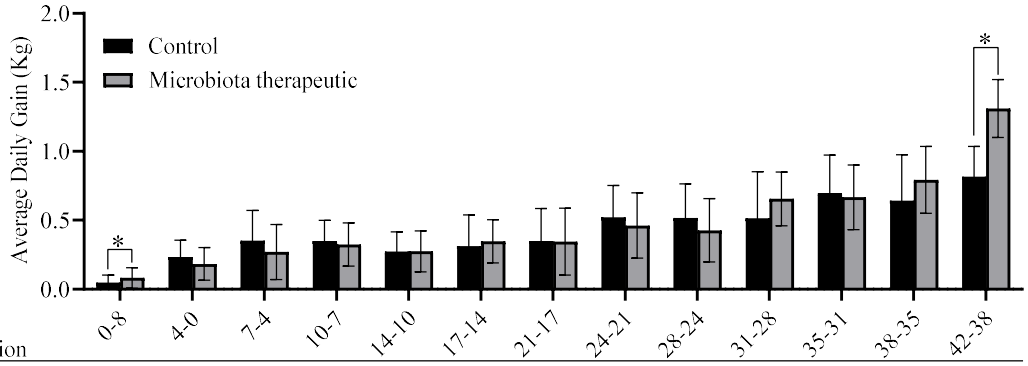


Figure 4.3 (A) Detection of antibody in microbiota therapeutic administered and control pigs. (B) Gross lung affected by pneumonia in pigs after PRRSV and PCV-2d co infection.

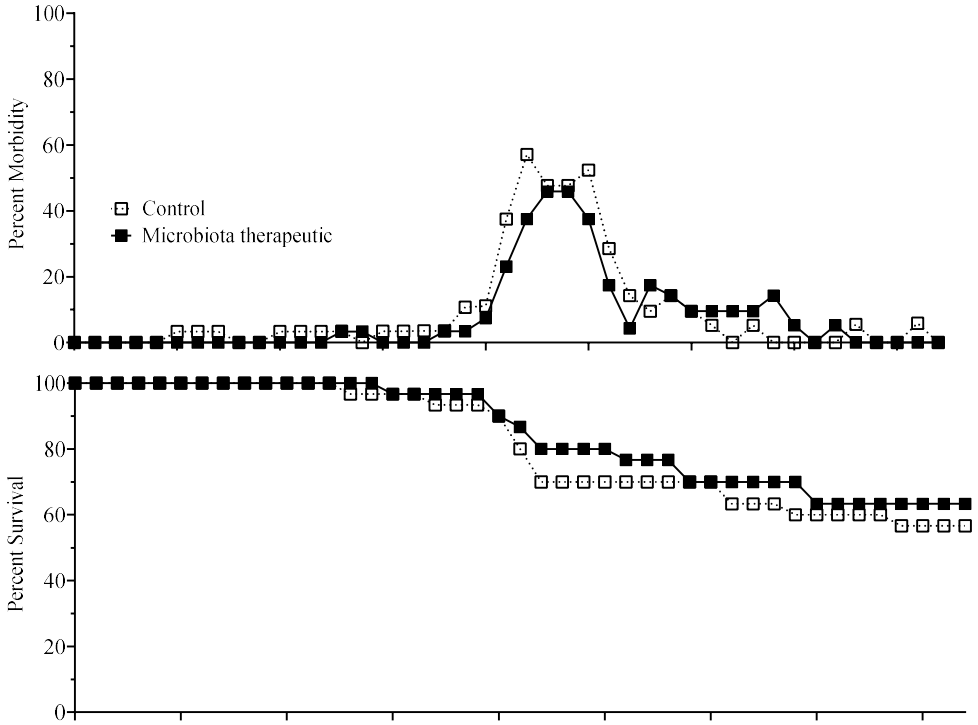
(A) Data is shown as the mean sample: positive ratio ± one standard deviation for PRRSV N protein. (B) The mean percent of lung affected was higher in microbiota therapeutic pigs (69.0 ± 13.7%) when compared to control pigs (55.5± 18.8%), but the differences were not statistically significant.



Day Post-Infection	0-8	4-0	7-4	10-7	14-10	17-14	21-17	24-21	28-24	31-28	35-31	38-35	42-38
No. of Control Pigs	30	30	30	30	29	29	26	21	21	21	18	18	17
No. of Therapeutic Pigs	30	30	30	30	30	29	27	24	23	21	21	19	19

Figure 4.4 Distribution of average daily gain (ADG) between -8 and 42 days post-infection (dpi).

Bars represent the mean ADG between two successive timepoints in the study. Data is shown as the mean ± standard deviation of control and microbiota therapeutic groups. Asterisks demarcate statistically significant differences (*$p<0.05$; unpaired *t*-test). The surviving pigs in each group are shown at the bottom of the figure over time.



Day Post-Infection	0	5	10	15	20	25	30	35	40
No. of Control Pigs	30	30	30	29	27	21	21	18	17
No. of Therapeutic Pigs	30	30	30	29	27	24	21	19	19

Figure 4.5 Morbidity and survival of pigs with and without microbiota therapeutic 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 antibiotics and non-steroidal anti-inflammatory drugs (NSAIDs) prescribed by the veterinarian due to moderate to severe clinical disease. B) Percent survival over time; data is shown as the percent of pigs in each group surviving severe clinical disease due to co-infection. The surviving pigs in each group are shown at the bottom of the figure over time.

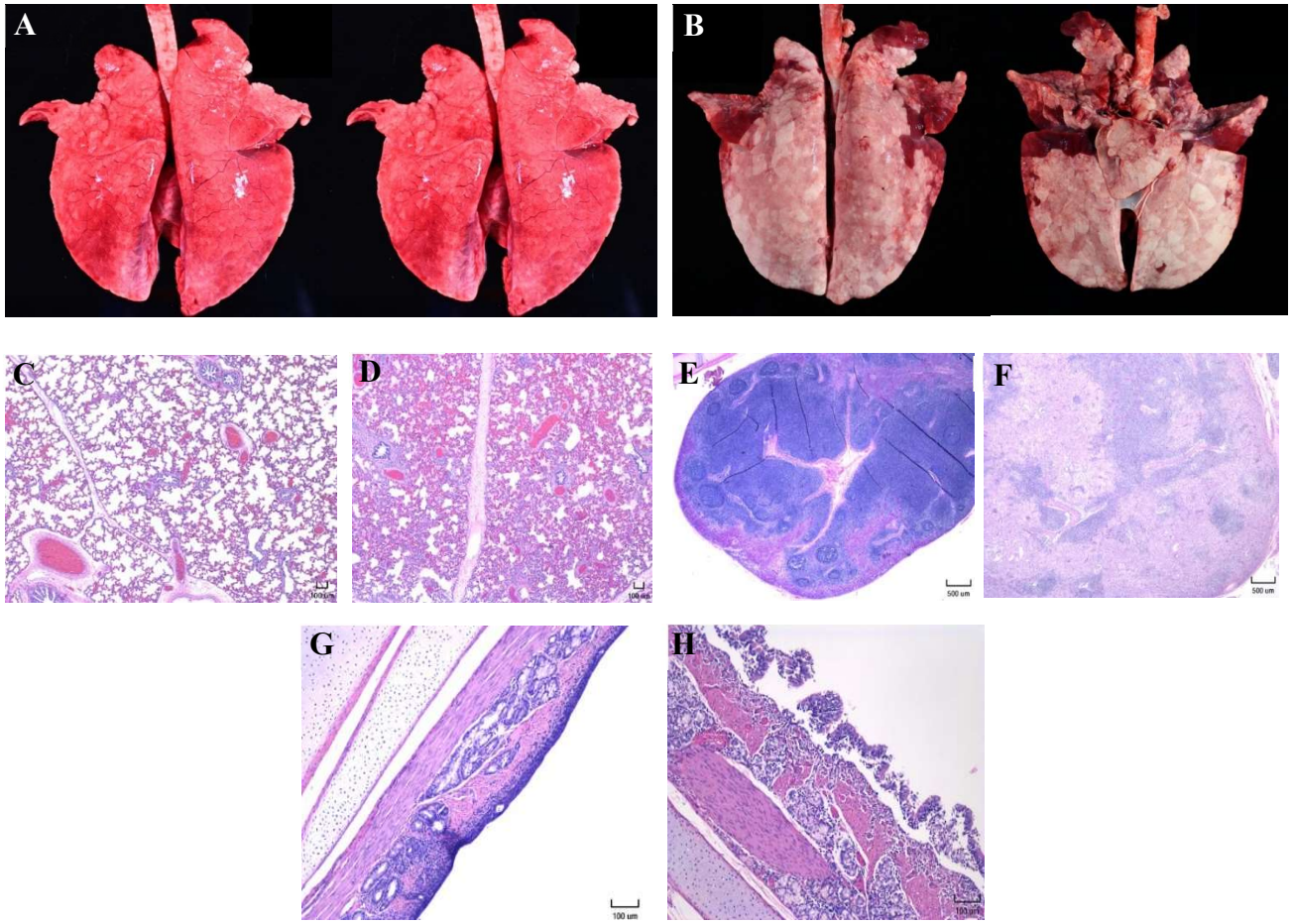


Figure 4.6 Representative gross and microscopic lesions associated with porcine circovirus associated disease (PCVAD).

Images shown are from representative PCVAD-affected pigs with severe clinical disease between 17 and 39 dpi or minimally-affected pigs for the purpose of comparison. **A)** Dorsal and ventral gross lung showing no lung lesions; normal lungs. **B)** Dorsal and ventral gross lung showing severe pneumonia with edema and congestion affecting approximately 100% of the lung. **C)** H&E-stained lung showing normal lung with no interstitial pneumonia. **D)** H&E-stained lung showing severe interstitial pneumonia with some neutrophil infiltration in alveoli affecting approximately 90% of lung. **E)** Immunohistochemical staining of a tracheobronchial lymph

node showing lymphoid follicles with no lymphoid depletion and no PCV-2 antigen staining. **F)** Immunohistochemical staining of a tracheobronchial lymph node showing severe lymphoid depletion associated with large amounts of PCV-2 antigen. **G)** H&E-stained trachea showing mild lymphoplasmacytic tracheitis with multifocal lymphocyte depletion. **H)** H&E-stained trachea showing severe lymphoplasmacytic tracheitis with necrosis.

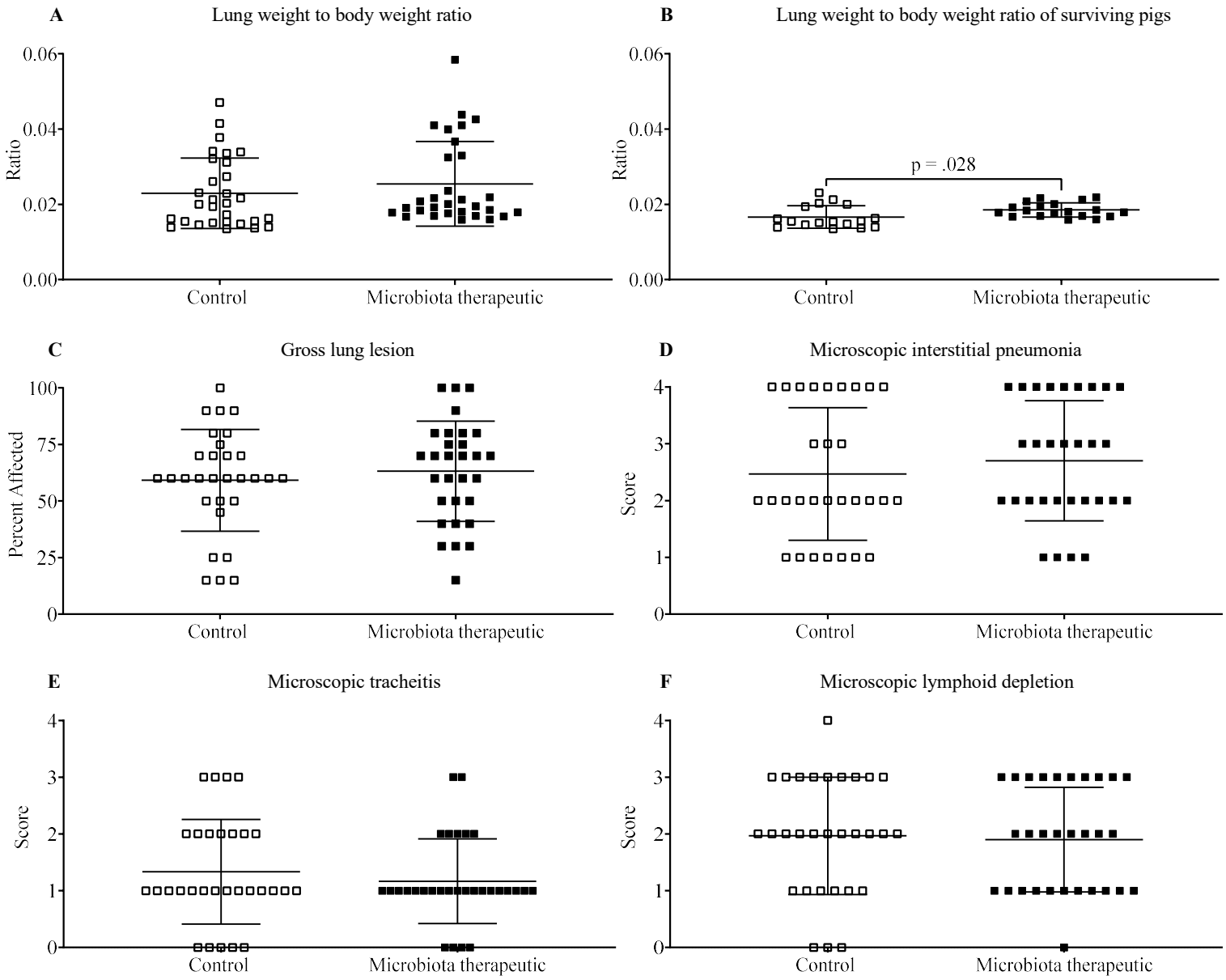

A
Lung weight to body weight ratio
Ratio
0.06
0.04
0.02
0.00
Control
Microbiota therapeutic
B
Lung weight to body weight ratio of surviving pigs
Ratio
p = .028
0.06
0.04
0.02
0.00
Control
Microbiota therapeutic
C
Gross lung lesion
Percent Affected
100
75
50
25
0
Control
Microbiota therapeutic
D
Microscopic interstitial pneumonia
Score
4
3
2
1
0
Control
Microbiota therapeutic
E
Microscopic tracheitis
Score
4
3
2
1
0
Control
Microbiota therapeutic
F
Microscopic lymphoid depletion
Score
4
3
2
1
0
Control
Microbiota therapeutic

Figure 4.7 Degree of the lung, trachea, and lymphoid lesions in pigs after PRRSV and PCV-2d co infection.

Data is shown as individual scores at the time of death with horizontal lines representing the mean ± 1 standard deviation for each group. **A.** Lung weight to body weight ratio at the time of necropsy was higher in microbiota therapeutic pigs () when compared to the control pigs (), but the differences were not statistically significant. **B.** Lung weight (with trachea) to body weight ratio of pigs that survived the entire study at the time of necropsy. It shows the microbiota therapeutic pigs that survived 42 dpi had significantly higher ratios ($p = 0.028$, unpaired t-test). **C.** Gross lung affected by pneumonia. The mean percent of lung affected was higher in microbiota therapeutic pigs (63.2 ± 22.2) when compared to control pigs (59.2 ± 22.6), but the differences were not statistically significant. **D.** Microscopic lung lesion. The mean lung lesion scores were higher in the microbiota therapeutic group (2.7 ± 1.1) when compared to the control group (2.5 ± 1.2), but the differences were not statistically significant. **E.** Microscopic trachea lesion. The mean trachea lesion scores were higher in the control group (1.3 ± 0.9) when compared to the microbiota therapeutic group (1.2 ± 0.7), but the differences were not statistically significant. **F.** Microscopic lymphoid depletion. The mean lymphoid depletion scores were higher in the control group (2.0 ± 1.0) when compared to the microbiota therapeutic group (1.9 ± 0.9), but the differences were not statistically significant.

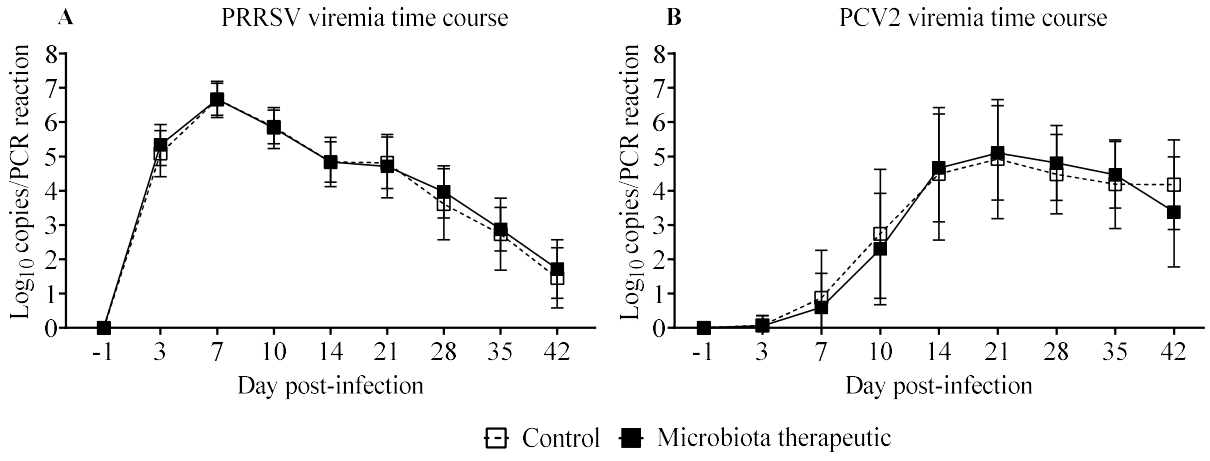


Figure 4.8 Time course of PRRSV and PCV-2d viremia.

(A) and (B) Data is shown as mean log_{10} serum viral copies/PCR reaction volume ± one standard deviation for each group.

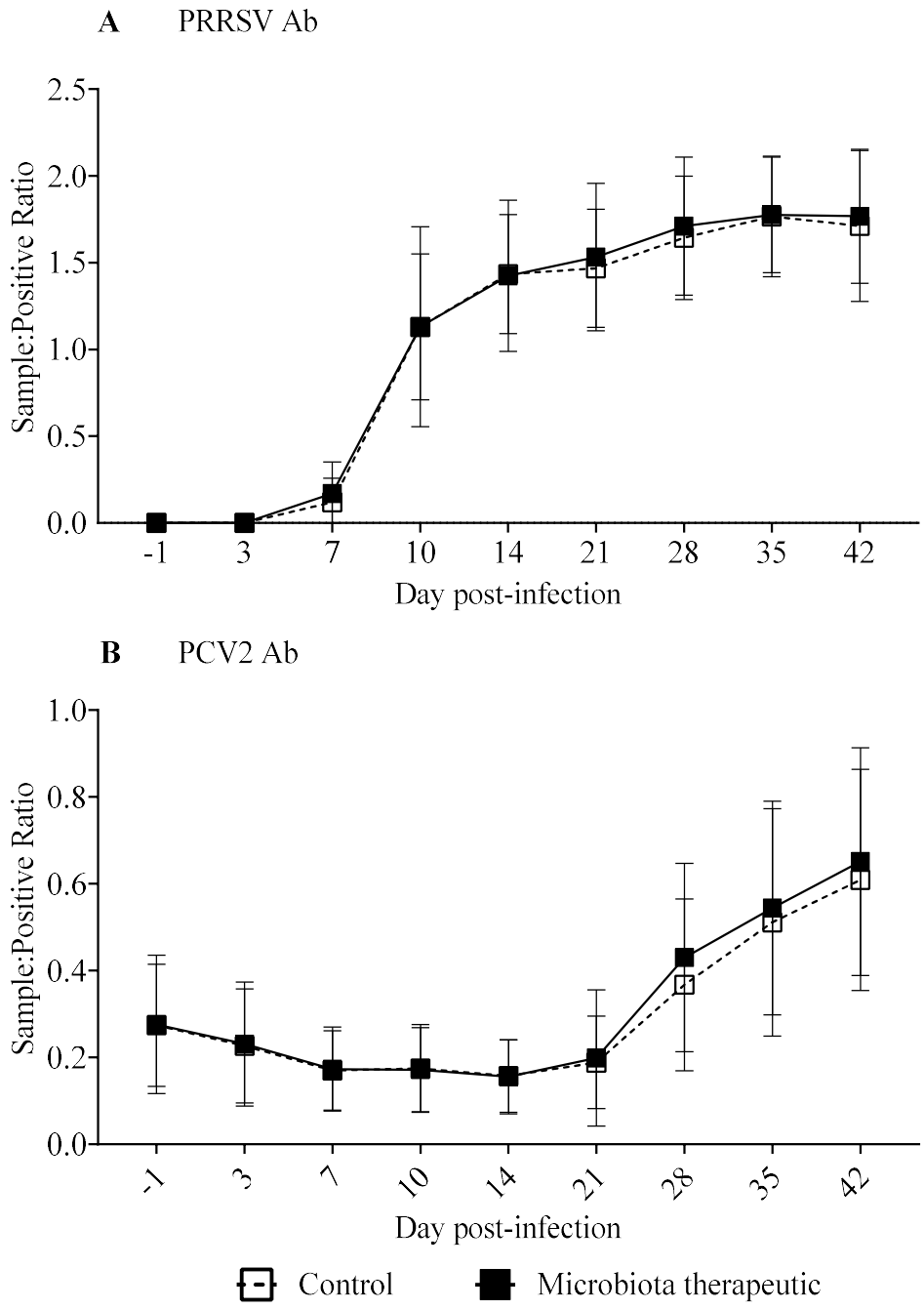


Figure 4.9 Detection of antibody in microbiota therapeutic administered and control pigs.

Data is shown as the mean sample: positive ratio ± one standard deviation for PRRSV N protein (A), PCV-2 whole capsid protein (B).

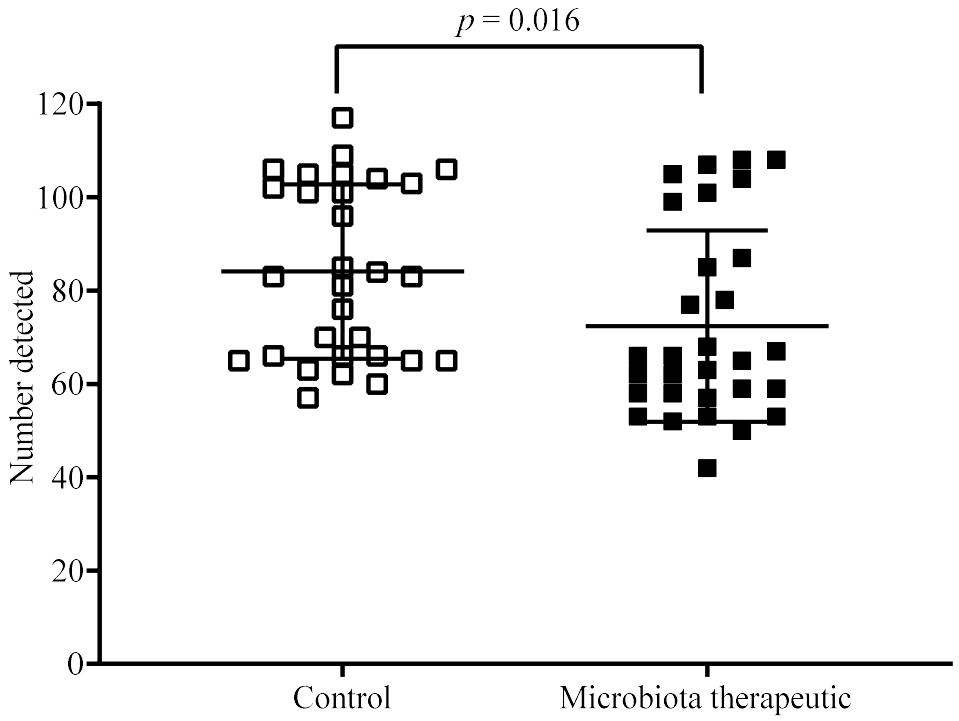


Figure 4.10 Fecal microbiome species diversity in microbiota therapeutic and control pigs prior to co-infection.

Data is shown as the total number of microbial species detected by the microarray after microbiota therapeutic or mock transplantation for individual pigs. Group mean ± one standard deviation is represented by solid horizontal lines. Significantly greater microbiome diversity was detected at species level in microbiota therapeutic pigs ($p = 0.016$, Mann-Whitney *U* test).

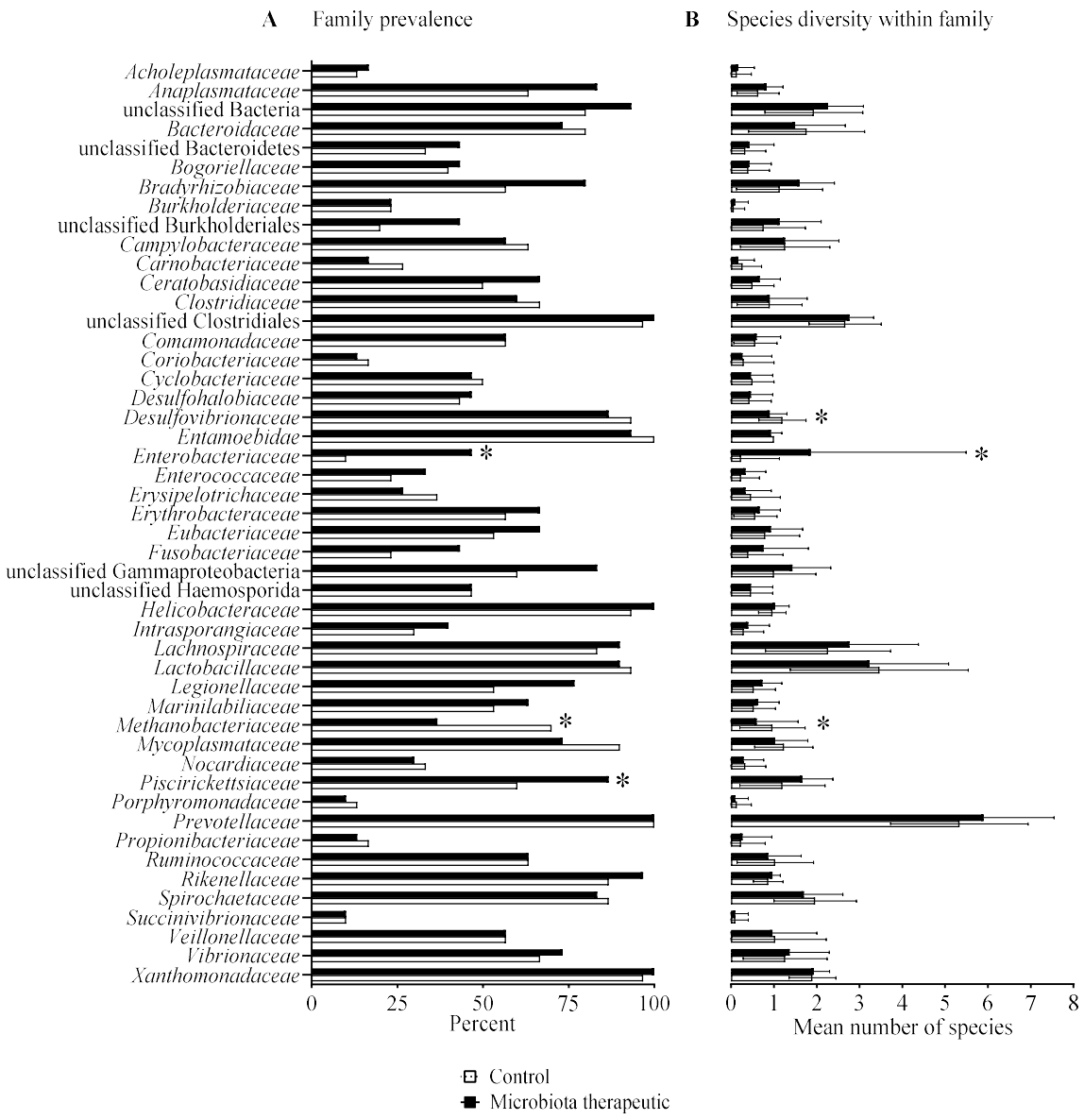


Figure 4.11 Fecal microbiome composition as detected by the pan-microbial array in microbiota therapeutic and control pigs after transplantation for 7 days.

(A) Microbiome family prevalence is shown as the percent of microbiota therapeutic pigs (n = 30) and control pigs (n = 30) with each family detected on the pan-microbial array. Families with a total prevalence of less than 10% between the microbiota therapeutic and control groups are not shown. There was a significantly higher prevalence of species within the families *Methanobacteriaceae* (p = 0.019, Fisher's exact test) in the control group and *Enterobacteriaceae* and *Piscirickettsiaceae* (p = 0.003, p = 0.039, respectively, Fisher's exact) in the microbiota therapeutic group. (B) Data is shown as the mean number of species detected ± one standard deviation in each family detected in the microbiota therapeutic and control pigs. Greater species diversity was noted within the families *Desulfovibrionaceae*, *Enterobacteriaceae* and *Methanobacteriaceae* (p = 0.015, p = 0.0017 and p = 0.025, respectively, Mann-Whitney U test).

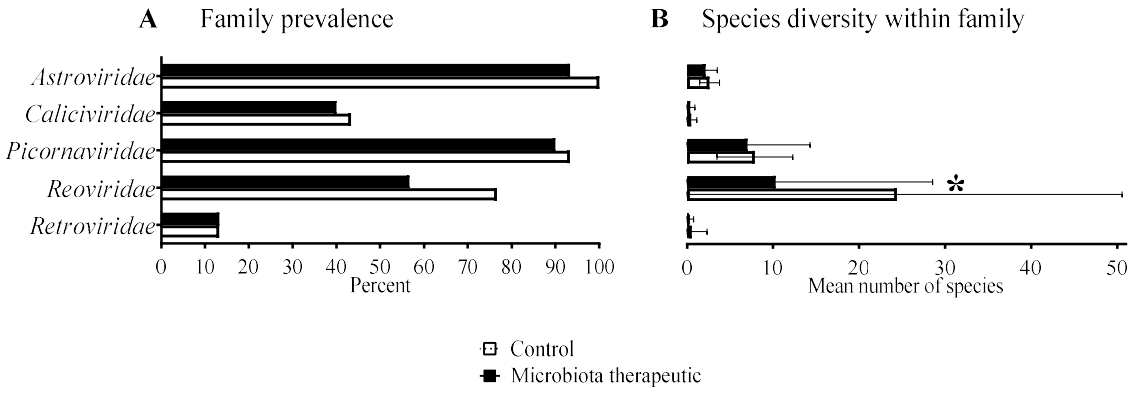


Figure 4.12 Composition of viral families in the fecal microbiome as detected by the pan-microbial array in microbiota therapeutic and control pigs after transplantation for 7 days.

Viral family prevalence and species diversity within each family was determined as explained above. Greater species diversity was noted within the *Reoviridae* family ($p = 0.036$, Mann-Whitney *U* test).

Chapter 5 – Conclusions and Future Directions

ASF remains a significant threat to global pork production, with high case fatality rates and no effective vaccine. Despite evidences of stability of ASFV in feed during transoceanic shipping conditions and environmental storage, diagnostic tools for surveillance and detection of ASFV in feed remain a challenge for the pig industry. We identified a new tool for the swine industry to detect and prevent the spread of ASFV through contaminated feed. Within the first study, we demonstrated the effectiveness of using feed dust swabs as a novel diagnostic surveillance tool for the detection and quantification of viral nucleic acid and infectious virus titer in ASFV-contaminated feed. To our knowledge, this is the first study providing proof of concept that feed dust from ASFV-contaminated feed can be used for ASFV detection. This tool could be used by the swine industry to screen feed ingredients for ASFV contamination, enabling early detection and prevention of the spread of the virus through contaminated feed which could ultimately help protect global pork production and the economy. However, our study also identifies potential limitations. Further investigations should focus on defining the sensitivity of feed dust as a diagnostic sample for ASFV-contaminated feed at lower inoculation doses, longer incubation periods, and varied environmental conditions.

The remaining three studies were dedicated to understanding potential role of gut microbiota to help control the onset and spread of PRRS. PRRS is the most common and costliest infectious disease in the US and globally. Although PRRS-MLV vaccines are widely utilized to reduce PRRS-associated losses, the currently available vaccines are considered inadequate for disease control. Alternative strategies to aid in the prevention and control of PRRS are actively investigated. Gut microbiotas are recognized for their beneficial role in infectious disease outcome, growth, and immunity. Additionally, gut microbiota-based

therapeutics like FMT are known to have therapeutic and prophylactic potential for diseases outside the gastrointestinal system. Hence, modulating them may serve as a promising alternative to antibiotics and growth promoters in food animal production while reducing the consequence of PRRS in the swine industry.

Within the second study, we demonstrated modulation of gut microbiome composition by the PRRS MLV vaccine in nursery pigs and that the gut microbiome characteristic associated with vaccination may improve the vaccine efficacy in pigs. To completely understand the effect of the vaccine on the gut microbiome, characterization of the gut microbiome at the beginning of the study is also valuable. Since all the pigs came from the same commercial source and were approximately at the same age, we assumed their microbiome composition is comparable. This could keep us from identifying absolute impact of vaccination on the gut microbiome composition. However, well-designed studies to characterize specific properties of individual families or species are needed further to make insightful predictions about their role in the development and regulation of host immune responses. This will help identify bacterial species that can provide better protection against different infectious diseases and can also help improve vaccine efficacy and duration of protection.

The third study was dedicated to characterizing FMT material shown beneficial to improve health outcomes in growing pigs with PCVAD. We demonstrated that FMT material can be characterized and pure cultures of cultivable fecal bacteria can be obtained using culture-based techniques. Additionally, the cultivable portion of the FMT material is dominated by anaerobes. This knowledge could help regulate the composition of FMT materials and understand the mechanisms behind the benefits of the transplant material. We used only six different types of media to isolate and propagate microbial species including blood agar, CNA

agar, MacConkey agar, and Brucella agar, MRS broth and BHI broth under aerobic and anerobic conditions so we may not have been able to fully characterize the FMT material. Future studies can include different culture media and cultivation methods shown to recover majority of the human fecal bacteria to identify bacterial species that could not be cultivated in the current study.

The fourth study was dedicated to developing and evaluating microbiota therapeutic as a potential alternative to FMT as measured by its effect on the clinical outcome of PRRSV-PCV-2 co-infection. Microbiota therapeutic included defined consortia of pure cultures of cultivable bacteria obtained in the third study also referred to as microbiota therapeutic. Since its composition is defined, microbiota therapeutic can be regulated and any detrimental pathogens can be eliminated that would otherwise be present in the FMT material. Within the fourth study we demonstrated microbiota therapeutic to be safe after administration with no adverse reactions reported. Additionally, the therapeutic increased average daily gain of pigs and increased PCV-2 clearance in the therapeutic administered pigs. In future studies, it would be relevant to transplant individual microbial species instead of a consortium to identify their roles in the improved clinical outcome of co-infected pigs.

Taken together, our findings are important to the swine industry because we identified a novel diagnostic tool for ASFV detection in feed ingredients arriving at different ports of entry throughout the US, the feed industries, commercial swine farms, and local producers. This will allow early detection and prevention of the spread of the virus through contaminated feed which could ultimately help protect global pork production and the economy. In addition, our next finding suggests that manipulating the gut microbiome could potentially improve the efficacy of PRRS MLV vaccines, which could lead to reduced economic losses. Furthermore, using gut

microbiome-based therapeutics as an alternative to antibiotics and growth promoters could be a more sustainable and effective approach to animal production.

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