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Assessing the Effects of Genetic Selection for Porcine Circovirus Type 2 Resistance or Feed Additives on Nursery and Grow-Finish Pig Performance

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ASSESSING THE EFFECTS OF GENETIC SELECTION FOR PORCINE CIRCOVIRUS TYPE 2 RESISTANCE OR FEED ADDITIVES ON NURSERY AND GROW-FINISH PIG PERFORMANCE

by

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The swine industry is ever evolving as it strives to produce healthy, wholesome pork for consumers. As such, the industry is constantly looking for ways to improve production and reduce costs. This includes using novel feed ingredients to reduce cost and improve animal health, genetic selection for increased performance, and vaccines to attenuate or prevent illness. Research plays a significant role in assessing the effectiveness of these strategies. Overall, the objective of this thesis was to assess how genetic selection, vaccines, and feed additives may impact growth performance, health, nutrient digestibility, and the microbiome.

In Chapter 2, pigs were either infected with or vaccinated for PCV2. Fecal samples taken from these pigs were used to assess changes in the microbiome. Overall, the microbiome did not differ at the phylum level, although some organisms were shown to be positively and negatively associated with growth and IgG production.

In Chapter 3, experiments were performed to assess the impact of genetic selection for PCV2 resistance on nursery and long-term performance of pigs. Pigs resistant to PCV2 infection had reduced viremia and IgG production. Despite no overall

effect on growth performance, the resistant genotypes were found to have lower production costs, especially when infected with PCV2.

In Chapter 4, pigs were supplemented with tryptophan to improve post-weaning response Tryptophan improved feed efficiency in experiment 1 and reduced production costs. Tryptophan did not provide the same benefit in experiment 2. Supplementation of tryptophan numerically increased *Lactobacillus* abundance in experiment 1, but not in experiment 2.

In Chapter 5, a series of cell culture experiments were performed to assess the effects of rhamnolipids on IPEC-J2 cells and jejunal explants. Rhamnolipid concentrations exceeding 0.01% were found to be cytotoxic. Lower concentrations were found to be less cytotoxic, but reduced transepithelial resistance in a dose dependent fashion. Secretion of interleukin-8, a marker of inflammation, was observed to be similar to control and LPS samples. Jejunum explants treated with 0.5% rhamnolipid had an IL-8 response higher than controls.

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CHAPTER 1: LITERATURE REVIEW

Introduction

With an ever-growing world population, the need for healthy and nutritious foods to feed this population continues to be an issue in 21st century. Producers continue to work towards improving efficiency and sustainability while maintaining a profitable enterprise. The current global population is estimated to be 7.6 billion people and growing at a rate of 1.09% per annum (Worldometers, 2018). Despite the great carrying capacity of the planet, effects of population growth (i.e., urban sprawl) has led to a loss of 31 million acres of agricultural land since 1981 (Sorensen et al., 2018). In addition, the effect of climate change on increasing severity of weather systems can potentially damage production of crops and animals and devastate infrastructure of affected areas. Thus, it is important for farmers and producers to continue pushing the efficiency of production to maximize land carrying capacity of agricultural products.

Swine Production

Pork production is the top meat commodity produced across the world, with beef and broiler (poultry) production being the other two. Global production of beef, broiler meat, and pork, and is expected to reach 63.0, 92.5, and 113.5 million tons, respectively, in 2018 (USDA-FAS, 2018). In regard to pork, according to USDA-FAS, production has been led by China, the European Union (EU), and the United States with 54.7, 23.3, and 12.2 million metric tons expected in 2018, respectively. Of the 8.3 million tons of pork

exported, the EU and US are expected to account for 35 and 32% of global exports, respectively, while China accounts for 20% of global imports. Hog production in the US is estimated to generate over $20 billion in gross income (NPPC, 2018). At present, December-February 2018, the U.S. pig inventory totals 72.9 million head. This includes a breeding inventory of 6.2 million head with an average litter rate of 10.58 piglets and a market hog inventory of 66.7 million head (USDA-NASS, 2018).

Swine production encompasses multiple parts. Breeding companies maintain nucleus herds for creating genetic lines to maximize desirable maternal traits (e.g., number of piglets born). Progeny from these groups are used to maintain nucleus herds while also being shipped to other sites to be used for multiplication. Gilts from these herds are than developed and bred using boar semen from terminal lines. These lines are selected for rapid growth and meat quality characteristics to produce market animals for slaughter. Thus, the focus on reproduction and propagation of favorable genetics (e.g., maternal traits, disease resistance, muscling) is important. The market side of swine production focuses on the growth and finishing of the terminal line pigs that will be sold to slaughter and ultimately generate profit for commercial swine producers.

Swine health is important in all facets of the industry. Stress and illness can increase producer's costs due to added treatments, decreased feed efficiency and added days on feed, or pig mortality. Over the years, strides have been made to decrease stress and improve herd health. Improvement in animal welfare training, monitoring, and regulation coupled with well-designed facilities, including sow housing and slaughter plants, has led to better animal handling practices, improved meat quality, and improved public perception of the swine industry (Grandin, 2017). Although it should be noted that

the latter continues to be challenge for the livestock industry as a whole. At the same time, herd health has improved as a result of using genetics, vaccines, and improved biosecurity. Additionally, improvement in the nutritional value of diets has facilitated the near maximal growth rates of pigs. Inclusion of feed additives in the diet has also aimed to improve performance and prevent health challenges. Despite these efforts, disease can still occur across all levels of production. In these cases, antibiotics may be given to help fight bacterial illness or prevent secondary infections that can occur.

Stress

Stress can be prominent factor in the health status of an animal. Causes of stress can be attributed to a number of sources including environment, social, handling, and transport to name a few (Campbell et al., 2013). A newly weaned piglet will typically experience several stressors in the 24-hour period post weaning. These include removal from the dam, handling, transport, thermal conditions in transit to nursery barn, and social hierarchy establishment in their new pens. Piglets need time to adjust to the new conditions and including finding feed troughs and waterers for consumption. Failure to adapt may result in pigs falling behind their cohorts leading to smaller body size, illness, or death. Stress can have a broad impact on host physiology regarding energy usage, hormone secretion, and tissue function. Intestinal function can be hampered by stress through decreases in gut barrier function, such as increased intestinal permeability, allowing bacteria and toxins to translocate across the barrier into other host tissue and blood (Moeser et al., 2017). Ultimately, this can lead to illness forcing an immune response, which will repartition nutrients and tissue proteins towards this response.

Disease

A large number of infectious diseases exist that can affect pigs. These diseases may originate from bacteria (*Streptococcus suis),* viruses (Porcine Epidemic Diarrhea Virus, PEDV; Porcine Respiratory and Reproductive Syndrome Virus, PRRSV), or other microbial origins. Disease may ultimately cost the industry millions of dollars in lost productivity (Schulz and Tonsor, 2015; Nathues et al., 2017). The severity of disease depends largely on the type of the disease, the severity of the strain, and the age of pigs when infected. Additionally ambient temperature may play a role as well with viruses generally being more prevalent in cooler months than hot ones (Pujols and Segalés, 2014). For example, PEDV, which caused diarrhea in pigs, was more likely to cause high mortality in prewean pigs, whereas older pigs display some illness but little to no mortality (Stevenson et al., 2013). On the other hand, PRRSV can negatively affect the feed efficiency, nutrient digestibility of grower pigs (Schweer et al., 2017a), but can reduce farrowing rate in sows (Rossow, 1998). Severity of disease is determined by the virulence of the strain as some strains may result in clinical signs and mortality whereas less virulent strains may only be subclinical, and animals appear to "walk through" the challenge (Brockmeier et al., 2017). Furthermore, diseases like PRRSV can alter the immune response, such as suppressing the interferon response (Patel et al., 2010), which is important in the antiviral response, subsequently leaving the animal open to secondary challenges.

Porcine Circovirus

Porcine circovirus (PCV) is a single stranded DNA virus of the family Circoviridae and is classified as type 1 or type 2, although a type 3 has recently been identified (Palinski et al., 2017). As reviewed by Segalés (2012), porcine circovirus type 2 (PCV2) is considered to be ubiquitous across the world, although the occurrence of clinical disease is lower, especially with PCV2 vaccines on the market. As a result, some herds may have subclinical PCV2 infections that reduce growth of animals without presenting clinical signs (Jacobsen et al., 2009). Porcine circovirus diseases may be labeled differently depending on the main clinical signs shown (Opriessnig et al., 2007). The most prominent, PCV2 systemic disease (PCV2-SD), has also been known as post weaning multisystemic wasting syndrome (PMWS), has been characterized by reduced weight gain or weight loss resulting in lowered performance in pigs (Rosell et al., 1999). It may be further coupled with respiratory and gastrointestinal clinical signs and lymphocyte depletion (Rosell et al., 1999). Additionally, respiratory or gastrointestinal tract issues may be also classified as PCV2 lung disease or PCV2 enteric disease respectively (Segalés, 2012). Like PCV2-SD, these types will also have a high titer of PCV2 and immune responses in the local tissues without microscopic lesions in the lymphoid tissues typically found in PCV2-SD (Segalés, 2012). Furthermore, PCV2 may also cause reproductive disease resulting in abortions or mummifications of the fetuses or alter return to estrus (Madson et al., 2009). In some cases, PCV2 in combination with other pathogens can cause porcine respiratory disease complex (PRDC), which often serves as a clinical sign of PCV2-SD (Hansen et al., 2010). In a review by Chae (2016), the summarized data showed that PCV2 viremia and lesions can be enhanced by an

earlier PRRSV infection or vaccination while the reverse, PCV2 infection or vaccination, has no effect on PRRSV. Similarly, *Mycoplasma hyopneumoniae* infections or vaccination can also increase PCV2 severity and lesions, but PCV2 infection or vaccination has no effect on *M. hyopneumoniae* illness. These responses are primarily due to PRRSV and *M. hyopneumoniae* infections stimulating proliferation of immune cells, a primary target of PCV2. Thus, in commercial herds where PCV2, PRRSV, and *M. hyopneumoniae* are prevalent, vaccination for PCV2 is a priority.

Clinical incidence of PCV2-related disease has largely been mitigated due to the development and use of PCV2 vaccines. Four vaccines are currently on the market for use in the United States and other areas in the world: Ingelvac CircoFLEX (Boehringer-Ingelheim), Circumvent PCV (Merck), Fostera (Zoetis), and Circovac (Merial). A recent meta-analysis by da Silva et al. (2014) found that all four vaccines are associated with increased growth in pigs from wean to finish. However, the estimated grow improvement varies with each vaccine.

Antimicrobials

Antimicrobials are a broad group of compounds that can have an effect on the microbes inhabiting the bodies of animals. Antibiotics are the classic and most commonly used antimicrobials due to their ability to kill or inhibit microbial growth (Gaskins et al., 2002). There are numerous classes of antibiotics such as penicillins, tetracyclines, and quinolones to name a few, but it is their mode of action, which is important. The majority of antibiotics are capable of disrupting one of three categories of microbial growth: nucleic acid synthesis, protein synthesis, or cell wall synthesis. Due to the commonality

of these processes, some antibiotics are considered broad spectrum for their ability to inhibit or kill a diverse group of organisms.

Antibiotics have been beneficial to the livestock industry as they were useful in controlling illness in herds and thus reducing morbidity and mortality at both low and high levels of disease incidence (Cromwell, 2002). An additional benefit was realized as antibiotics could help suppress subclinical illness when fed independently of specific disease resulting in increased growth performance (Cromwell, 2002). This led to continued use of antibiotics for growth promotion purposes. Usage of antibiotics has been beneficial for swine reproduction as it generally improved sow farrowing rate, piglets born alive, and improved piglet survivability to weaning (Cromwell, 2002). Overall, usage of antibiotics has been useful for herd health and saving the producer $2.99, 0.69, 0.30 per pig in the grow-finish, breeding and lactation periods respectively (Cromwell, 2002). Despite all the benefits, one consequence of prolonged usage of antibiotics, regardless of human or animal use, has been the development of antibiotic resistance strains of disease, such as drug resistant *E. coli* and *Salmonella,* and methicillin-resistant *Staphylococcus aureus* (MRSA), some of the more common resistant pathogenic bacteria found in humans (Marshall and Levy, 2011). Due to the increase in incidence of resistant bacteria, there has been concern about poorly regulated use of antibiotics in the livestock industry and limiting the use of "medically important" antibiotics needed for human health. This is especially important with last resort antibiotics such as vancomycin, because currently, there is a risk of vancomycin resistant *S. aureus* (McGuinness et al., 2017).

The Animal Drug Availability Act of 1996 (ADAA) was designed to increase the number of newly approved animal drugs with support from the FDA and animal industry groups (FDA, 1996). The ADAA allowed for the creation of a new drug category called "Veterinary Feed Directive (VFD) Drugs", which deals with the approval and use of new animal drugs in animal feed. However, a licensed veterinarian can only administer a VFD drug through prescription. Overall, the goal of the VFD is reduce off label use of drugs, to facilitate judicious use of medically important antimicrobials, and to allow better tracking of drug use. The VFD no longer allows the use of VFD drugs for growth promotion purposes. Usage of medicated feeds had been common practice to help ease the weaning transition in pigs both for disease and growth promotion purposes (Cromwell, 2002). This has further added pressure to find new or improve other antimicrobials to fill the gap left by the loss of antibiotics (Dębski, 2016).

Antibiotic Alternatives

Antibiotic alternatives are antimicrobials that are being used in lieu of antibiotics. Many alternatives have been researched and used prior to the VFD going into full effect. A list of common antibiotic alternatives can be found in Table 1.1. Alternatives vary in terms of direct or indirect action. Lysozyme, minerals, and essential oils typically have more direct action against bacteria (Cromwell, 2002; Oliver and Wells, 2015; Omonijo et al., 2018). Indirect action relies on the growth of beneficial bacteria that can out compete pathogenic bacteria either by supplying them directly (probiotics/DFMs) or feeding them via prebiotics, oligosaccharides, or fiber. Additionally, metabolites produced by the gut bacteria may stimulate antimicrobial peptide secretions from the host Paneth cells

(Ouellette, 2010). Pigs are believed to not have Paneth cells (Trautman and Fiebiger, 1952), although some histological (Myer, 1982) and transcription factor (Gonzalez et al., 2013) evidence of hallmark Paneth expression has been published.

Although numerous antibiotic alternatives are on the market and have been researched, the efficacy of these products has been highly variable in terms of response. This could be due to genetics, conditions, and dosage rate. A meta-analysis by Schweer et al. (2017b) looked at the impact of antibiotic alternatives on pig ADG relative to controls (Figure 1.1). Over a total of 2034 trials, antibiotic alternatives exhibited a positive impact on average daily gain, average daily feed intake, and feed efficiency occurred at a rate of 28.6, 14.3, and 17.3%, respectively. The majority of trials saw no change in growth and very few studies observed negative results (< 3.4%), although this could be a limited by the analysis method or presence of published studies.

Table 1.1. List of antibiotic alternatives and descriptions[1]	
Probiotics/Direct Fed Microbials (DFM)	Bacterial organisms (*Lactobacillus, Bacillus*) considered beneficial by competing against other bacteria or through secondary metabolite production (Allen et al., 2013)
Prebiotics/oligosaccharides	Carbohydrates (fructo-, galacto-, mannan-oligosaccharides, inulin) or other compounds used by beneficial gut microbes for growth or secondary metabolites (Allen et al., 2013)
Organic Acids	SCFAs decrease environmental pH, can reduce spoilage in feed, reduce pathogen survivability (Allen et al., 2013)
Botanicals	Essential oils derived from plants with antimicrobial, anti-inflammatory, anti-oxidant properties (Omonijo et al., 2018)
Yeast	Fed as live or various cell and/or extract preparations to promote prebiotic or probiotic benefits
Starch/Fiber	Resistant starch or fiber from foods that can be fermented into SCFAs and serve as prebiotics
Minerals Zinc/Copper	Fed at pharmacological doses that elicit antimicrobial effects (Cromwell, 2002)
Lysozyme	Naturally occurring enzyme that breaks down peptidoglycan layer of bacterial cell wall (Oliver and Wells, 2015)
[1]All items comprehensively reviewed by Liu et al. (2018)	

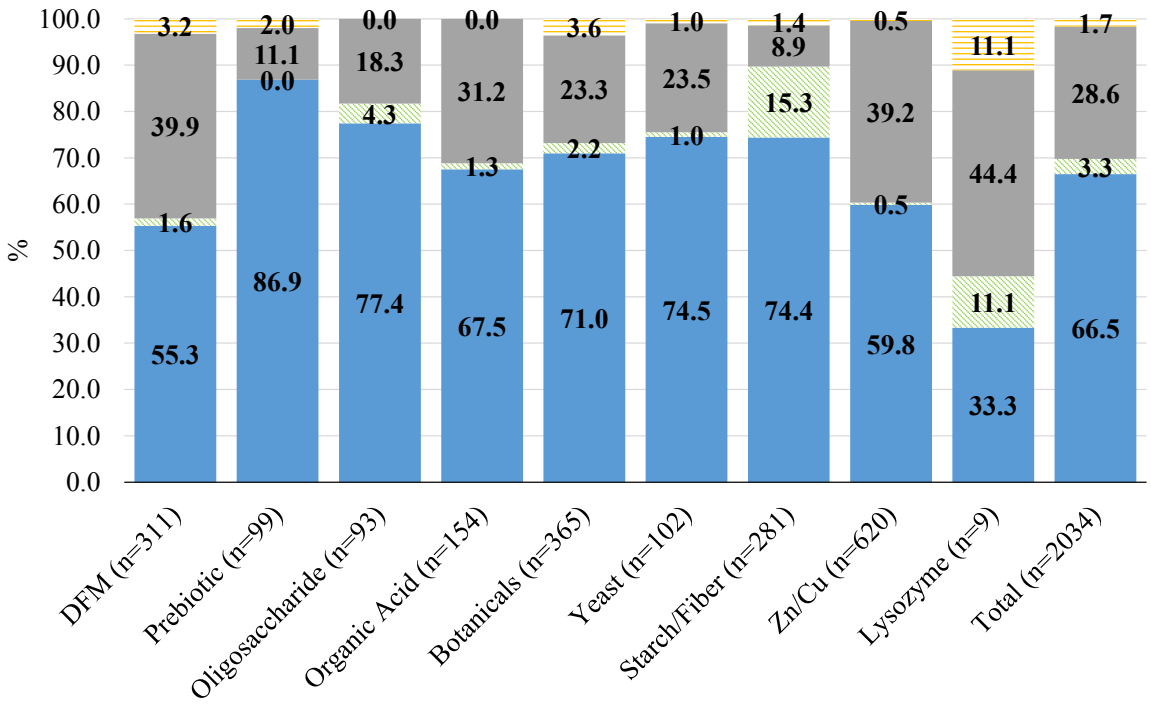

Figure 1.1. Percentage of outcomes for average daily gain when using antibiotic alternatives. No response (blue), negative response (green), positive response (grey), and no response reported (yellow) for outcomes. Adapted from Schweer et al., (2017b).

Rhamnolipids

Rhamnolipids are glycolipids that are anionic amphiphilic molecules containing hydrophilic mono- or di-rhamnose heads and lipophilic beta-hydroxyalkanoic acid tails with a typical length of eight to twelve carbons (Figure 1.2). This structure allows rhamnolipids to function as biosurfactants capable of reducing surface tension of liquids, thus making them good emulsifiers. Rhamnolipids are produced by numerous species of bacteria, but bacteria of the genus *Pseudomonas* tend to be prolific producers of rhamnolipids and *P. aeruginosa* is the predominate organism used due to its consistent and high production of rhamnolipid in fermentation settings (Chong and Li, 2017). Rhamnolipids serve a broad number of functions for their host organisms by enhancing motility, biofilm formation, and facilitate nutrient uptake of low solubility substrates

(Abdel-Mawgoud et al., 2010). Additionally, rhamnolipids appear to have antimicrobial effects against some Gram positive and negative bacteria, filamentous and phytopathogenic fungi, and limited activity against other microbial types (Abdel-Mawgoud et al., 2010). Furthermore, these molecules play a role in virulence of *P. aeruginosa* by inducing histamine release from mast cells, suppressing phagocytic action of macrophages, or increasing permeability by opening up the tight junctions between epithelial cells, to name a few (Abdel-Mawgoud et al., 2010). *Pseudomonas aeruginosa* is a common, problematic colonizer of cystic fibrosis patients as it exacerbates the patient's condition due to virulence factors (Abdel-Mawgoud et al., 2010). Rhamnolipids may also play a role in flagellin-mediated immune stimulation by inducing flagellin removal from the cell membrane (Gerstel et al., 2009). Rhamnolipids from a small number of species may have endotoxin like activity, but lacks the potency typically seen with lipopolysaccharide (Andrä et al., 2006) and comes at the expense of antimicrobial activity (Benincasa et al., 2004).

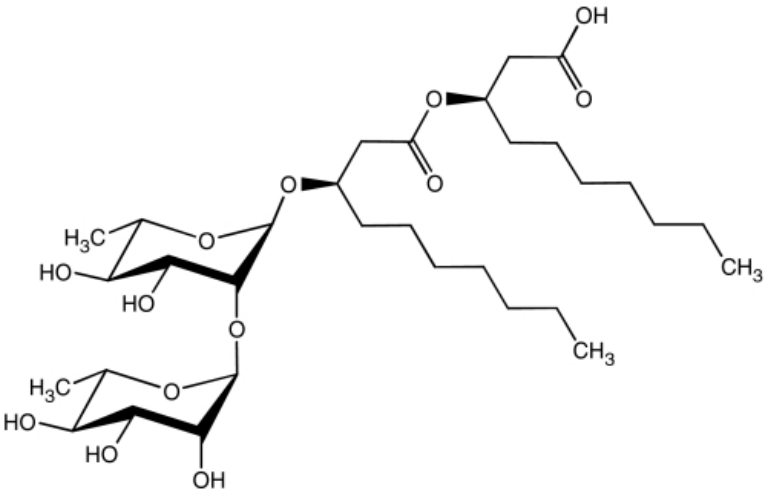

Figure 1.2. Structure of a di-rhamnolipid with rhamnose and lipid motieties on the left and right respectively.

A considerable amount of the literature regarding rhamnolipids is dedicated towards *P. aeruginosa* pathology and industrial applications (Chong and Li, 2017). The amphipathic nature of rhamnolipids makes them useful as emulsifiers and they have been used for bioremediation of sites contamination with petroleum, heavy metals or pesticides. Rhamnolipids have been considered for use as biopesticides on crops to reduce phytopathogens or in food processing to reduce growth of food-borne pathogens or biofilm production (Chong and Li, 2017). Additionally, these antimicrobial properties are beneficial for reducing medical device biofilms and inhibiting growth of cancer. As rhamnolipids increase epithelial cell permeability, they have been considered for excipient use in the pharmaceutical industry (Jiang et al., 2013; Jiang et al., 2014).

The ability of rhamnolipids to function as an emulsifier and antimicrobial makes it an interesting compound for an animal. The emulsification action may be beneficial to animals, especially those with reduced absorption capacity due to illness for increase nutrient uptake in the intestine. The antimicrobial nature could be useful for reducing pathogenic bacteria in the gut and for stimulating mucin production to enhance barrier function. Unfortunately to date, no research has been published on rhamnolipid use in animal models for its effects on growth performance and gut health. We aim to address some of this with research in Chapter 5 and in future research studies.

Tryptophan

Tryptophan (Trp) is an essential amino acid. Like other amino acids, tryptophan is necessary for incorporation into body tissue via protein synthesis. Tryptophan can play a role with cellular signaling and function by differentially regulating tight junction

proteins in intestinal cells (Wang et al., 2015; Tossou et al., 2016) and promoting activation of mTOR for protein synthesis, cell growth and decreased protein degradation (Wang et al., 2015). Tryptophan also serves as a precursor molecule for secondary substrates in the body (e.g., serotonin, kynurenine, and indole derivatives). Serotonin is a tryptophan metabolite that functions as a neurotransmitter in neurological tissues. Kynurenine serves as precursor to niacin, needed to synthesize NAD and NADP for cellular metabolism. Kynurenine also can interact with the aryl hydrocarbon receptor (AhR) (Opitz et al., 2011). Indole derivatives (e.g., indole-3-aldehyde, indole-3 acetic acid, and indole-3-acetylaldehyde) like kynurenine, are capable of interacting with AhR (Chung and Gadupudi, 2011). The AhR serves as a transcription factor for numerous genes encoding drug-metabolizing enzymes including cytochrome P450 (Beischlag et al., 2008). In the gut, AhR ligands activate IL-22 production by group 3 innate lymphoid cells (ILC3), which play a role in microbiota tolerance (Lee et al., 2012), that stimulates tissue protection, survival, and differentiation in epithelial cells (Eyerich et al., 2017). Phosphorylation of STAT3 through IL-22 activity is necessary for inducing antimicrobial factors offering protection against enteric viral challenges in vitro in IPEC-J2 cells (Xue et al., 2017).

Tryptophan is considered one of the first four limiting amino acids in the diets of pigs, the others being lysine, threonine, and methionine. To ensure adequate concentrations of tryptophan in the diet, tryptophan may be supplemented in crystalline form to meet dietary needs. The essential amino acids are fed relative to lysine (Lys) content in the diet on standardized ileal digestibility (SID) basis. In the swine NRC (2012), the recommended SID Trp:Lys ratio was 0.163 or 16.3%. The requirement was

estimated to be higher than 19.5% by Guzik et al. (2005) and a meta-analysis by Simongiovanni et al. (2012) found increasing the requirement from 17% to 22% improved ADG by 8%. Goncalves et al. (2015) estimated the SID Trp:Lys requirement at 20.4% and 23.9% for G:F and ADG, respectively, in 6 to 11 kg BW pigs, and 16.6% and 21.2% for G:F and ADG, respectively, in 11 to 20 kg BW pigs. Different types of stressors can adversely affect animal health. Health challenges certainly alter pig homeostasis due to activation of the immune system and repartitioning nutrients (Le Floc'h et al., 2009) away from tissue deposition to production of acute phase proteins, immune cell proliferation, and antibody production (Reeds et al., 1994). Tryptophan has been shown to be the first limiting amino acid during an acute phase response indicating an increase need for and possibly incorporation of tryptophan into acute phase proteins (Preston et al., 1998). When tryptophan is limited in the diet, stimulation of the immune system reduces N retention and protein deposition efficiency of tryptophan, suggesting a 7% increase in requirement (de Ridder et al., 2012). An increase in dietary tryptophan was shown to improve feed efficiency in pigs infected with enterotoxigenic *Escherichia coli* (ETEC) (Capozzalo et al., 2012; Capozzalo et al., 2015). Pigs susceptible to intestinal adhesion by ETEC saw a decrease in microbial richness, which was attenuated with supplemental tryptophan (Messori et al., 2013). Supplementing both threonine and tryptophan mitigated lung damage from a PRRS modified live vaccine and improved the immune response in pigs (Xu et al., 2014). Supplementation of threonine, tryptophan, and methionine not only improved growth performance in poor sanitary conditions, but also decreased incidences of damaging behaviors (e.g., tail and ear biting) (van der Meer et al., 2016; 2017). A mouse study by Zelante et al. (2013) found supplemental tryptophan

to be beneficial in reducing *Candida* infections in the stomach, however, this was mediated by indole-derivative activation of AhR via *Lactobacillus* metabolism of tryptophan. Altogether, tryptophan plays an important role in host tissue deposition and immune function, but understanding where the optimal SID Trp:Lys ratio is will be important for cost effective pig production.

Gut Microbiome

The microbiome is the collection of microbes that colonize the surfaces (e.g., skin, gastrointestinal, urogenital) of the body. The composition of the microbiome differs from site to site as the environment and access to nutrients change (The Human Microbiome Project et al., 2012) (Figure 1.3). This section will focus on the gastrointestinal or gut microbiome, specifically the pig, where possible. The population of bacterial cells in and on the human body is nearly the same number as human cells (Sender et al., 2016) with a substantial amount of these being found in the cecum and colon across animal species (Whitman et al., 1998). The commensal microbiota within the gastrointestinal tract play an important role in the health and homeostasis of animals (Nicholson et al., 2012). The microbiome in the gut trains the immune system to tolerate commensal organisms, aids in the defense of pathogens, utilizes nutrients that our bodies cannot directly breakdown (e.g., fiber), and provides volatile fatty acids (VFAs) to serve as an energy source (Nicholson et al., 2012; Arnolds and Lozupone, 2016). The microbiome contains a highly diverse population that may vary from individual to individual, but provides functional redundancy through similar gene profiles (The Human Microbiome Project et al., 2012). Individuals may have niche microbes and functions that

allow for special and unique microbial arrangements that can be masked due to broad classifications of microbes and genes (Lozupone et al., 2012). Our understanding of the composition and role that the microbiome plays has increased in the last few decades with advances in genome sequencing and the "-omics" (e.g., metabolomics, proteomics, and transcriptomics). With this knowledge has come the understanding that the relationship between the host and microbiome is a complex one as composition may not only affect nutrient breakdown and gut health, but neuroendocrine signaling from the gut to the brain of the host (Lyte, 2013).

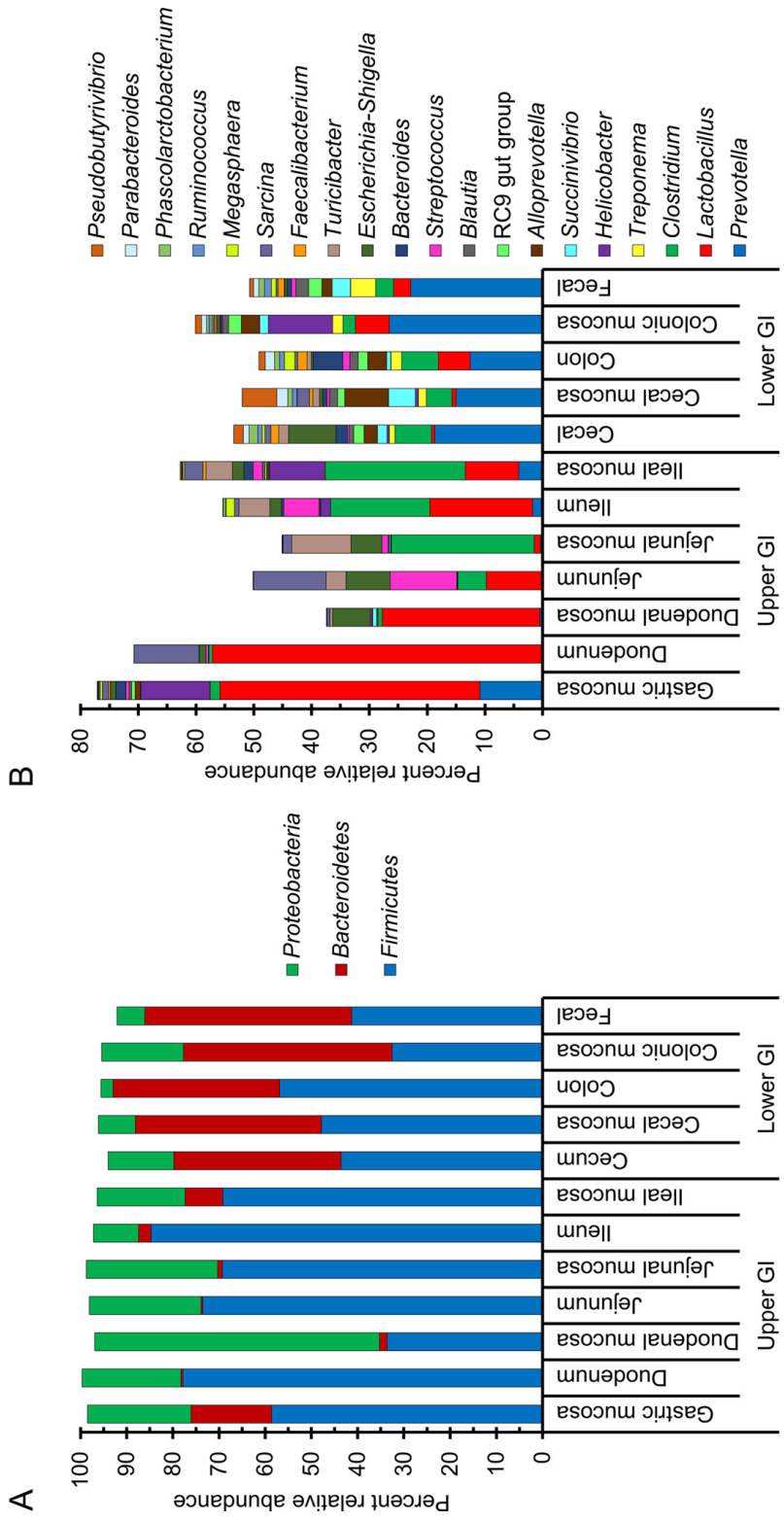

Figure 1.3. Percent relative abundances of the three most abundant phyla (A) and 20 most abundant genera (B) by gastrointestinal tract sample type from pigs. (Holman et al., 2017)

The microbiome encompasses nine phyla: *Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, Fusobacteria, Tenericutes, Spirochaetes, Cyanobacteria*, and *Verrucomicrobia*. *Firmicutes* and *Bacteroidetes* are typically the most abundant (> 85%) phyla in pigs (Holman et al., 2017), although *Proteobacteria* and *Actinobacteria* may be more abundant depending on breed, diet, and location. Microbial phyla and genera may differ in composition throughout the gastrointestinal tract (Holman et al., 2017), but expression of metabolic pathways between gastrointestinal locations and species is largely consistent (The Human Microbiome Project et al., 2012; Mao et al., 2015).

For most animal species, the microbiome is established with parturition as the progeny receive their first inoculum via exposure to the mother's vaginal microbiome. In the case of progeny born via Cesarean section, first exposure is likely to come from the skin microbiome (Dominguez-Bello et al., 2010). As a result, the initial gut microbiome will be a reflection of mother's, just dependent on site of birth. Interestingly, this difference may have long-term impact on the progeny's health and risk for disease (Johnson and Versalovic, 2012) although diet, antibiotic exposure, and illness may also play an important role. Subsequently, lactation will serve as another point of exposure for inoculation and provide substrate for growth of the animal and influence the evolution of the piglet's microbiome. Pannaraj et al. (2017) found that children predominantly breastfed through 30 days post birth received a greater proportion of their microbiome from the breast milk and skin. Likewise, exposure to the environment and other animals may also play an influential role in the development of the microbiome. Age plays a

partial role in the changes that may occur, but more so than that, diet will be the largest driver of microbial change and diversity (Frese et al., 2015). For mammalian livestock, this is the transition from mother's milk to solid feed and forage that will provoke the largest and most sustained changes. For the microbiome, bacteria will see a shift from milk proteins and sugars towards plant proteins, sugars, and fiber. Bacteria members such as *Prevotella* typically increase post weaning (Pajarillo et al., 2014) due to their ability to degrade xylans (Lamendalla et al., 2011). As the animal grows, fiber concentration in the diet typically increases as adult pigs have greater capacity for degrading fiber (Varel, 1987) although increasing the fiber content of the diet may increase cellulolytic bacteria numbers also (Varel and Pond, 1985). Variety in the diet can play a large role in shaping the microbiome as a more diverse diet can result in a more diverse microbiome and one that may handle perturbations more readily (Heiman and Greenway, 2016). Ultimately, a diverse microbiome may result in better health outcomes with large capacity for functional redundancy, but also specificity (Le Chatelier et al., 2013). Functional specificity enables organisms to utilize uncommon nutrients for their metabolism or facilitate part of missing pathway (Lozupone et al., 2012). *Oxalobacter formigenes* is a bacteria known for using oxalate as its sole energy source (Allison et al., 1986), however, loss of this organism through antibiotic usage can result in an increased risk for oxalate based kidney stones (Kelly et al., 2011). Thus, loss of functional gene specificity may not have a large impact but may result in long-term consequences.

The gut microbiome plays an important role in host nutrition by enhancing energy capture in the diet and up-regulating lipogenesis in the host leading to greater adipose deposition and decreased feed intake relative to germ-free mice (Bäckhed et al., 2004). It

was further demonstrated that the gut microbiome influences signaling for energy partitioning (Backhed et al., 2007). To a point, the host serum metabolite profile can be considered a reflection of the microbial metabolism occurring in the gut (Velagapudi et al., 2010).

The relationship between the immune system and the gut microbiota is as important as it is complex, due to the interaction between the two. In the absence of microbes, the immune system is woefully under developed with reductions in the size of lymphatic tissues, cell number (e.g., T cells), and expression of cell surface markers important for immune function (Round and Mazmanian, 2009). Microbial tolerance appears to be initially driven by a blunted immune response in neonates to suppress inflammatory signals in lieu of regulatory signals allowing for development of the immune system and lymphoid tissues (Belkaid and Hand, 2014). Exposure to microbial antigens leads to the induction of immune cells, but importantly, the induction of T regulatory cells is necessary for regulating the inflammatory response (Weiner et al., 2011). Regulation of T helper 1 and 17 cells via T regulatory cells or anergy and deletion play a significant role in microbial tolerance and attenuation of the immune response (Weiner et al., 2011). Besides training the immune system, the commensal organisms themselves serve as barrier to pathogens directly by providing competition by overwhelming pathogens with numbers, using up available substrate, and modifying the environment (e.g. pH, inhibitory compounds) (Kamada et al., 2013). The microbiome can also compete indirectly by stimulating barrier function properties (e.g. mucins, antimicrobial peptides, RegIIIγ) via the immune system and T helper 17 cells (Kamada et al., 2013). When gut dysbiosis occurs, due to health challenges or use of antibiotics, this

can decrease competition and enable pathogens to grow unchecked and overwhelm the gut causing illness. For example, *Clostridium difficile* is an opportunistic pathogen that is normally held to small numbers, but will flourish in environments where antibiotics or illness have compromised the commensal population (Dicks et al., 2018).

Alteration by Diet

Diet is the biggest driver of change in the microbiome throughout the life of an animal (Bauer et al., 2007). Piglets initially consume the dam's milk for nutrition, but prior to and after weaning, introduction to solid feed will occur. The nursery diets are typically high in crude protein and easily digestible. As the pig grows and moves on to grower and finish diets, crude protein (CP) will be reduced in the diet and fiber will typically be increased. The microbiome will see its largest adjustment with the transition to solid feed and will remain fairly constant for its adult life (Frese et al., 2015), barring an significant health challenges, antibiotics use, or physiologic changes (e.g., pregnancy). The following sections will go into more detail in regard to protein, lipid, and carbohydrate effects on the microbiome and growth. It should be noted that while the focus is to use papers focused on using the pig as the model of choice, a lack of literature requires using other species for reference.

Protein

Proteins, or more specifically, amino acids, are necessary for tissue deposition and bodily function. Sow's milk is estimated to contain an average of 5.16% CP (NRC, 2012). Amino acid requirements in the NRC (2012) suggest that piglets start at 22.69% CP. Although the amino acid requirements increase with size, crude protein density

decreases as AA requirements are met with an increase in feed consumption. By the late finishing phase, CP drops to 10.41%.

To ease weaning stress and get piglets started, prestarter and early nursery diets contain a diversity of protein sources that are easily digestible to facilitate growth and maturation of the gut to promote downstream nutrient absorption and growth. Although diets can contain higher CP levels than those suggested in the NRC 2012, too much CP can be concern for causing post-weaning diarrhea (Prohászka and Baron, 1980). Decreasing CP in nursery diets reduced growth performance, but also resulted in lower plasma urea and ammonia in the digesta with no effect on the microbiome (Nyachoti et al., 2006; Lynch et al., 2009). Wellock et al. (2007) observed that higher CP in the diet increased the fluidity and coliform counts of the feces while reducing *Lactobacillus* counts. Similarly, the increase in CP also increased ADG and G:F without any effect on ADFI. Opapeju et al. (2009) showed similar growth performance results, but after challenging with ETEC, found that higher CP did not have a better growth response compared to lower CP, but maintained a higher bacterial diversity in the colon and had a higher percentage of pigs shedding the ETEC at d 3 and 7 post challenge. Decreasing dietary CP resulted in a reduction in biogenic amine concentration in the colon while a decrease from 16 to 13% CP led to increased expression of occludin, a tight junction protein (Fan et al., 2017). Interestingly, a further reduction to 10% CP reduced expression of occludin, biomarkers for intestinal stem cells, and ileal morphology. Work by Peng et al. (2017) showed that CP reduction did not result in a linear decrease in biogenic amines in digesta from ileum, cecum, or colon, but linear decreases in ammonia, and cecal and colonic SCFA occurred. Changes in intestinal SCFA production were not affected by

reduction in CP shown by Bikker et al. (2006), which may have resulted from using younger aged pigs. Furthermore, while there was no difference in *Lactobacillus* counts between CP levels, *Bifidobacterium* decreased linearly with CP content (Peng et al., 2017). A nursery pig study showed that *Lactobacillus* spp. increased as CP content increased in ileal digesta regardless of protein source (Rist et al., 2014). It is worth noting that changes in *Lactobacillus* counts appear to vary with CP content and fluctuation may be due to differences in diet, genetics, or even the starting microbiome.

Comparing the effects of dietary protein sources on the microbiota has been minimally studied. Work by Cao et al. (2016a), found that pigs fed SBM and fishmeal had discriminately different microbial profiles compared with cottonseed meal (CSM) or SBM-CSM combination. Likewise, fishmeal, SBM, and SBM-CSM had greater bacterial diversity compared with feeding solely CSM. Interestingly, where the majority of diets showed the *Firmicutes* phyla to be the predominate one, *Proteobacteria* was the dominate phyla in the fishmeal-based diet. It was further shown that CSM-based diets had increased abundance of *Lactobacillus* spp. and may be beneficial for intestinal health. On the other hand, fishmeal had increased abundance of *Escherichia* and *Shigella* species demonstrating that fishmeal may promote an environment with increased susceptibility to post-weaning diarrhea.

Lipid

Due to their energy density, dietary lipids have mostly been a concern as an energy source and less emphasis on lipid composition. Studies assessing the effect of dietary fat level are skewed towards high fat Western diet-obesity models. Additionally, these models use higher fat inclusions than typically seen in commercial diets.

Furthermore, many of these models also incorporate the use of fiber, prebiotics, or other anti-obeseogenic compounds for determination of weight loss or decreased fat deposition (Yan et al., 2013; Heinritz et al., 2016). Other literature has covered lipid composition, identifying the effects of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) on the growth and physiology of pigs.

Usage of DDGS at high levels has been of concern in grow-finish production as the higher unsaturated fats derived from DDGS have been known to cause soft bellies during meat processing, specifically bacon slicing (Whitney et al., 2006; Leick et al., 2010; Cromwell et al., 2011). However, Xu et al. (2010) reported that belly firmness can be increased by withdrawing DDGS from the diet a few weeks prior to slaughter. Feeding conjugated linoleic acid was reported to help improve belly firmness in pigs fed DDGS (White et al., 2009).

Due to their potential anti-inflammatory effects, omega-3 fatty acids have also been considered in nutrition studies. Gestation and lactation diets supplemented with PUFA enhanced glucose uptake in intestinal tissue and glycogen storage in weanling pigs (Gabler et al., 2007). Diets with PUFA supplementation appear to decrease intestinal endotoxin transport, endotoxemia, and TLR-4 activation when compared with SFA supplementation (Liu et al., 2012; Mani et al., 2013). Inclusion of omega-3 PUFA appeared to decrease *Bacteroides* species in the cecum of pigs without any effect of diet or microbiome on fat accumulation (Andersen et al., 2011). Supplementation of a high fat diet with PUFA decreased abundance of *Streptococcus*, *Clostridium*, and pathogenic *Enterobacteriaceae* while also increasing bacterial diversity in premature infants (Younge et al., 2017). A mice model found that omega-6 PUFA induced inflammation

prone organisms, *Clostridia* sp. and *Enterobacteriaceae,* whereas omega-3 PUFA suppressed inflammatory organisms while promoting *Lactobacillus* and *Bifidobacteria* (Ghosh et al., 2013). Altogether, omega-3 PUFA supplementation may be useful for gut health and modulating the microbiome to favor beneficial commensal organisms over pathogenic bacteria. In human studies, supplementation of omega-3 PUFA generally did not cause a change at the phylum level, although some changes did occur at the genus level, but there was no consistency in which bacteria were up or down regulated due to the variation in study type (Costantini et al., 2017). However, the authors note that supplementation of omega-3 may still be beneficial in promoting eubiosis of the gut.

Carbohydrates

Carbohydrates range from simple mono- and disaccharides to the prebiotic oligosaccharides and polysaccharides, which include starch and nonstarch polysaccharides, the latter being commonly referred to as dietary fiber. This section will primarily focus on nonstarch polysaccharides. While fiber does serve a role as bulking agent in diets, it does provide benefits to the gut and gut microbiota. In young pigs, higher fiber content promoted *Lactobacillus* abundance in the small intestine and VFA formation in the hindgut (Bikker et al., 2006). Diets devoid of fiber or just containing prebiotic carbohydrates were found to dramatically shift the microbiome of mice by promoting mucus degrading bacteria compared to those on fiber rich diets (Desai et al., 2016). This resulted in a decreased mucus thickness of the colon while increasing susceptibility to *Citrobacter rodentium,* an enteric pathogen in mice, leading to increased shedding, weight loss, and death.

A recent report has suggested that insoluble fiber content from a 30% DDGS diet not only shifts the microbiome by reducing the *Firmicutes:Bacteroidetes* ratio and *Lactobacillus* abundance, but this reduction along with alterations in the metabolome may leave pigs susceptible to colitis (Burrough et al., 2015). When using fiber derived from different grain products in nursery diets, Chen et al. (2013) found that wheat bran fiber increased villus height and villus:crypt depth ratio in the ileum and ileum and colon Goblet cell number compared with maize and soybean fiber. Soybean fiber resulted in higher colon VFA concentrations relative to maize fiber, but similar VFA concentrations were noted in wheat bran and pea fiber diets. Abundance of *Lactobacillus* and *Bifidobacterium* appeared to increase due to pea, maize, and wheat bran fiber inclusion, but decreased in control and soybean fiber diets. Wheat bran fiber diets reduced *E. coli* counts in ileal and colonic digesta, whereas soybean fiber had increased numbers of these bacteria. Wheat bran fiber also increased transcription of tight junction proteins zonula occludens 1 and occludin, and TLR2, which could translate to improved barrier function. A follow up study by Chen et al. (2014) with grow-finish pigs found that wheat bran improved ileal villus:height ratio and sucrose production when compared with soybean fiber also. Soybean fiber inclusion promoted acetate production in both the ileum and cecum when compared to the control diet although no difference was noted in total VFA production. Wheat bran inclusion resulted in increased cecal butyrate compared to all treatments, suggesting potential for improving cell tissue health similar to data reported by Molist et al. (2009). Interestingly, alfalfa and pure cellulose were shown to increase gene expression for genes related to butyrate production in the lumen of the cecum, but not the mucosa when compared against wheat bran (Mu et al., 2017). Alfalfa increased

total VFAs in the proximal colon compared with wheat bran, but was not different in the cecum or distal colon.

Similar to their previous data (Chen et al., 2013), Chen et al. (2014) found that pea fiber and wheat bran increased ileal *Bifidobacterium*, numerically, and *Lactobacillus* while decreasing *E. coli* concentrations with the opposite occurring in soybean fiber. Similar trends were noted in the colonic digesta. Pea fiber increased jejunal GLUT2 gene expression compared with the control and soybean fiber diets. Pea fiber and wheat bran both increased ileal glucagon and GLUT2 gene expression over maize and soybean fibers, while only wheat bran improved SGLT1 expression.

Molist et al. (2009) found that insoluble fiber from wheat bran had less unbound water in the colonic digesta compared with control or sugar beet pulp diets suggesting higher water binding capacity, allowing for increased substrate for large intestine microflora. Milled wheat bran was shown to increase fecal score and *E. coli* concentration while decreasing total VFA and acetic acid production compared with coarse wheat bran after being challenged with *E. coli* (Molist et al., 2011). The coarse wheat bran had similar fecal scores to the antibiotic control, reduction in *E. coli,* and an increase in VFA production.

Cao et al. (2016b) reported Lantang pigs fed a low fiber diet had increased methane production compared with pigs fed a high fiber diet. This methane production was positively correlated with higher density of methanogenic bacteria although the mode of action of rice hulls in the high fiber diet on decreasing methane production was unclear. Likewise, inclusion of pea fiber increased the diversity of methanogenic bacteria

in weanling and finisher pigs (Luo et al., 2017), but it was not reported if this altered methane production.

Antibiotics and Alternatives

Antibiotics and alternatives have been highly researched for their effects on swine growth performance and modulation of the microbiome. Antibiotics have been shown to modulate the gut microbiome (Holman and Chénier, 2015), although this modulation is highly variable due to breed of pigs used, location, and antibiotics used. Which antibiotics are used can play a role as many classes (e.g., tylosin, lincomycin, and penicillin) target gram positive bacteria (Gaskins et al., 2002). Other antibiotic classes are broad spectrum (e.g., carbadox, tetracycline, and sulfonamides) targeting both gram negative and positive and are commonly used throughout the swine industry. Antibiotic alternatives, besides lysozyme, are less likely to target specific microbes but more likely drive a response due to promoting commensal bacteria to outcompete pathogenic organisms (Verstegen and Williams, 2002).

Reviewing the current literature for all antibiotic and antibiotic alternative studies would be a daunting task. Rather, it would be more worthwhile to mention that the complexity of the microbiome increases as we add more variables to it. Overall, antibiotic- or antibiotic alterative-mediated changes to the microbiome may not be apparent at the phylum level or for alpha diversity, but more noticeable at the family or genus level and with beta diversity (Soler et al., 2018). Studies reviewed by Holman and Chénier (2015) have shown that diversity may decrease, increase, or stay the same with antibiotic treatment. However, the authors did see that feed additive supplementation

increased abundance of *Bacillus* and *Lactobacillus* species compared to control or antibiotic supplemented animals.

Antibiotics benefit growth promotion through prevention and tempering of infection while suppressing bacteria, commensal or pathogenic, that may divert nutrients away from the host for their own use (Brüssow, 2015). The usage of probiotics or commensal stimulating prebiotic compounds may benefit the host by stabilizing the gut microbiome (Brüssow, 2017). The plethora of antibiotic alternative studies reviewed by (Schweer et al., 2017b) may corroborate this as few have positive impact on growth performance and those that do may not see major alterations in taxa, save for a few species. It is also noted that besides the differences in diets, breeds, locations and experimental designs, the large differences in methodology used to characterize the microbial communities can be inconsistent or lacking in depth (Allen et al., 2013).

Conclusions

The swine industry is a vast one working to continue producing wholesome and healthy pork that is affordable for consumers. This can be challenging as feed prices, production costs, government regulations, and consumer perceptions drive the evolution of the industry. Antibiotics have been useful for improving the efficiency of the industry and even with the VFD, will still be critical for treating disease so that herds can remain healthy and productive. Continued research into the microbiome and antibiotic alternatives will be critical for understanding host health, the functions and improving swine performance.

Literature Cited

Abdel-Mawgoud, A. M., F. Lépine, and E. Déziel. 2010. Rhamnolipids: Diversity of structures, microbial origins and roles. Appl Microbiol Biotechnol 86:1323-1336.

Allen, H. K., U. Y. Levine, T. Looft, M. Bandrick, and T. A. Casey. 2013. Treatment, promotion, commotion: Antibiotic alternatives in food-producing animals. Trends in Microbiology 21:114-119.

Allison, M. J., H. M. Cook, D. B. Milne, S. Gallagher, and R. V. Clayman. 1986. Oxalate degradation by gastrointestinal bacteria from humans. The Journal of nutrition 116:455-460.

Andersen, A. D., L. Mølbak, T. Thymann, K. F. Michaelsen, and L. Lauritzen. 2011. Dietary long-chain n-3 pufa, gut microbiota and fat mass in early postnatal piglet development—exploring a potential interplay. Prostaglandins, Leukotrienes and Essential Fatty Acids 85:345-351.

Andrä, J., J. Rademann, J. Howe, H. J. Koch Michel, H. Heine, U. Zähringer, and K. Brandenburg. 2006. Endotoxin-like properties of a rhamnolipid exotoxin from burkholderia (pseudomonas) plantarii: Immune cell stimulation and biophysical characterization Biological Chemistry No. 387. p 301.

Arnolds, K. L., and C. A. Lozupone. 2016. Striking a balance with help from our little friends – how the gut microbiota contributes to immune homeostasis. The Yale Journal of Biology and Medicine 89:389-395.

Bäckhed, F., H. Ding, T. Wang, L. V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich, and J. I. Gordon. 2004. The gut microbiota as an environmental factor that regulates fat storage. Proceedings of the National Academy of Sciences of the United States of America 101:15718.

Backhed, F., J. K. Manchester, C. F. Semenkovich, and J. I. Gordon. 2007. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. PNAS 104:979-984.

Bauer, E., B. A. Williams, H. Smidt, R. Mosenthin, and M. W. A. Verstegen. 2007. Influence of dietary components on development of the microbiota in single-stomached species. Nutrition Research Reviews 19:63-78.

Belkaid, Y., and Timothy W. Hand. 2014. Role of the microbiota in immunity and inflammation. Cell 157:121-141.

Benincasa, M., A. Abalos, I. Oliveira, and A. Manresa. 2004. Chemical structure, surface properties and biological activities of the biosurfactant produced by pseudomonas aeruginosa lbi from soapstock. Antonie van Leeuwenhoek 85:1-8.

Bikker, P., A. Dirkzwager, J. Fledderus, P. Trevisi, I. le Huërou-Luron, J. P. Lallès, and A. Awati. 2006. The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets1. Journal of Animal Science 84:3337-3345.

Brockmeier, S. L., C. L. Loving, M. V. Palmer, A. Spear, T. L. Nicholson, K. S. Faaberg, and K. M. Lager. 2017. Comparison of asian porcine high fever disease isolates of porcine reproductive and respiratory syndrome virus to united states isolates for their ability to cause disease and secondary bacterial infection in swine. Veterinary Microbiology 203:6-17.

Brüssow, H. 2015. Growth promotion and gut microbiota: Insights from antibiotic use. Environmental Microbiology 17:2216-2227.

Brüssow, H. 2017. Adjuncts and alternatives in the time of antibiotic resistance and in-feed antibiotic bans. Microbial Biotechnology 10:674-677.

Burrough, E. R., B. L. Arruda, J. F. Patience, and P. J. Plummer. 2015. Alterations in the colonic microbiota of pigs associated with feeding distillers dried grains with solubles. PLOS ONE 10:e0141337.

Campbell, J. M., J. D. Crenshaw, and J. Polo. 2013. The biological stress of early weaned piglets. Journal of Animal Science and Biotechnology 4:19-19.

Cao, K. F., H. H. Zhang, H. H. Han, Y. Song, X. L. Bai, and H. Sun. 2016a. Effect of dietary protein sources on the small intestine microbiome of weaned piglets based on high-throughput sequencing. Letters in Applied Microbiology 62:392-398.

Cao, Z., J. B. Liang, X. D. Liao, A. D. G. Wright, Y. B. Wu, and B. Yu. 2016b. Effect of dietary fiber on the methanogen community in the hindgut of lantang gilts. animal 10:1666-1676.

Capozzalo, M. M., J. C. Kim, J. K. Htoo, C. F. de Lange, B. P. Mullan, C. F. Hansen, J.-W. Resink, P. A. Stumbles, D. J. Hampson, and J. R. Pluske. 2012. An increased ratio of dietary tryptophan to lysine improves feed efficiency and elevates plasma tryptophan and kynurenine in the absence of antimicrobials and regardless of infection with enterotoxigenic escherichia coli in weaned pigs. J Anim Sci 90:191-193.

Capozzalo, M. M., J. C. Kim, J. K. Htoo, C. F. M. de Lange, B. P. Mullan, C. F. Hansen, J.-W. Resink, P. A. Stumbles, and J. R. Pluske. 2015. Effect of increasing the dietary tryptophan to lysin ratio on plasma levels of tryptophan, kynurenine and urea and on production traits in weaner pigs experimentally infected with an enterotoxigenic strain of escherichia coli. Arch Anim Nutr 69:17-29.

Chae, C. 2016. Porcine respiratory disease complex: Interaction of vaccination and porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, and mycoplasma hyopneumoniae. The Veterinary Journal 212:1-6.

Chen, H., X. Mao, J. He, B. Yu, Z. Huang, J. Yu, P. Zheng, and D. Chen. 2013. Dietary fibre affects intestinal mucosal barrier function and regulates intestinal bacteria in weaning piglets. British Journal of Nutrition 110:1837-1848.

Chen, H., X. B. Mao, L. Q. Che, B. Yu, J. He, J. Yu, G. Q. Han, Z. Q. Huang, P. Zheng, and D. W. Chen. 2014. Impact of fiber types on gut microbiota, gut environment and gut function in fattening pigs. Animal Feed Science and Technology 195:101-111.

Chong, H., and Q. Li. 2017. Microbial production of rhamnolipids: Opportunities, challenges and strategies. Microbial Cell Factories 16:137.

Chung, K.-T., and G. S. Gadupudi. 2011. Possible role of excess tryptophan metabolites in cancer. Environ Mol Mutagen 52:81-104.

Costantini, L., R. Molinari, B. Farinon, and N. Merendino. 2017. Impact of omega-3 fatty acids on the gut microbiota. International Journal of Molecular Sciences 18.

Cromwell, G. L. 2002. Why and how antibiotics are used in swine production. Animal Biotechnology 13:7-27.

Cromwell, G. L., M. J. Azain, O. Adeola, S. K. Baidoo, S. D. Carter, T. D. Crenshaw, S. W. Kim, D. C. Mahan, P. S. Miller, and M. C. Shannon. 2011. Corn distillers dried grains with solubles in diets for growing-finishing pigs: A cooperative study12. Journal of Animal Science 89:2801-2811.

da Silva, N., A. Carriquiry, K. O'Neill, T. Opriessnig, and A. M. O'Connor. 2014. Mixed treatment comparison meta-analysis of porcine circovirus type 2 (pcv2) vaccines used in piglets. Preventive Veterinary Medicine 117:413-424.

de Ridder, K., C. L. Levesque, J. K. Htoo, and C. F. M. de Lange. 2012. Immune system stimulation reduces the efficiency of tryptophan utilization for body protein deposition in growing pigs. J Anim Sci 90:3485-3491.

Dębski, B. 2016. Supplementation of pigs diet with zinc and copper as alternative to conventional antimicrobials. Polish Journal of Veterinary Sciences 19:917-924.

Desai, M. S., A. M. Seekatz, N. M. Koropatkin, N. Kamada, C. A. Hickey, M. Wolter, N. A. Pudlo, S. Kitamoto, N. Terrapon, A. Muller, V. B. Young, B. Henrissat, P. Wilmes, T. S. Stappenbeck, G. Núñez, and E. C. Martens. 2016. A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. Cell 167:1339-1353.e1321.

Dicks, L. M. T., L. S. Mikkelsen, E. Brandsborg, and H. Marcotte. 2018. Clostridium difficile, the difficult "kloster" fuelled by antibiotics. Current Microbiology.

Dominguez-Bello, M. G., E. K. Costello, M. Contreras, M. Magris, G. Hidalgo, N. Fierer, and R. Knight. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences 107:11971.

Eyerich, K., V. Dimartino, and A. Cavani. 2017. Il-17 and il-22 in immunity: Driving protection and pathology. European Journal of Immunology 47:607-614.

Fan, P., P. Liu, P. Song, X. Chen, and X. Ma. 2017. Moderate dietary protein restriction alters the composition of gut microbiota and improves ileal barrier function in adult pig model. 7:43412.

FDA. 1996. Animal drug availability act of 1996. https://http://www.fda.gov/AnimalVeterinary/GuidanceComplianceEnforcement/ActsRulesRegulations/ucm105940.htm (Accessed November 12 2018.)

Frese, S. A., K. Parker, C. C. Calvert, and D. A. Mills. 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome 3:28.

Gabler, N. K., J. D. Spencer, D. M. Webel, and M. E. Spurlock. 2007. In utero and postnatal exposure to long chain (n-3) pufa enhances intestinal glucose absorption and energy stores in weanling pigs. J. Nutr. 137:2351-2358.

Gaskins, H. R., C. T. Collier, and D. B. Anderson. 2002. Antibiotics as growth promotants:Mode of action. Animal Biotechnology 13:29-42.

Gerstel, U., M. Czapp, J. Bartels, and J.-M. Schröder. 2009. Rhamnolipid-induced shedding of flagellin from pseudomonas aeruginosa provokes hbd-2 and il-8 response in human keratinocytes. Cellular Microbiology 11:842-853.

Ghosh, S., D. DeCoffe, K. Brown, E. Rajendiran, M. Estaki, C. Dai, A. Yip, and D. L. Gibson. 2013. Fish oil attenuates omega-6 polyunsaturated fatty acid-induced dysbiosis and infectious colitis but impairs lps dephosphorylation activity causing sepsis. PLOS ONE 8:e55468.

Goncalves, M. A. D., S. Nitikanchana, M. D. Tokach, S. S. Dritz, N. M. Bello, R. D. Goodband, K. J. Touchette, J. L. Ursy, J. M. DeRouchey, and J. C. Woodworth. 2015. Effects of standardized ileal digestible tryptophan:Lysine ratio of growth performance of nursery pigs. J Anim Sci 93:3909-3918.

Gonzalez, L. M., I. Williamson, J. A. Piedrahita, A. T. Blikslager, and S. T. Magness. 2013. Cell lineage identification and stem cell culture in a porcine model for the study of intestinal epithelial regeneration. PLOS ONE 8:e66465.

Grandin, T. 2017. On-farm conditions that compromise animal welfare that can be monitored at the slaughter plant. Meat science 132:52-58.

Guzik, A. C., M. J. Pettitt, E. Beltranena, L. L. Southern, and B. J. Kerr. 2005. Threonine and tryptophan ratios fed to nursery pigs*. Journal of Animal Physiology and Animal Nutrition 89:297-302.

Hansen, M. S., S. E. Pors, H. E. Jensen, V. Bille-Hansen, M. Bisgaard, E. M. Flachs, and O. L. Nielsen. 2010. An investigation of the pathology and pathogens associated with porcine respiratory disease complex in denmark. Journal of Comparative Pathology 143:120-131.

Heiman, M. L., and F. L. Greenway. 2016. A healthy gastrointestinal microbiome is dependent on dietary diversity. Molecular Metabolism 5:317-320.

Heinritz, S., E. Weiss, M. Eklund, T. Aumiller, C. Heyer, S. Messner, A. Rings, S. Louis, S. Bischoff, and R. Mosenthin. 2016. Impact of a high-fat or high-fiber diet on intestinal microbiota and metabolic markers in a pig model. Nutrients 8:317.

Holman, D. B., B. W. Brunelle, J. Trachsel, and H. K. Allen. 2017. Meta-analysis to define a core microbiota in the swine gut. mSystems 2.

Holman, D. B., and M. R. Chénier. 2015. Antimicrobial use in swine production and its effect on the swine gut microbiota and antimicrobial resistance. Canadian Journal of Microbiology 61:785-798.

Jacobsen, B., L. Krueger, F. Seeliger, M. Bruegmann, J. Segalés, and W. Baumgaertner. 2009. Retrospective study on the occurrence of porcine circovirus 2 infection and associated entities in northern germany. Veterinary Microbiology 138:27-33.

Jiang, L., X. Long, and Q. Meng. 2013. Rhamnolipids enhance epithelial permeability in caco-2 monolayers. International Journal of Pharmaceutics 446:130-135.

Jiang, L., C. Shen, X. Long, G. Zhang, and Q. Meng. 2014. Rhamnolipids elicit the same cytotoxic sensitivity between cancer cell and normal cell by reducing surface tension of culture medium. Appl Microbiol Biotechnol 98:10187-10196.

Johnson, C. L., and J. Versalovic. 2012. The human microbiome and its potential importance to pediatrics. Pediatrics 129:950.

Kamada, N., G. Y. Chen, N. Inohara, and G. Núñez. 2013. Control of pathogens and pathobionts by the gut microbiota. Nature immunology 14:685-690.

Kelly, J. P., G. C. Curhan, D. R. Cave, T. E. Anderson, and D. W. Kaufman. 2011. Factors related to colonization with oxalobacter formigenes in u.S. Adults. Journal of Endourology 25:673-679.

Lamendalla, R., J. W. Santo Domingo, S. Ghosh, J. Martinson, and D. B. Oerther. 2011. Comparative fecalmetagenomics unveils unique functional capacity of the swine gut. BMC Microbiology 11:103.

Le Chatelier, E., T. Nielsen, J. Qin, E. Prifti, F. Hildebrand, G. Falony, M. Almeida, M. Arumugam, J. M. Batto, S. Kennedy, P. Leonard, J. Li, K. Burgdorf, N. Grarup, T. Jorgensen, I. Brandslund, H. B. Nielsen, A. S. Juncker, M. Bertalan, F. Levenez, N. Pons, S. Rasmussen, S. Sunagawa, J. Tap, S. Tims, E. G. Zoetendal, S. Brunak, K. Clement, J. Dore, M. Kleerebezem, K. Kristiansen, P. Renault, T. Sicheritz-Ponten, W. M. de Vos, J. D. Zucker, J. Raes, T. Hansen, P. Bork, J. Wang, S. D. Ehrlich, and O. Pedersen. 2013. Richness of human gut microbiome correlates with metabolic markers. Nature 500:541-546.

Le Floc'h, N., L. LeBellego, J. J. Matte, D. Melchior, and B. Sève. 2009. The effect of sanitary status degradation and dietary tryptophan content on growth rate and tryptophan metabolism in weaning pigs1. Journal of Animal Science 87:1686-1694.

Lee, J., M. Cella, K. McDonald, C. Garlanda, G. D. Kennedy, M. Nukaya, A. Mantovani, R. Kopan, C. A. Bradfield, R. Newberry, and M. Colonna. 2012. Ahr drives the development of gut ilc22 cells and postnatal lymphoid tissues via pathways dependent on and indepedent of notch. Nat Immunol 13:144-151.

Leick, C. M., C. L. Puls, M. Ellis, J. Killefer, T. R. Carr, S. M. Scramlin, M. B. England, A. M. Gaines, B. F. Wolter, S. N. Carr, and F. K. McKeith. 2010. Effect of distillers dried grains with solubles and ractopamine (paylean) on quality and shelf-life of fresh pork and bacon. Journal of Animal Science 88:2751-2766.

Liu, Y., F. Chen, J. Odle, X. Lin, S. K. Jacobi, H. Zhu, Z. Wu, and Y. Hou. 2012. Fish oil enhances intestinal integrity and inhibits tlr4 and nod2 signaling pathways in weaned pigs after lps challenge. The Journal of nutrition 142:2017-2024.

Liu, Y., C. D. Espinosa, J. J. Abelilla, G. A. Casas, L. V. Lagos, S. A. Lee, W. B. Kwon, J. K. Mathai, D. M. D. L. Navarro, N. W. Jaworski, and H. H. Stein. 2018. Non-antibiotic feed additives in diets for pigs: A review. Animal Nutrition 4:113-125.

Lozupone, C. A., J. I. Stombaugh, J. I. Gordon, J. K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489:220.

Luo, Y., H. Chen, B. Yu, J. He, P. Zheng, X. Mao, G. Tian, J. Yu, Z. Huang, J. Luo, and D. Chen. 2017. Dietary pea fiber increases diversity of colonic methanogens of

pigs with a shift from methanobrevibacter to methanomassiliicoccus-like genus and change in numbers of three hydrogenotrophs. BMC Microbiology 17:17.

Lynch, B., J. J. Callan, and J. V. O'Doherty. 2009. The interaction between dietary crude protein and fermentable carbohydrate source on piglet post weaning performance, diet digestibility and selected faecal microbial populations and volatile fatty acid concentration. Livest. Sci. 124:93-100.

Lyte, M. 2013. Microbial endocrinology in the microbiome-gut-brain axis: How bacterial production and utilization of neurochemicals influence behavior. PLoS pathogens 9:e1003726.

Madson, D. M., A. R. Patterson, S. Ramamoorthy, N. Pal, X. J. Meng, and T. Opriessnig. 2009. Reproductive failure experimentally induced in sows via artificial insemination with semen spiked with porcine circovirus type 2. Veterinary Pathology 46:707-716.

Mani, V., J. H. Hollis, and N. K. Gabler. 2013. Dietary oil composition differentially modulates intestinal endotoxin transport and postprandial endotoxemia. Nutrition & Metabolism 10:6.

Mao, S., M. Zhang, J. Liu, and W. Zhu. 2015. Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: Membership and potential function. Scientific Reports 5:16116.

Marshall, B. M., and S. B. Levy. 2011. Food animals and antimicrobials: Impacts on human health. Clinical Microbiology Reviews 24:718.

McGuinness, W. A., N. Malachowa, and F. R. DeLeo. 2017. Vancomycin resistance in staphylococcus aureus . The Yale journal of biology and medicine 90:269-281.

Messori, S., P. Trevisi, A. Simongiovanni, D. Priori, and P. Bosi. 2013. Effect of susceptibility to enterotoxigenic escherichia coli f4 and of dietary tryptophan on gut microbiota diversity observed in healthy young pigs. Vet Microbiol 162:173-179.

Moeser, A. J., C. S. Pohl, and M. Rajput. 2017. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. Animal Nutrition 3:313-321.

Molist, F., A. G. de Segura, J. Gasa, R. G. Hermes, E. G. Manzanilla, M. Anguita, and J. F. Pérez. 2009. Effects of the insoluble and soluble dietary fibre on the physicochemical properties of digesta and the microbial activity in early weaned piglets. Animal Feed Science and Technology 149:346-353.

Molist, F., E. G. Manzanilla, J. F. Pérez, and C. M. Nyachoti. 2011. Coarse, but not finely ground, dietary fibre increases intestinal firmicutes:Bacteroidetes ratio and reduces diarrhoea induced by experimental infection in piglets. British Journal of Nutrition 108:9-15.

Mu, C., L. Zhang, X. He, H. Smidt, and W. Zhu. 2017. Dietary fibres modulate the composition and activity of butyrate-producing bacteria in the large intestine of suckling piglets. Antonie van Leeuwenhoek 110:687-696.

Myer, M. S. 1982. The presence of paneth cells confimed in the pig. Onderstepoort J Vet Res 49:131-132.

Nathues, H., P. Alarcon, J. Rushton, R. Jolie, K. Fiebig, M. Jimenez, V. Geurts, and C. Nathues. 2017. Cost of porcine reproductive and respiratory syndrome virus at individual farm level – an economic disease model. Preventive Veterinary Medicine 142:16-29.

Nicholson, J. K., E. Holmes, J. Kinross, R. Burcelin, G. Gibson, W. Jia, and S. Pettersson. 2012. Host-gut microbiota metabolic interactions. Science (New York, N.Y.) 336:1262.

NPPC. 2018. Pork facts. http://nppc.org/pork-facts/ (Accessed July 10 2018.)

NRC. 2012. Nutrient requirements of swine. 11th Ed. ed. Natl. Acad. Press, Washington, DC.

Nyachoti, C. M., F. O. Omogbenigun, M. Rademacher, and G. Blank. 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets1. Journal of Animal Science 84:125-134.

Oliver, W. T., and J. E. Wells. 2015. Lysozyme as an alternative to growth promoting antibiotics in swine production. Journal of Animal Science and Biotechnology 6:35.

Omonijo, F. A., L. Ni, J. Gong, Q. Wang, L. Lahaye, and C. Yang. 2018. Essential oils as alternatives to antibiotics in swine production. Animal Nutrition 4:126-136.

Opapeju, F. O., D. O. Krause, R. L. Payne, M. Rademacher, and C. M. Nyachoti. 2009. Effect of dietary protein level on growth performance, indicators of enteric health, and gastrointestinal microbial ecology of weaned pigs induced with postweaning colibacillosis12. Journal of Animal Science 87:2635-2643.

Opitz, C. A., U. M. Litzenburger, F. Sahm, M. Ott, I. Tritschler, S. Trump, T. Schumacher, L. Jestaedt, D. Schrenk, M. Weller, M. Jugold, G. J. Guillemin, C. L. Miller, C. Lutz, B. Radlwimmer, I. Lehmann, A. von Deimling, W. Wick, and

M. Platten. 2011. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. Nature 478:197-203.

Opriessnig, T., X.-J. Meng, and P. G. Halbur. 2007. Porcine circovirus type 2–associated disease: Update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. Journal of Veterinary Diagnostic Investigation 19:591-615.

Ouellette, A. J. 2010. Paneth cells and innate mucosal immunity. Current Opinion in Gastroenterology 26.

Pajarillo, E. A., J. Chae, M. P. Balolong, H. B. Kim, and D. Kang. 2014. Assessment of fecal bacterial diversity among healthy piglets during the weaning transition. J Gen Appl Microbiol 60:140-146.

Palinski, R., P. Piñeyro, P. Shang, F. Yuan, R. Guo, Y. Fang, E. Byers, and B. M. Hause. 2017. A novel porcine circovirus distantly related to known circoviruses is associated with porcine dermatitis and nephropathy syndrome and reproductive failure. Journal of Virology 91.

Pannaraj, P. S., F. Li, C. Cerini, and et al. 2017. Association between breast milk bacterial communities and establishment and development of the infant gut microbiome. JAMA Pediatrics 171:647-654.

Patel, D., Y. Nan, M. Shen, K. Ritthipichai, X. Zhu, and Y.-J. Zhang. 2010. Porcine reproductive and respiratory syndrome virus inhibits type i interferon signaling by blocking stat1/stat2 nuclear translocation. Journal of Virology 84:11045.

Peng, Y., K. Yu, C. Mu, S. Hang, L. Che, and W. Zhu. 2017. Progressive response of large intestinal bacterial community and fermentation to the stepwise decrease of dietary crude protein level in growing pigs. Appl Microbiol Biotechnol 101:5415-5426.

Preston, T., C. Slater, D. C. McMillan, J. S. Falconer, A. Shenkin, and K. C. H. Fearon. 1998. Fibrinogen synthesis is elevated in fasting cancer patients with an acute phase response. The Journal of nutrition 128:1355-1360.

Prohászka, L., and F. Baron. 1980. The predisposing role of high dietary protein supplies in enteropathogenic e. Coli infections of weaned pigs. Zentralblatt für Veterinärmedizin Reihe B 27:222-232.

Pujols, J., and J. Segalés. 2014. Survivability of porcine epidemic diarrhea virus (pedv) in bovine plasma submitted to spray drying processing and held at different time by temperature storage conditions. Veterinary Microbiology 174:427-432.

Reeds, P. J., C. R. Fjeld, and F. Jahoor. 1994. Do the differences between the amino acid compositions of acute-phase and muscle proteins have a bearing on nitrogen loss in traumatic states? The Journal of nutrition 124:906-910.

Rist, V. T. S., E. Weiss, N. Sauer, R. Mosenthin, and M. Eklund. 2014. Effect of dietary protein supply originating from soybean meal or casein on the intestinal microbiota of piglets. Anaerobe 25:72-79.

Rosell, C., J. Segalés, J. Plana-Durán, M. Balasch, G. M. Rodríguez-Arrioja, S. Kennedy, G. M. Allan, F. McNeilly, K. S. Latimer, and M. Domingo. 1999. Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (pmws) in pigs. Journal of Comparative Pathology 120:59-78.

Rossow, K. D. 1998. Porcine reproductive and respiratory syndrome. Veterinary Pathology 35:1-20.

Round, J. L., and S. K. Mazmanian. 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nature Reviews Immunology 9:313.

Schulz, L. L., and G. T. Tonsor. 2015. Assessment of the economic impacts of porcine epidemic diarrhea virus in the united states. Journal of Animal Science 93:5111-5118.

Schweer, W., K. Schwartz, J. F. Patience, L. Karriker, C. Sparks, M. Weaver, M. Fitzsimmons, T. E. Burkey, and N. K. Gabler. 2017a. Porcine reproductive and respiratory syndrome virus reduces feed efficiency, digestibility, and lean tissue accretion in grow-finish pigs1. Translational Animal Science 1:480-488.

Schweer, W. P., J. F. Patience, K. Schwartz, D. Linhares, C. Rademacher, H. Allen, C. Loving, A. Ramirez, and N. K. Gabler. 2017b. A review and evaulation of (growth promoting) antibiotic alternatives in the literature. In: Midwest Section ASAS, Omaha, NE

Segalés, J. 2012. Porcine circovirus type 2 (pcv2) infections: Clinical signs, pathology and laboratory diagnosis. Virus Research 164:10-19.

Sender, R., S. Fuchs, and R. Milo. 2016. Revised estimates for the number of human and bacteria cells in the body. PLOS Biology 14:e1002533.

Simongiovanni, A., E. Corrent, N. Le Floc'h, and J. van Milgen. 2012. Estimation of the tryptophan requirement in piglets by meta-analysis. Animal 6:594-602.

Soler, C., T. Goossens, A. Bermejo, L. Migura-García, A. Cusco, O. Francino, and L. Fraile. 2018. Digestive microbiota is different in pigs receiving antimicrobials or a feed additive during the nursery period. PLOS ONE 13:e0197353.

Sorensen, A. A., J. Freedgood, J. Dempsey, and D. M. Theobald. 2018. Farms under threat: The state of america's farmland. American Farmland Trust, Washington, DC.

Stevenson, G. W., H. Hoang, K. J. Schwartz, E. R. Burrough, D. Sun, D. Madson, V. L. Cooper, A. Pillatzki, P. Gauger, B. J. Schmitt, L. G. Koster, M. L. Killian, and K. J. Yoon. 2013. Emergence of porcine epidemic diarrhea virus in the united states: Clinical signs, lesions, and viral genomic sequences. Journal of Veterinary Diagnostic Investigation 25:649-654.

The Human Microbiome Project, C., C. Huttenhower, D. Gevers, R. Knight, S. Abubucker, J. H. Badger, A. T. Chinwalla, H. H. Creasy, A. M. Earl, M. G. FitzGerald, R. S. Fulton, M. G. Giglio, K. Hallsworth-Pepin, E. A. Lobos, R. Madupu, V. Magrini, J. C. Martin, M. Mitreva, D. M. Muzny, E. J. Sodergren, J. Versalovic, A. M. Wollam, K. C. Worley, J. R. Wortman, S. K. Young, Q. Zeng, K. M. Aagaard, O. O. Abolude, E. Allen-Vercoe, E. J. Alm, L. Alvarado, G. L. Andersen, S. Anderson, E. Appelbaum, H. M. Arachchi, G. Armitage, C. A. Arze, T. Ayvaz, C. C. Baker, L. Begg, T. Belachew, V. Bhonagiri, M. Bihan, M. J. Blaser, T. Bloom, V. Bonazzi, J. Paul Brooks, G. A. Buck, C. J. Buhay, D. A. Busam, J. L. Campbell, S. R. Canon, B. L. Cantarel, P. S. G. Chain, I. M. A. Chen, L. Chen, S. Chhibba, K. Chu, D. M. Ciulla, J. C. Clemente, S. W. Clifton, S. Conlan, J. Crabtree, M. A. Cutting, N. J. Davidovics, C. C. Davis, T. Z. DeSantis, C. Deal, K. D. Delehaunty, F. E. Dewhirst, E. Deych, Y. Ding, D. J. Dooling, S. P. Dugan, W. Michael Dunne, A. Scott Durkin, R. C. Edgar, R. L. Erlich, C. N. Farmer, R. M. Farrell, K. Faust, M. Feldgarden, V. M. Felix, S. Fisher, A. A. Fodor, L. J. Forney, L. Foster, V. Di Francesco, J. Friedman, D. C. Friedrich, C. C. Fronick, L. L. Fulton, H. Gao, N. Garcia, G. Giannoukos, C. Giblin, M. Y. Giovanni, J. M. Goldberg, J. Goll, A. Gonzalez, A. Griggs, S. Gujja, S. Kinder Haake, B. J. Haas, H. A. Hamilton, E. L. Harris, T. A. Hepburn, B. Herter, D. E. Hoffmann, M. E. Holder, C. Howarth, K. H. Huang, S. M. Huse, J. Izard, J. K. Jansson, H. Jiang, C. Jordan, V. Joshi, J. A. Katancik, W. A. Keitel, S. T. Kelley, C. Kells, N. B. King, D. Knights, H. H. Kong, O. Koren, S. Koren, K. C. Kota, C. L. Kovar, N. C. Kyrpides, P. S. La Rosa, S. L. Lee, K. P. Lemon, N. Lennon, C. M. Lewis, L. Lewis, R. E. Ley, K. Li, K. Liolios, B. Liu, Y. Liu, C.-C. Lo, C. A. Lozupone, R. Dwayne Lunsford, T. Madden, A. A. Mahurkar, P. J. Mannon, E. R. Mardis, V. M. Markowitz, K. Mavromatis, J. M. McCorrison, D. McDonald, J. McEwen, A. L. McGuire, P. McInnes, T. Mehta, K. A. Mihindukulasuriya, J. R. Miller, P. J. Minx, I. Newsham, C. Nusbaum, M. O'Laughlin, J. Orvis, I. Pagani, K. Palaniappan, S. M. Patel, M. Pearson, J. Peterson, M. Podar, C. Pohl, K. S. Pollard, M. Pop, M. E. Priest, L. M. Proctor, X. Qin, J. Raes, J. Ravel, J. G. Reid, M. Rho, R. Rhodes, K. P. Riehle, M. C. Rivera, B. Rodriguez-Mueller, Y.-H. Rogers, M. C. Ross, C. Russ, R. K. Sanka, P. Sankar, J. Fah Sathirapongsasuti, J. A. Schloss, P. D. Schloss, T. M. Schmidt, M. Scholz, L. Schriml, A. M. Schubert, N. Segata, J. A. Segre, W. D. Shannon, R. R. Sharp, T. J. Sharpton, N. Shenoy, N. U. Sheth, G. A. Simone, I. Singh, C. S.

Smillie, J. D. Sobel, D. D. Sommer, P. Spicer, G. G. Sutton, S. M. Sykes, D. G. Tabbaa, M. Thiagarajan, C. M. Tomlinson, M. Torralba, T. J. Treangen, R. M. Truty, T. A. Vishnivetskaya, J. Walker, L. Wang, Z. Wang, D. V. Ward, W. Warren, M. A. Watson, C. Wellington, K. A. Wetterstrand, J. R. White, K. Wilczek-Boney, Y. Wu, K. M. Wylie, T. Wylie, C. Yandava, L. Ye, Y. Ye, S. Yooseph, B. P. Youmans, L. Zhang, Y. Zhou, Y. Zhu, L. Zoloth, J. D. Zucker, B. W. Birren, R. A. Gibbs, S. K. Highlander, B. A. Methé, K. E. Nelson, J. F. Petrosino, G. M. Weinstock, R. K. Wilson, and O. White. 2012. Structure, function and diversity of the healthy human microbiome. Nature 486:207.

Tossou, M. C. B., H. Liu, M. Bai, S. Chen, Y. Cai, V. Duraipandiyan, H. Liu, T. O. Adebowale, N. A. Al-Dhabi, L. Long, H. Tarique, A. O. Oso, G. Liu, and Y. Yin. 2016. Effect of high dietary tryptophan on intestinal morphology and tight juntion protein of weaned pig. Biomed Res Int.

Trautman, A., and J. Fiebiger. 1952. Fundamentals of the histology of domestic animals. Comstock Pub., Cornell Univ. Press, Ithaca, N. Y.

USDA-FAS. 2018. Livestock and poultry: World markets and trade. In: F. A. Service (ed.). p 1-29. United States Department of Agriculture.

USDA-NASS. 2018. Quarterly hogs and pigs. http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1086 (Accessed May 11 2018.)

van der Meer, Y., W. J. J. Gerrits, A. J. M. Jansman, B. Kemp, and J. E. Bolhuis. 2017. A link between damaging behaviour in pigs, sanitary conditions, and dietary protein and amino acid supply. PLOS ONE 12:e0174688.

van der Meer, Y., A. Lammers, A. J. M. Jansman, M. M. J. A. Rijnen, W. H. Hendriks, and W. J. J. Gerrits. 2016. Performance of pigs kept under different sanitary conditions affected by protein intake and amino acid supplementation1,2. Journal of Animal Science 94:4704-4719.

Varel, V. H. 1987. Activity of fiber-degrading microorganisms in the pig large intestine. Journal of Animal Science 65:488-496.

Varel, V. H., and W. G. Pond. 1985. Enumeration and activity of cellulolytic bacteria from gestating swine fed various levels of dietary fiber. Applied and Environmental Microbiology 49:858.

Velagapudi, V. R., R. Hezaveh, C. S. Reigstad, P. Gopalacharyulu, L. Yetukuri, S. Islam, J. Felin, R. Perkins, J. Boren, M. Oresic, and F. Backhed. 2010. The gut microbiota modualtes host energy and lipid metabolism in mice. J. Lipid Res. 51:1101-1112.

Verstegen, M. W. A., and B. A. Williams. 2002. Alternatives to the use of antibiotics as growth promoters for monogastric animals. Animal Biotechnology 13:113-127.

Wang, H., Y. Ji, G. Wu, K. Sun, Y. Sun, W. Li, B. Wang, B. He, Q. Zhang, Z. Dai, and Z. Wu. 2015. L-tryptophan activates mammalian target of rapamycin and enhances expression of tight junctions protein in intestinal procine epithelial cells. The Journal of nutrition 145:1156-1162.

Weiner, H. L., A. P. da Cunha, F. Quintana, and H. Wu. 2011. Oral tolerance. Immunological Reviews 241:241-259.

Wellock, I. J., P. D. Fortomaris, J. G. M. Houdijk, and I. Kyriazakis. 2007. The effect of dietary protein supply on the performance and risk of post-weaning enteric disorders in newly weaned pigs. Animal Science 82:327-335.

White, H. M., B. T. Richert, J. S. Radcliffe, A. P. Schinckel, J. R. Burgess, S. L. Koser, S. S. Donkin, and M. A. Latour. 2009. Feeding connugated linoleic acid partially recovers carcass quality in pigs fed dried corn distillers grains with solubles. J. Anim. Sci. 87:157-166.

Whitman, W. B., D. C. Coleman, and W. J. Wiebe. 1998. Prokaryotes: The unseen majority. Proceedings of the National Academy of Sciences 95:6578.

Whitney, M. H., G. C. Shurson, L. J. Johnston, D. M. Wulf, and B. C. Shanks. 2006. Growth performance and carcass characteristics of grower-finisher pigs fed high-quality corn distillers dried grain with solubles originating from a modern midwestern ethanol plant1. Journal of Animal Science 84:3356-3363.

Xu, G., S. K. Baidoo, L. J. Johnston, D. Bibus, J. E. Cannon, and G. C. Shurson. 2010. The effects of feeding diets containing corn distillers dried grains with solubles, and withdrawal period of distillers dried grains with solubles, on growth performance and pork quality in grower-finisher pigs. J. Anim. Sci. 88:1388-1397.

Xu, S., Y. Zhao, J. Shen, Y. Lin, Z. Fang, L. Che, and D. Wu. 2014. Threonine and tryptophan supplementation enhance porcine respiratory and reproductive syndrome (prrs) vaccine-induced immune responses of growing pigs. Anim Sci J 86:294-304.

Xue, M., J. Zhao, L. Ying, F. Fu, L. Li, Y. Ma, H. Shi, J. Zhang, L. Feng, and P. Liu. 2017. Il-22 suppresses the infection of porcine enteric coronaviruses and rotavirus by activating stat3 signal pathway. Antiviral Research 142:68-75.

Yan, H., R. Potu, H. Lu, V. Vezzoni de Almeida, T. Stewart, D. Ragland, A. Armstrong, O. Adeola, C. H. Nakatsu, and K. M. Ajuwon. 2013. Dietary fat content and fiber

type modulate hind gut microbial community and metabolic markers in the pig. PLOS ONE 8:e59581.

Younge, N., Q. Yang, and P. C. Seed. 2017. Enteral high fat-polyunsaturated fatty acid blend alters the pathogen composition of the intestinal microbiome in premature infants with an enterostomy. The Journal of Pediatrics 181:93-101.e106.

Zelante, T., R. G. Iannitti, C. Cunha, A. De Luca, G. Giovannini, G. Pieraccini, R. Zecchi, C. D'Angelo, C. Massi-Benedetti, F. Fallarino, A. Carvalho, P. Puccetti, and L. Romani. 2013. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity 39:372-385.

CHAPTER 2. ALTERATION OF THE PIG INTESTINAL MICROBIOME WHEN VACCINATED AGAINST OR INOCULATED WITH PORCINE CIRCOVIRUS 2 USING A MULTIVARIATE ANALYSIS MODEL[1]

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ABSTRACT: This study assessed the effect of porcine circovirus 2 (**PCV2**) vaccination compared to inoculation on the intestinal microbiome of piglets. Ninety-six weaned barrows (age 27 to 40 d; 7.1 kg BW) were either vaccinated (**VAC**) for or inoculated (**PCV**) with PCV2 on d 0. Fecal samples were collected at d 0, 14, and 28 (n=6/treatment). Microbial community structure was analyzed using Ion Torrent technology by sequencing the 16S rRNA gene. No differences ($P > 0.10$) were detected at the genus level between d 0 samples. *Prevotella* spp. decreased from d 14 to 28 in VAC pigs ($P < 0.05$) and was reduced compared with PCV at d 28 ($P < 0.10$). A core set of 383 operational taxonomic units (**OTU**), present in >80% of samples, were analyzed using a multivariate analysis by linear models. A total of 58 OTUs were correlated with at least one phenotypic trait or fixed effect i.e., BW, day, PCV-status, ($P <0.05$, $q < 0.05$). Of these, 15 OTUs were determined to be of the genus *Prevotella*. Abundance of *P. stercorea* ($r = 0.21$) and *Solitalea koreenis* ($r = 0.41$) were shown to be associated with serum IgG concentration, while *Intestinimonas butyriciproducens* ($r = -0.07$) and *Oscillbacter valericigenes* ($r = -0.09$) were inversely associated with serum IgM concentration. Presence of *P. stercorea* ($r = -0.11$) was negatively associated with VAC pigs by d 28; whereas, *Ruminiclostridium thermosuccinogenes* ($r = 0.11$) presence increased. These data suggest that *Prevotella* spp. flourish during a PCV challenge, irrespective of PCV-status (vaccinated or inoculated), but decrease sooner in VAC pigs than PCV pigs. However, an age-dependent shift in the microbiome may also play a role.

Keywords: microbiome, pig, Porcine Circovirus 2, vaccination

INTRODUCTION

Porcine Circovirus 2 (**PCV2**) is one of the top diseases causing economic loss to the pork industry. Pigs affected by PCV2 infection often have increased susceptibility to co-infections (Gillespie et al., 2009; Takada-Iwao et al., 2011; Opriessing and Halbur, 2012), which is a necessary factor to form PCV2 associated diseases (**PCVAD**; e.g., post-weaning multi-systemic wasting syndrome). The prevention and treatment of PCVAD result in significant financial loss in terms of the vaccination, management intervention, and reduced performance associated with PCVAD (Alacron et al., 2013). It has been estimated that the cost associated with PCVAD can range from 3 to 20 dollars/pig culminating in a total loss of up to 2 billion dollars for the U.S pork industry (Gillespie et al., 2009).

In recent years, research into the microbiome has dramatically increased. The microbiome plays a large role in intestinal health, digestion of undigested feed particles, and immune modulation, to name a few (Kosiewicz et al., 2011). As such, dysbiosis of the microbiome can enable pathogens to flourish causing intestinal disturbances such as diarrhea, inflammation, and activation of the immune system (Kosiewicz et al., 2011). The goal of this study was to assess if pigs vaccinated against PCV2 display an altered microbiome compared with those inoculated with PCV2.

MATERIALS AND METHODS

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Nebraska, Lincoln.

Animals and Experimental Design

Initially, a total of 160 weaned crossbred barrows (Large White × Landrace) were screened for PCV2-specific IgG and IgM by ELISA (Ingenasa, Madrid, Spain) from blood samples obtained at 14 d of age at the Agricultural Research and Development Center Swine Unit (Mead, NE). Individuals (n = 32) with a sample-to-positive ratio lower than 0.3 for passive IgG (all pigs were IgM negative) were included in the current experiment. At 16 to 17 d of age, one half (n = 16) of the pigs were vaccinated for PCVAD with a single dose of Ingelvac CircoFLEX vaccine (Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany). At weaning, all pigs were fed a standard corn-soybean meal diet (without antibiotics) until approximately 30 d of age at which time all pigs were transferred to the University of Nebraska-Lincoln animal science complex (Lincoln, NE) where the experimental infection was conducted. Upon arrival at the University of Nebraska-Lincoln animal science complex, pigs (n = 32; average 33.5 d of age; 7.1 kg average BW) were sorted by initial BW and PCV status (vaccinated for PCV2 [VAC] or inoculated with PCV2 [PCV]) and randomly assigned to 8 pens (4 pigs/pen; 4 pens/treatment) 2 d prior to the beginning of the experiment. Pens were fed a simple corn-soybean meal diet with no added antibiotics that met or exceeded NRC requirements. During the 28-d experiment, pigs were housed in a common room in 24 identical pens with a combination of slatted and solid surface flooring. The pens provided approximately 0.65 m^2 of floor space per pig. All pigs were allowed ad libitum access to feed and water.

Experimental PCV2 Inoculation

The PCV2b isolate used in the experimental infection was recovered from a pig that had symptoms characteristic of PCV2 infection and is the same isolate used in

previous experiments. On d 0 (average 33.5 d of age) all naïve pigs (*n* = 16) were intranasally (2 mL) and intramuscularly (1 mL) infected with a titer of 10^4 median tissue culture infective dose/mL. Pigs were observed daily for clinical signs of infection and facial thermometers were used to monitor daily body temperature (data not shown) for 7 d post-inoculation. Serum samples were collected via jugular venipuncture on d 0, 7, 14, 21, and 28 to assess serum IgG and IgM using method described above.

Growth Performance

Pig BW and feed disappearance measurements were obtained at the beginning of the experiment and weekly thereafter (d 0, 7, 14, 21, and 28). Pig BW and feed disappearance data were used to calculate ADG, ADFI, and G:F.

Fecal Sample Collection and Extraction

Fecal samples were obtained from the pigs on d 0, 14, and 28, using clean and disinfected plastic loops interested into the rectum for sampling. For each treatment and time point, samples were collected from the same pigs (n = 6). Samples were placed in autoclaved 2 mL tubes and stored at -20˚C for later use.

Fecal samples were extracted for genomic DNA following the procedure described by Martínez et al. (2009). The DNA pellet was resuspended in Tris-HCl buffer (10 mM pH 8.0). Concentration and purity of DNA was measure using a Nanodrop spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE). Extracted DNA samples were checked for quality using a 1% agarose gel. Samples were stored at -20˚C until later use.

PCR Amplification and Sequencing of 16S rRNA

Samples were processed following a protocol previously reported by Tran et al. (2018). Samples were amplified using the V3 region of the 16SrRNA gene using 341F and 518R barcoded primers (Whiteley et al., 2012). A 25 uL PCR mixture was used and contained 0.25 uL of Terra PCR Direct polymerase (Clontech Laboratories, Moutainview, CA), 12.5 uL of 2X PCR Direct reaction buffer, 0.5 uL of 341 forward primers (25 umol), 1 uL of 518 reverse primers (10 umol) 0.25 uL of BSA, and 2 uL of DNA (20-50 ng). Conditions for amplification were as follows: 98˚C for 3 m; 30 cycles of 98˚C for 30 s, 53˚C for 30 s and 68˚C for 40 s; and a single final extension step at 68˚C for 4 m. Amplified DNA was run on a 2% agarose gel and band intensity was determined using GeneTools 1D (Syngene, Frederick, MD) gel analysis. Afterwards, amplicons were pooled to equal concentrations and purified using the MinElute PCR Purification Kit (Qiagen, Valencia, CA). Fragments of DNA containing the V3 region in the pooled sample were selected using an E-gel SizeSelect 2% agarose (Invitrogen, Life Technologies, South San Francisco, CA). Quality and concentration were assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Samples were diluted to 15 pM for templating to Ion Sphere particles and subsequent emulsion PCR using the Ion OneTouch 2 instrument (life Technologies, South San Francisco, CA). Templated-Ion Sphere particles were sequenced on a 316-chip kit with the Ion Torrent Personnel Genome Machine (Life Technologies, Carlsbad, CA) according to manufacturer's instructions.

Quality Control of Sequences and Analysis

Initial processing of obtained sequences was done as described by Tran et al. (2018). An operational taxonomic unit (**OTU**) table was constructed using Quantitative Insights Into Microbial Ecology (**QIIME**, 1.8.0) (Caporaso et al., 2012) and reverse complemented with Mothur (1.34.1) (Schloss et al., 2009). Sequences were clustered into OTU of >97% similarity using the UPARSE pipeline in USEARCH (Edgar, 2010, 2013). Taxonomic information was added using the 12_10 Greengenes database release in QIIME. Cyanobacteria were filtered out of the OTU table prior to further analysis. Sequences were aligned using The Ribosomal Database Project (RDP) Aligner tool (Cole et al., 2014). A distance matrix and phylogenetic tree were created in Mothur using the alignment file from RDP and the dist.seqs() and clearcut commands. Total number of sequences varied between samples, as such, samples were subsampled to the lowest sequences (2,137) using QIIME. Singletons were removed from the OTU table. Using QIIME, core sequences were identified as those belonging to 83% of the treatment samples. Total, singleton, and core sequence sets were rarefied for Chao1, Shannon, and Simpson indices of alpha diversity. Beta diversity and jackknifed beta diversity were performed to generate weighted and unweighted Unifrac plots. Additionally, all three sequence sets had taxa summary plots generated. The core OTU table was averaged by treatment x time point to generate new plots for readability.

Statistical Analysis

Analysis was performed using Multivariate Association with Linear Models (Huttenhower, 2014) previously described by Morgan et al. (2015) by finding associations between metadata and microbial community abundance utilizing the

distribution of OTUs in samples. Metadata used included day, treatment (PCV and VAC), BW, IgG, IgM, and day × treatment interaction. Associations were considered significant with a *P*- and *q*-value ≤ 0.05 (minimum false discovery rate). Significantly associated OTU were then submitted to National Center for Biotechnology Basic Local Alignment Search Tool (BLAST) for bacterial identification.

RESULTS AND DISCUSSION

To determine the microbial profile in our PCV or VAC pigs, fecal samples were sequenced and analyzed (Fig. 1). Within d-0 samples, there were no differences between PCV- or VAC-treated pigs, indicating that vaccination for PCV2 resulted in little to no change in the microbiome relative to unvaccinated contemporaries. The microbiome profile in our study is similar to those reported by Pajarillo et al. (2014) and Frese et al. (2015) for weaned pigs. Overall, the majority of shifts in the microbiome occurred in the Bacteroidales, Clostridiales, and Lactobacillales orders. The proportion of Lactobacillaceae and Bacteroidales decreased, whereas Clostridiales increased over the experimental period. The decrease in Lactobacillaceae abundance is may be the result of weaning and the absence of sow's milk or milk-derived products in the diet fed. Prevotellaceae increased from approximately 31% in abundance from d-0 PCV and VAC pigs to 45% in d-14 VAC pigs but decreased to 24% in PCV pigs. However, by d 28, Prevotellaceae abundance had decreased substantially to 14% in VAC pigs and increased to 42% in PCV pigs. In a human study conducted by Qin et al. (2015), *Prevotella* has been previously shown by (Lamendalla et al., 2011) to be a major contributor to the microbiome in post weaned swine due to ability to degrade plant fibers seen in the diet.

Unclassified Bacteroidales and Ruminococcaceae were increased in d-14 PCV and d-28 VAC samples but were decreased in d-14 VAC and d-28 PCV samples.

Using MaAsLin (Huttenhower, 2014), both positive and negative associations were identified between sequenced OTU and metadata (Table 1). *Oscillibacter valericigenes* (-0.094) and *Intestimonas butyriciproducens* (-0.073) were associated with blood IgM whereas *Prevotella stercorea* (0.214) and *Solitalea koreensis* (0.407) were associated with blood IgG. Interestingly, *Prevotella* were prominently featured in associations with metadata; however, only those with a coefficient greater than 0.05, positive or negative are shown here. These data show that *Prevotella salivae* and *Prevotella scopos*, associate positively and negatively with increased and decreased *Prevotella* abundance as would be expected, but *P. stercorea* showed a negative association when *Prevotella* abundance was increased, suggesting that individual species may modulate differently than other members of the genus.

These data suggest *Prevotella* spp. may flourish during a PCV challenge, irrespective of PCV status (vaccinated or inoculated), but decrease sooner in VAC pigs than PCV pigs. However, an age-dependent shift may also play a role. Additional time points during the 28-d period and beyond may be required to elucidate the fluctuations occurring within the microbiome over the course of a PCV2 challenge. Further data is needed to determine if PCV2 or other viral challenges select for specific microbial species or phenotypes and their impact on short- and long-term animal health.

LITERATURE CITED

Alacron, P., J. Rushton, H. Nathues, and B. Wieland. 2013. Economic efficiency analysis of different strategies to control post-weaning multi-systemic wasting syndrome and porcine circovirus type 2 subclinical infection in 3-weekly batch system farms. Prev Vet Med 110:103-118.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Ownes, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. D. Knight. 2012. Qiime allows analysis of high-throughput community sequencing data. Nat. Methods 7:335-336.

Cole, J. R., Q. Wang, J. A. Fish, B. Chai, D. M. McGarrell, Y. Sun, C. T. Brown, A. Porras-Alfaro, C. R. Kuske, and J. M. Tiedje. 2014. Ribosomal database project: Data and tools for high throughput rrna analysis. Nucl. Acids Res. 42:D633-D642.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than blast. Bioinformatics 26:2460-2461.

Edgar, R. C. 2013. Uparse: Highly accurate otu sequences from microbial amplicon reads. Nat Methods 10:996-998.

Frese, S. A., K. Parker, C. C. Calvert, and D. A. Mills. 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome 3:28.

Gillespie, J., T. Opriessing, X. J. Meng, K. Pelzer, and V. Buechner-Maxwell. 2009. Porcine circovirus type 2 and porcine circovirus-associated disease. J Vet Intern Med 23:1151-1163.

Huttenhower. 2014. Maaslin. http://huttenhower.sph.harvard.edu/maaslin.)

Kosiewicz, M. M., A. L. Zirnheld, and P. Alard. 2011. Gut microbiota, immunity, and disease: A complex relationship. Front Microbiol 2:180.

Lamendalla, R., J. W. Santo Domingo, S. Ghosh, J. Martinson, and D. B. Oerther. 2011. Comparative fecalmetagenomics unveils unique functional capacity of the swine gut. BMC Microbiology 11:103.

Martínez, I., G. Wallace, C. Zhang, R. Legge, A. K. Benson, T. P. Carr, E. N. Moriyama, and J. Walter. 2009. Diet-induced metabolic improvements in a hamster model of hypercholesterolemia are strongly linked to alterations of the gut microbiota. Applied and Environmental Microbiology 75:4175.

Morgan, X. C., B. Kabakchiev, L. Waldron, A. D. Tyler, T. L. Tickle, R. Milgrom, J. M. Stempak, D. Gevers, R. J. Xavier, M. S. Silverberg, and C. Huttenhower. 2015. Associations between host gene expression, the mucosal microbiome, and clinical

outcome in the pelvic pouch of patients with inflammatory bowel disease. Genome Biology 16:67.

Opriessing, T., and P. G. Halbur. 2012. Concurrent infections are important for expression of porcine circovirus associated disease Viral Research 164:20-32.

Pajarillo, E. A., J. Chae, M. P. Balolong, H. B. Kim, and D. Kang. 2014. Assessment of fecal bacterial diversity among healthy piglets during the weaning transition. J Gen Appl Microbiol 60:140-146.

Qin, N., B. Zheng, J. Yao, L. Guo, J. Zuo, L. Wu, J. Zhou, L. Liu, J. Guo, S. Ni, A. Li, Y. Zhu, W. Liang, Y. Xiao, S. D. Ehrlich, and L. Li. 2015. Influence of h7n9 virus infection and associated treatment on human gut microbiota. Scientific Reports 5:14771.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn, and C. F. Weber. 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comapring microbial communities. Appl. Environ. Microbiol. 75:7537-7541.

Takada-Iwao, A., M. Nakanishi, J. Souma, S. Chikata, Y. Okuda, Y. Imai, and S. Sato. 2011. Porcine circovirus type 2 potentiates morbidity of salmonella enterica serovar choleraesuis in cesarean-derived, colostrum-deprived pigs. Vet Microbiol 154:104-112.

Tran, H., C. L. Anderson, J. W. Bundy, S. C. Fernando, P. S. Miller, and T. E. Burkey. 2018. Effects of spray-dried porcine plasma on fecal microbiota in nursery pigs. Journal of Animal Science 96:1017-1031.

Whiteley, A. S., S. Jenkins, I. Waite, N. Kresoje, H. Payne, B. Mullan, R. Allcock, and A. O'Donnell. 2012. Microbial 16s rrna ion tag and community metagenome sequencing using the ion torrent (pgm) platform. Journal of Microbiological Methods 91:80-88.

Table 2.1. Associations of operational taxonomic units (OTU) with phenotypic data using multivariate analysis by linear associations (MaAsLin; Huttenhower, 2014)[1]

Variable	Feature	Value[2]	Phylum	Family	Genus	Species	Coefficient	*P*.value	*q*-value
day*PCV	OTU102	`day*PCV`d14_I	*Bacteroidetes*	*Prevotellaceae*	*Prevotella*	*salivae*	-0.063	0.001	0.025
day*PCV	OTU102	`day*PCV`d28_V	*Bacteroidetes*	*Prevotellaceae*	*Prevotella*	*salivae*	-0.084	0.001	0.030
IgM	OTU11	IgM	*Firmicutes*	*Oscillospiraceae*	*Oscillibacter*	*valericigenes*	-0.094	0.005	0.034
day*PCV	OTU154	`day*PCV`d14_V	*Firmicutes*	*Clostridiaceae*	*Clostridium*	*saccharobutylicum*	-0.071	0.001	0.023
day*PCV	OTU196	`day*PCV`d28_V	*Firmicutes*	*Ruminococcaceae*	*Ruminococcus*	*bromii*	0.067	0.000	0.000
day*PCV	OTU231	`day*PCV`d28_V	*Firmicutes*	*Ruminococcaceae*	*Ruminiclostridium*	*thermosuccinogenes*	0.105	0.000	0.015
day*PCV	OTU364	`day*PCV`d0_V	*Firmicutes*	*Lachnospiraceae*	*Anaerostipes*	*caccae*	0.062	0.000	0.001
day*PCV	OTU364	`day*PCV`d28_I	*Firmicutes*	*Lachnospiraceae*	*Anaerostipes*	*caccae*	0.059	0.000	0.002
day*PCV	OTU364	`day*PCV`d28_V	*Firmicutes*	*Lachnospiraceae*	*Anaerostipes*	*caccae*	0.051	0.000	0.009
day*PCV	OTU37	`day*PCV`d14_I	*Firmicutes*	*Clostridiaceae*	*Clostridium*	*autoethanogenum*	0.051	0.000	0.011
day*PCV	OTU37	`day*PCV`d28_V	*Firmicutes*	*Clostridiaceae*	*Clostridium*	*autoethanogenum*	0.071	0.001	0.019
IgM	OTU39	IgM	*Firmicutes*	-	*Intestinimonas*	*butyriciproducens*	-0.073	0.002	0.013
day*PCV	OTU426	`day*PCV`d0_V	*Firmicutes*	*Oscillospiraceae*	*Oscillibacter*	*ruminatium*	0.079	0.000	0.001
day*PCV	OTU426	`day*PCV`d14_I	*Firmicutes*	*Oscillospiraceae*	*Oscillibacter*	*ruminatium*	0.068	0.000	0.012
day*PCV	OTU426	`day*PCV`d28_V	*Firmicutes*	*Oscillospiraceae*	*Oscillibacter*	*ruminatium*	0.063	0.000	0.013
day*PCV	OTU45	`day*PCV`d14_V	*Bacteroidetes*	*Prevotellaceae*	*Prevotella*	*stercorea*	-0.089	0.000	0.009
day*PCV	OTU45	`day*PCV`d28_V	*Bacteroidetes*	*Prevotellaceae*	*Prevotella*	*stercorea*	-0.114	0.001	0.023
IgG	OTU45	IgG	*Bacteroidetes*	*Prevotellaceae*	*Prevotella*	*stercorea*	0.214	0.007	0.041
day*PCV	OTU50	`day*PCV`d14_V	*Bacteroidetes*	*Prevotellaceae*	*Prevotella*	*denticola*	0.066	0.000	0.002
day*PCV	OTU52	`day*PCV`d28_I	*Firmicutes*	*Clostridiaceae*	*Saccharofermentans*	*acetigenes*	-0.056	0.001	0.028
day*PCV	OTU713	`day*PCV`d14_V	*Bacteroidetes*	*Prevotellaceae*	*Prevotella*	*scopos*	0.063	0.000	0.006
day*PCV	OTU713	`day*PCV`d28_I	*Bacteroidetes*	*Prevotellaceae*	*Prevotella*	*scopos*	0.058	0.000	0.013
day*PCV	OTU801	`day*PCV`d14_I	*Firmicutes*	*Veillonellaceae*	*Megasphaera*	*elsdenii*	0.063	0.000	0.010
day*PCV	OTU801	`day*PCV`d14_V	*Firmicutes*	*Veillonellaceae*	*Megasphaera*	*elsdenii*	0.060	0.000	0.017
day*PCV	OTU801	`day*PCV`d28_I	*Firmicutes*	*Veillonellaceae*	*Megasphaera*	*elsdenii*	0.062	0.000	0.011
day*PCV	OTU814	`day*PCV`d0_V	*Bacteroidetes*	*Cytophagaceae*	*Cytophaga*	*xylanolytica*	0.060	0.001	0.044
IgG	OTU9	IgG	*Bacteroidetes*	*Sphingobacteriacea*	*Solitalea*	*koreensis*	0.407	0.002	0.014

[1]All OTU are >1% of OTU relative abundance.

[2]V = vaccinated; I = inoculated with porcine circovirus 2

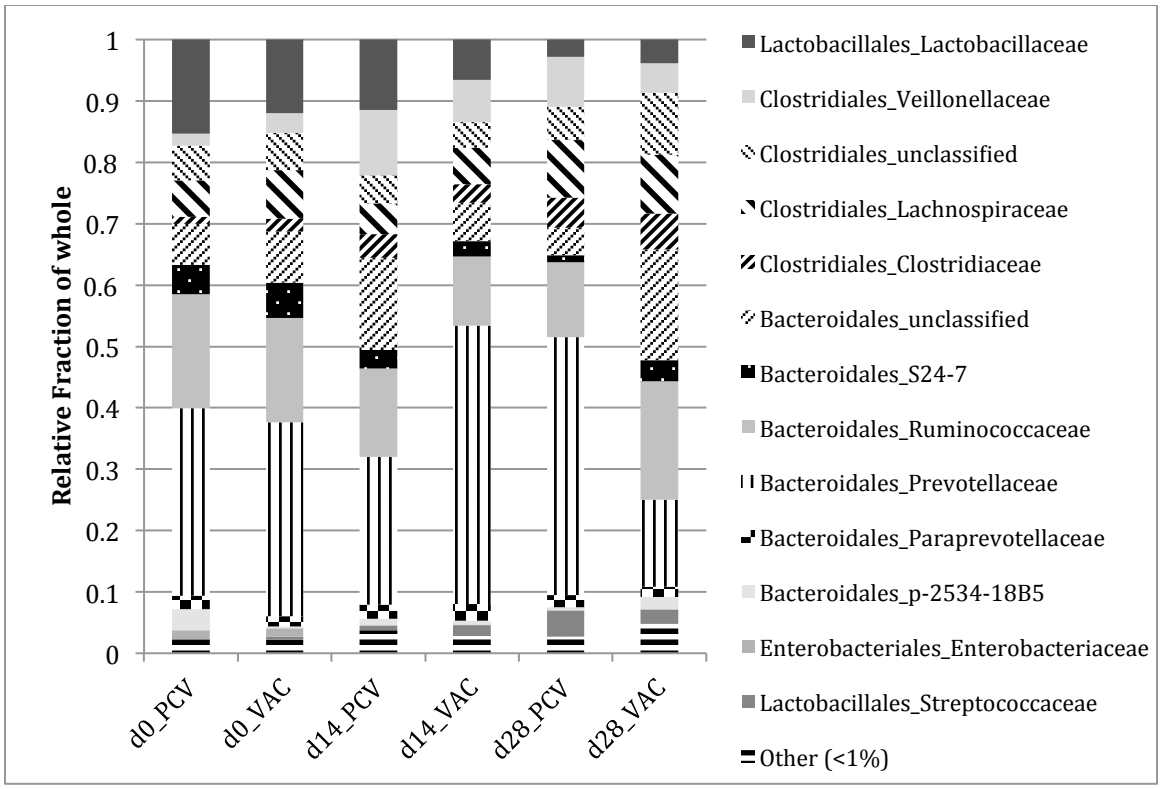

Figure 2.1. Proportion of microbial sequences relative to total averaged across treatment. Other categories are individual sequences totaling < 1% of total population combined together. Microbial groups are labeled with order and family names. PCV = inoculated with porcine circovirus 2; VAC = vaccinated for porcine circovirus 2.

CHAPTER 3. EVALUATION OF A QTL FOR PORCINE CIRCOVIRUS TYPE 2B (PCV2) VIRAL LOAD ON NURSERY AND LONG-TERM GROWTH PERFORMANCE AND NUTRIENT DIGESTIBILITY IN INOCULATED OR VACCINATED PIGS FOR PCV2

A manuscript prepared for submission to the *Journal of Animal Science*

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ABSTRACT: A prior experiment identified a PCV2-susceptibility marker QTL (*C*, resistant; *T*, susceptible) in pigs infected with PCV2. Thus, an experiment was conducted to test the effect of this QTL marker in a nursery setting and through grow-finish. For Exp. 1, a total of 156 crossbred barrows were genotyped and screened for PCV2 specific immunoglobulin G (**IgG**) and M (**IgM**). Pigs with a sample-to-positive ratio (S/P) lower than 1.26 for passive IgG, and 1.0 for passive IgM were used for the study. Piglets were either vaccinated for PCV2 prior to experiment starting (VAC) or inoculated with PCV2 (PCV) at the beginning of the experiment. Pigs (n =111; 7.1 kg) were sorted by initial body weight (**BW**) and PCV status and randomly assigned to 24 pens (4-5 pigs/pen) with the following treatments: CC-PCV, CT-PCV, TT-PCV, CT-VAC, and TT-VAC. At 34 d of age, PCV pigs were inoculated with PCV2. Growth performance, viremia, IgG, and IgM were monitored over six weeks. For Exp. 2, 8 pigs from each treatment in Exp. 1 (31.5±1.26 kg BW) were selected and housed 2 pigs per pen and fed a four-phase grow-finish diet. Growth performance, digestibility, and carcass traits were assessed. For Exp. 1, no differences in growth performance were detected. Viremia was reduced in CC-PCV compared with CT-PCV and TT-PCV from d 7 to 21 and d 7 to 28 respectively ($P < 0.05$). Titers of IgG were also reduced in CC-PCV pigs compared to both CT-PCV and TT-PCV pigs at d 28 ($P < 0.05$). Total tract digestibility for gross energy and dry matter at 14 dpi was increased ($P < 0.05$) in PCV pigs compared to VAC pigs. For Exp. 2, average daily feed intake was similar for both treatment groups with the exception that feed intake was greater ($P < 0.01$) for PCV pigs during the finisher 1 (wk 6 to 10) phase compared to VAC pigs. For feed efficiency, VAC pigs had greater ($P < 0.05$) efficiency compared to PCV pigs during the last three grow-finish phases. No differences were

observed in dry matter or gross energy digestibility with the exception that VAC pigs had greater ($P < 0.05$) dry matter and gross energy apparent total tract digestibility compared to PCV pigs at the end of the Finisher 1 (wk 10) phase. No differences were observed for carcass traits. Despite no differences in growth performance, analysis of production costs observed that the presence of the C allele reduced production costs from nursery to finish, regardless of PCV-status. Together these data show that selecting for PCV resistance is beneficial during a clinical infection and for long-term production costs.

Keywords: digestibility, health, pig, porcine circovirus type 2

INTRODUCTION

Porcine circovirus type 2 (PCV2) is a single stranded DNA virus that is known for causing severe lymphoid depletion leading to coinfection with secondary diseases known by many names: post weaning multisystemic wasting syndrome (PMWS), PCV2-associated diseases (PCVAD), or PCV2 systemic disease (PCV2-SD) (Segalés, 2012). Infection with PCVAD causes weight loss and lethargy and may cause diarrhea and respiratory symptoms in growing pigs (Gillespie et al., 2009; Segalés, 2012). Additionally, lymphocyte depletion and inflammation of lymphoid tissues along with high expression of PCV2 in these cites may also be seen histologically (Segalés, 2012). Infections may cause low to moderate morbidity and mortality, but morbidity losses have been estimated from $3 to 20 per pig or a total of $2 billion annually (Gillespie et al., 2009). Although PCV2 is considered ubiquitous across the world, occurrence of clinical disease has largely been reduced due to the use of vaccines (da Silva et al., 2014). However, despite the use of vaccines, they do not completely prevent or eliminate the

spread of disease, but allow for reduction in viremia, clinical symptoms, and viral shedding (Beach and Meng, 2012).

Pigs vaccinated for PCV2 show an improvement in growth performance compared against non-vaccinated animals (Jacela et al., 2011; Shelton et al., 2012; Potter et al., 2014). Increasing the standardized ileal digestibility ratio of lysine to metabolizable energy improved feed efficiency regardless of PCV2 vaccination status (Shelton et al., 2012). Surprisingly, there is no published research on PCV2 affecting nutrient digestibility. However, as diarrhea has been reported as possible clinical sign (Segalés, 2012) and the gut functions as the largest lymphatic organ (Burkey et al., 2009), it is likely that a PCV2 response in the gut-associated lymphatic tissue may cause some intestinal dysbiosis resulting in diarrhea.

Previously, the genetics of crossbred pigs were analyzed for variation in PCVAD susceptibility (Engle et al., 2014a). Two QTLs were shown to be associated with viral load. The first QTL, identified on *Sus scrofa* chromosome (SCC) 7 as swine leukocyte antigen II was shown to account for 2.8% of the variation (Engle et al., 2014b) while the second QTL, found on SCC 12 (*UNLPCV2.2009*), accounted for 11.5% of the variation. Presence of a *T* or *C* allele on the SNP within the SSC 12 QTL was correlated with greater or reduced susceptibility, respectively. However, this data did not elucidate any other response criteria besides viremia and ADG. We hypothesized that pigs with the favorable *C* allele will display an attenuated immune response and reduced reduction in growth performance compared to presence of the *T* allele. Thus, a study was conducted to determine the phenotypic difference between the SNP alleles for growth performance and digestibility in the nursery and grow-finish phases of production.

MATERIALS AND METHODS

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Nebraska, Lincoln.

Animals and Experimental Design.

Experiment 1. Initially, a total of 156 crossbred barrows (Large White x Landrace) were screened for PCV2 specific immunoglobulin (**Ig)** G and M by ELISA (Ingenasa, Madrid, Spain) from blood samples obtained at 14 d (± 4 d) of age at the UNL ENREC Swine Unit (Mead, NE). Pigs were also genotyped for SSC12 SNP alleles. Pigs (n = 111) with a sample-to-positive ratio (S/P) lower than 1.26 for passive IgG, and 1.0 for passive IgM were used for the study. At d 17 of age, 40 piglets were vaccinated for PCVAD with a single dose of Ingelvac CircoFLEX vaccine (Boehringer Ingelheim). The remaining 71 pigs were not vaccinated for PCVAD.

At weaning, all pigs were fed a standard corn-soybean meal diet (without antibiotics) until approximately 30 d of age at which time all pigs were transferred to the UNL Animal Science Complex (Lincoln, NE) where the experimental infection was conducted. Upon arrival at the UNL Animal Science Complex, pigs (n =111; 7.1 kg BW; 34.3 d of age) were sorted by initial BW and PCV status (vaccinated or inoculated) and genotype (CC, CT, and TT) for the following treatments (pigs per treatment): CC-PCV (11), CT-PCV (32), TT-PCV (28), CT-VAC (20), and TT-VAC (20) (**Table S3.1**). Pigs were assigned to 24 pens (4-5 pigs/pen) such that VAC and PCV may interact through nose-to-nose contact.

During the 42-d experiment, pigs were housed in a common room in 24 identical pens with a combination of slatted and solid surface flooring. The pens provided approximately 0.65 m^2 of floor space per pig. All pigs were allowed ad libitum access to feed and water. Pigs were fed a corn-soybean meal diet (Table 1) that met or exceeded NRC (2012) requirements with no antibiotic inclusion. Titanium dioxide was added into the experimental diet at 0.5% for use in assessing nutrient digestibility.

Experiment 2 and 3. The pigs from experiment 1 were selected using a statistical analysis to determine growth residuals between actual and predicted final BW using initial and final BW, day of infection, litter, pen, and maternal IgG from experiment 1.

For experiment 2, eight pigs from each of the previous five treatments (average BW, 31.5±1.26 kg), with a low net residual BW were selected, and were housed two pigs/pen by treatment for a total of 40 pigs (**Table S3.2**). For experiment 3, eight pigs from each of the previous treatments, excluding the CC-PCV group, were selected from experiment 1 pigs (**Table S3.2**). The pigs selected for experiment 3 included an equal number of pigs with high (positive) BW residuals (average BW, 33.6±3.22 kg) and with low (negative) residuals (average BW, 27.6±4.48), greater or lesser final BW compared with predicted BW, respectively. Therefore, experiment 3 was a 2x2x2 factorial arrangement for genotype (CT or TT), PCV status (PCV or VAC), and residual (high or low). These 32 pigs were individually housed in a different room within the UNL Animal Science Complex.

For experiment 2 and 3, animals had ad libitum access to water and feed. Pigs were sequentially fed the grower 1 diet for 2 weeks, and grower 2, finisher 1 and finisher 2 diets for 4 weeks (Table 2). Diets were corn-soybean meal based and designed to meet or

exceed NRC (2012) requirements. In the last week of each phase, diets with 0.5% titanium dioxide were used for digestibility analysis.

Experimental PCV2 Inoculation. The PCV2b isolate used in the experimental infection was recovered from a pig that had symptoms characteristic of PCV2 infection and is the same isolate used in previous experiments (McKnite et al., 2014). On d 0 (34.3 d of age) all naïve pigs (n = 71) were infected intranasally (2 mL) and intramuscularly (1 mL) with a titer of 10^4 $TCID_{50}$/mL. Additionally, pen design allowed for nose-to-nose contact to occur between VAC and PCV pigs. Quantification of PCV2 viral titer was done according to the protocol previously reported by McKnite et al. (2014).

Growth Performance. Pig BW and feed disappearance were measured at the beginning of experiment 1 and weekly thereafter for the 6 wk trial. For experiments 2 and 3, pig BW and feed disappearance were measured every 2 weeks. Pig BW and feed disappearance were used to calculate ADG, ADFI, and G:F.

Blood Collection. Blood samples were obtained from each pig (5 to 9 mL) via jugular venipuncture in serum separator tubes at d 0, 7, 14, 21, 28, 35, and 42 of experiment 1 and at the end of each diet phase in experiments 2 and 3. Tubes containing blood samples were immediately placed on ice and allowed to clot overnight before harvesting serum by centrifugation (1,500 × g for 20 min at 4°C). Serum samples were aliquoted and stored at -80^{o}C for subsequent analyses.

Genotyping. Genomic DNA was extracted from docked tails using the DNeasy tissue kit (Qiagen, CA). Quantification of DNA was performed using a NanoDrop Spectrophotometer (Thermo Scientific) and quality was assessed using agarose gel electrophoresis. All experimental animals were genotyped using second generation of the

porcine SNP60K BeadArray (Illumina), which included 61,565 SNPs respectively. Mapping of common SNPs to a porcine reference assembly and genome-wide association analyses was done according McKnite et al. (2014).

Digestibility Analysis. Proximate analysis was carried out on feed and pooled fecal samples as previously described (Patience et al., 2009; Jacobs et al., 2011). On d 14, 28, 42, fecal samples were collected from each pen for experiment 1. For experiments 2 and 3, fecal samples were collected by pen and individually, respectively, at the end of each diet phase. All samples were frozen at -20˚C for later analysis. Samples were later dried in a 100˚C forced-air oven for 3 d and then ground afterwards. Samples were analyzed for DM and gross energy using bomb calorimetry (Parr, Moline, IL). Total tract digestibility coefficients were calculated using indigestible marker methodology (Kerr et al., 2010).

Carcass Traits. At the end of the finisher 2 phase, animals from Exp. 2 and Exp. 3 were analyzed for loin-eye area (LEA) and backfat at the 10^{th} rib using ultrasound probing. Animals were then sent to a commercial slaughter facility where hot carcass weight (HCW) was provided. To calculate lean content, the procedure 6 equation from Burson (2006) was used: Lb. lean = 5.7769 + (0.401 x HCW, lbs) – (18.838 x 10^{th} rib fat depth, in.) + (4.357 x 10^{th} rib LEA, sq. in.) + (1.006 x sex of pig) (barrow = 1, gilt = 2). Lean was further converted from lbs to kg. Lean percentage was calculated as lean, percent = lean, kg / HCW, kg * 100.

Economic Analysis. For experiment 1, feed cost per kg of BW gain was calculated. Pen feed disappearance was multiplied by the feed cost of $0.31 per kg to determine pen feed cost. This was then divided by the net BW gain per pen to determine feed cost per kg of

BW gain. For experiments 2 and 3, slope and intercepts were determined for individual pigs based on weekly BW and days. Using a linear model, these values were then used to predict the number of days on feed each pig needed to reach a market weight of 128 kg or, if all pigs were marketed on the same day, their respective BW at day 167. Feed intake was calculated based on days on feed in each respective phase and at an average cost of $0.33 per kg. Barn cost was $0.10 per pig day. Carcass value was determined either at 128 kg for same weight marketing, or at calculated weight for market on the same day. Weights were converted to pounds and a dressing percentage of 75.6 was used, and finally multiplied by $54.95 per cwt based on the National Base Average price reported 12 December 2015. Barn and feed costs were subtracted from the carcass value to determine net profit.

Statistics. Data were analyzed with the MIXED procedure of SAS using LSMEANS with the Tukey-Kramer adjustment. In Exp. 1, pen was used as the experimental unit for growth performance and digestibility while BW at wk 0 served as a covariate. For viremia, IgM, and IgG, pig was used as the experimental unit with covariates of age at infection and d0 IgG, and pen and litter as random effects. In Exp. 2, pen was used as the experimental unit for all data. Both Exp. 1 and Exp. 2 were analyzed for the primary effects of PCV-status, genotype, and the interaction of both. The CC-PCV group was excluded from these effects although contrast statements and pairwise comparisons were made to discern the effect of genotype in the PCV group. In Exp. 3, pig was used as the experimental unit for all data and BW wk 0 as a covariate. The primary of effects of genotype, PCV-status, residual, and the corresponding interactions were used for Exp. 3.

RESULTS

Experiment 1. To determine the infectious status of the pigs, PCV2 viremia was measured in the serum. As expected, PCV pigs expressed higher PCV2 viremia than VAC counterparts from 7 to 42 days post infection (**dpi**) (**Figure 1**). No difference was observed between genotypes in the VAC group, whereas differences were observed in the PCV group. As expected, PCV pigs carrying at least one C allele had numerically decreased viremia compared to the TT genotype. The CC genotype showed reduced viremia compared from 7 to 28 dpi compared with TT genotype ($P < 0.05$) and 14 to 21 dpi with the CT genotype. Immunoglobulin response was measured next for PCV2 specific IgM and IgG. No difference was observed for genotype in either the PCV or VAC group. Similar to viremia, IgG was no different in the VAC group whereas the PCV group had a genotypic response. Post 14 dpi, IgG was numerically lower for PCV pigs presenting at least one C allele. The CC pigs had the lowest IgG levels of the PCV group, although only significantly different at 28 dpi ($P < 0.05$). Despite the differences in viremia and IgG titer, growth performance was not impacted by genotype, PCV status, or the interaction of genotype and PCV status (**Table 3.3**). Likewise, total tract digestibility for GE or DM was mostly unaffected by treatments, although the PCV group had increased ($P < 0.025$) digestibility during wk 2 compared with the VAC group (**Figure 3.3**). In terms of feed cost per kg of gain (**Figure 3.4**), genotype had no effect on cost for VAC pigs. Cost was largely decreased for CC-PCV pigs compared to the TT-PCV group ($2.27 v. 2.61); however, contrary to our expectations, the CT-PCV group had a highest cost ($2.81) out of the three genotypes.

Experiment 2. Pigs representing all 5 genotype-PCV status groups were fed through the grow-finish period (**Table 3.4**). Body weight and ADG showed no differences or trends through the period. Overall, ADFI was numerically higher for the TT-PCV group compared to other treatments. Specifically, TT-PCV had greater ADFI than CC-PCV (Grower 1) and CT-PCV (Finisher 1) ($P < 0.05$). Genotype had no effect on G:F, but the VAC pigs were more efficient than the PCV pigs from Grower 2 to Finisher 2 ($P <0.10$). Genotype was observed to effect nutrient digestibility during Grower 1 through Finisher 1, in favor of the C allele genotypes ($P < 0.05$). Vaccinated pigs appeared to have numerically higher digestibility, but only Finisher 1 was significant. Despite the previous differences in ADFI and ATTD, there were no differences in backfat, loin eye area or lean percentage for any of the treatments (**Table 3.5**). With respect to cost of production (**Table 3.6**), regardless of pigs slaughtered at same weight or age, the PCV2 infection increased cost compared with VAC pigs. Interestingly, genotype had a beneficial response by decreasing feed usage and by proxy cost and profit. Similar to Exp. 1, we saw favorable genotypes increase profit with the exception of the CT-PCV group, which had the highest feed cost and lowest profit of all treatment groups ($P < 0.05$).

Experiment 3. Pigs from the TT and CT genotypes with either PCV-status were split into high and low residuals and were fed individually through the grow-finish period (**Table 3.7**). Similar to the previous experiments, no difference in BW was observed. The low residual group had increased ADG during the finisher 1 phase compared to the high residual pigs ($P < 0.05$), while the PCV pigs had increased BW during the finisher 2 phase over the VAC group ($P < 0.10$). The VAC group was observed to have increased ADFI during grower 1 ($P < 0.05$), whereas the low residual was increased during the

finishing phase ($P < 0.05$). The low residual group also benefitted from improved feed efficiency during the grower 2 and finisher 1 phases ($P < 0.05$). No differences were observed for DM or GE ATTD during the experiment (**Table 3.8**). Lean weight and percentage ($P < 0.05$) were both increased, while backfat decreased ($P < 0.07$) in the CT pigs compared with TT pigs. Production costs were numerically lower in the CT-PCV group compared with the TT-PCV group by $3.00, but this difference was not present in the VAC pigs (**Table 3.9**). Unsurprisingly, pigs from the low residual group saw increased production costs either due to combination of increased feed intake and lower carcass yield compared to the high residual group ($P < 0.01$).

DISCUSSION

The objective of the experiments described was to assess the effects of genotypic selection at the SSC12 QTL (*ALGA0122080*) on nursery and long-term growth performance after a PCV2 infection or vaccination. This QTL, previously identified by Engle et al. (2014a), was one of two QTL found to be associated with viral load. Pigs with high viral loads showed a decrease in ADG during a four-week experimental PCV2b infection. At week 4, ADG in high viral load pigs remained 33% lower compared to low viral load pigs. However, this data was reflective of both QTLs reported, whereas our study only looked at SSC12 and encompassed the highest and lowest 10% of pig viral load. In the current experiment, pigs infected with PCV2 showed a phenotypic response for viremia, IgG, and IgM as expected among the different genotypes represented. In addition, VAC pigs began show an increase in PCV2 specific IgG and IgM after d 35 indicating the potential for PCV2 infection, however, this was not further investigated.

Data previously reported by McKnite et al. (2014) observed a reduction in viral load as the total number of favorable alleles, across multiple SNP sites, increased. However, we did not see an increase in ADG for pigs carrying the favorable C allele throughout our study. This differs from previous work (McKnite et al., 2014) as pigs carrying at least one favorable allele were observed to have a 30% increase in ADG; however, this occurred over three different SNP sites. Additionally, our pigs were not genotyped for the previously reported SNPs *ALGA0050315* (SSC9) and *MARC0001766* (SSC12) (McKnite et al., 2014) or swine leukocyte antigen II (SSC7) (Engle et al., 2014b). Thus, the lack of variation between treatments could be a result of the presence, or lack therefore, of these favorable alleles. Long-term growth performance over Exp. 2 and 3 showed no difference between genotypes, however, ATTD increased with favorable allele presence in infected pigs. Over the course of the three experiments, genotype had no impact on the growth performance or digestibility of pigs vaccinated for PCV2, implying that genotypic effects may be largely a result of experimental PCV2 infection as opposed to PCV vaccination. Interestingly, despite the lack of differences in growth performance, analysis of the data for production costs appears to indicate that presence of the favorable alleles may reduce production costs by $4-10 per head. These savings appear to be largely driven by decrease in ADFI, which, when also considering viremia and IgG production, would indicate a decreased immune response, and thus a reduction in energy and amino acids needed to fuel that response (Pastorelli et al., 2011).

This study did not explore the use of a co-infection model, but as PCVAD is known to include additional pathogens with PCV2 (Segalés, 2012), utilizing a co-infection model will be needed. Chae (2016) summarized that PCV2 viremia can be

enhanced by prior PRRS or *Mycoplasma hyopneumoniae* vaccination or infection and that PVC2 vaccination should be a priority in herds. Vaccination for PCV2 was shown to improve pig performance in commercial settings (Jacela et al., 2011; Shelton et al., 2012; Potter et al., 2014). Although there are a number of effective PCV2 vaccines on the market (da Silva et al., 2014) that may preclude the need for this work, the *UNLPCV2.2009* SNP may be beneficial to pigs experiencing PCVAD conditions.

Overall, these data show that pigs containing favorable alleles at the *UNLPCV2.2009* SNP have decreased viremia, and through immunoglobulin concentration or titers, a decreased immune response, allowing them to handle a PCV2 challenge more effectively. While this does not necessarily improve growth performance during the challenge or subsequent growth and finish phases, it may have an impact on reducing production cost for producers.

LITERATURE CITED

Beach, N. M., and X.-J. Meng. 2012. Efficacy and future prospects of commercially available and experimental vaccines against porcine circovirus type 2 (pcv2). Virus Research 164:33-42.

Burkey, T. E., K. A. Skjolaas, and J. E. Minton. 2009. Board-invited review: Porcine mucosal immunity of the gastrointestinal tract1. Journal of Animal Science 87:1493-1501.

Burson, D. 2006. Procedures for estimating pork carcass composition, Pork Information Gateway.

Chae, C. 2016. Porcine respiratory disease complex: Interaction of vaccination and porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, and mycoplasma hyopneumoniae. The Veterinary Journal 212:1-6.

da Silva, N., A. Carriquiry, K. O'Neill, T. Opriessnig, and A. M. O'Connor. 2014. Mixed treatment comparison meta-analysis of porcine circovirus type 2 (pcv2) vaccines used in piglets. Preventive Veterinary Medicine 117:413-424.

Engle, T., E. Jobman, T. Moural, A. McKnite, S. Barnes, E. Davis, J. Qiu, J. Bundy, J. Tart, T. Johnson, M. F. Rothschild, J. Galeota, R. K. Johnson, G. Plastow, S. Kachman, and D. C. Ciobanu. 2014a. Genome-wide analysis of the differential response to experimental challenges with porcine circovirus 2b, 10th World Congress on Genetics Applied to Livestock Production.

Engle, T. B., E. E. Jobman, T. W. Moural, A. M. McKnite, J. W. Bundy, S. Y. Barnes, E. H. Davis, J. A. Galeota, T. E. Burkey, G. S. Plastow, S. D. Kachman, and D. C. Ciobanu. 2014b. Variation in time and magnitude of immune response and viremia in experimental challenges with porcine circovirus 2b. BMC Veterinary Research 10:286.

Gillespie, J., T. Opriessing, X. J. Meng, K. Pelzer, and V. Buechner-Maxwell. 2009. Porcine circovirus type 2 and porcine circovirus-associated disease. J Vet Intern Med 23:1151-1163.

Jacela, J. Y., S. S. Dritz, J. M. DeRouchey, M. D. Tokach, R. D. Goodband, and J. L. Nelssen. 2011. Field evaluation of the effects of a porcine circovirus type 2 vaccine on finishing ig growth performance, carcass characterisitics, and mortality rate in a herd with a history of porcine circovirus-associated disease. J Swine Health Prod 19:10-18.

Jacobs, B. M., J. F. Patience, W. A. Dozier 3rd, K. J. Stalder, and B. J. Kerr. 2011. Effects of drying methods on nitrogen and energy concentrations in pig feces and urine, and poultry excreta. J. Anim. Sci. 89:2624-2630.

Kerr, B. J., T. E. Weber, P. S. Miller, and L. L. Southern. 2010. Effect of phytase on apparent total tract dgestibility of phosphorus in corn-soybean meal diets fed to finishing pigs. J. Anim. Sci. 88:238-247.

McKnite, A. M., J. W. Bundy, T. W. Moural, J. K. Tart, T. P. Johnson, E. E. Jobman, S. Y. Barnes, J. K. Qiu, D. A. Peterson, S. P. Harris, M. F. Rothschild, J. A. Galeota, R. K. Johnson, S. D. Kachman, and D. C. Ciobanu. 2014. Genomic analysis of the differential response to experimental infection with porcine circovirus 2b. Animal Genetics 45:205-214.

Patience, J. F., A. D. Beaulieu, and N. H. Williams. 2009. Response to dietary digestible energy concentration in growing pigs fed cereal grain-based diets. Journal of Animal Science 87:965-976.

Potter, M. L., J. Y. Jacela, J. R. Bergstrom, S. S. Dritz, M. D. Tokach, J. M. DeRouchey, R. D. Goodband, and J. L. Nelssen. 2014. Direct comparison of commercial porcine circovirus type 2 vaccines under field conditions. The Professional Animal Scientist 30:80-85.

Segalés, J. 2012. Porcine circovirus type 2 (pcv2) infections: Clinical signs, pathology and laboratory diagnosis. Virus Research 164:10-19.

Shelton, N. W., M. D. Tokach, S. S. Dritz, R. D. Goodband, J. L. Nelssen, J. M. DeRouchey, and J. L. Usry. 2012. Effects of porcine circovirus type 2 vaccine and increasing standardized ileal digestible lysine:Metabolizable energy ratio on growth performance and carcass composition of growing and finishing pigs1. Journal of Animal Science 90:361-372.

Table 3.1. Diet formulation and chemical composition for experiment 1 (%, as-fed basis).[1]

Ingredients, %	Diet
Corn	58.99
SBM, 47.7% CP	33.40
Dicalcium phosphate, 18.5%	1.73
Limestone	0.35
Salt	0.30
Vitamin premix[2]	0.25
Trace mineral premix[3]	0.15
Corn oil	3.85
Lysine-HCl	0.30
DL-Methionine	0.11
L-Threonine	0.07
Titanium dioxide	0.50
Calculated composition, %	
SID Lys	1.22
SID Thr	0.72
SID Trp	0.23
SID Met	0.39
CP	21.16
ME, kcal/kg	3.56
Ca	0.86
Available P	0.43

[1]Formulated using NRC (2012)

[2]Vitamin premix supplied per kg of diet: vitamin A (as retinyl acetate), 5500 IU; vitamin D (as cholecalciferol), 550 IU; vitamin E (as tocopheryl acetate), 30 IU; vitamin K (as menadione dimethylpyrimidinol bisulfate), 4.4 mg; riboflavin, 11.0 mg; d-pantothenic acid, 22.05 mg; niacin, 33.0 mg; vitamin B12 (as cyanocobalamin), 33.0 mg

[3]Trace mineral premix containing: copper (as CuSO4H2O), 10 mg/kg; iodine (as Ca(IO3) · H2O), 0.25 mg/kg; iron (as FeSO4 · 2H2O), 125 mg/kg; manganese (MnO), 15 mg/kg; selenium (Na2SeO3), 0.3 mg/kg; zinc (ZnSO4 · H2O), 125 mg/kg

Table 3.2. Diet formulation and predicted composition for experiments 2 and 3[1].

	Grower 1	Grower 2	Finisher 1	Finisher 2
Ingredients, %				
Corn[2]	59.92	65.14	68.36	74.55
Soybean meal, 46.5% CP	33.00	28.00	25.00	19.00
Dicalcium phosphate, 18.5% P	1.00	0.90	0.70	0.55
Limestone	0.85	0.75	0.73	0.70
Salt	0.30	0.30	0.30	0.30
Vitamin premix[3]	0.25	0.25	0.25	0.25
Trace mineral premix[4]	0.15	0.15	0.15	0.15
Corn Oil	4.00	4.00	4.00	4.00
Lysine·HCl	0.30	0.30	0.30	0.30
DL-Methionine	0.08	0.06	0.05	0.04
L-Threonine	0.15	0.15	0.15	0.14
L-Trp	0.00	0.00	0.01	0.02
Calculated composition, %				
CP	17.63	15.89	14.86	12.78
ME, kcal/kg	3474	3485	3496	3508
Ca	0.67	0.60	0.53	0.47
STTD P	0.32	0.29	0.25	0.22
SID Lys	1.22	1.09	1.02	0.87
SID Met	0.36	0.32	0.30	0.26
SID Thr	0.80	0.73	0.69	0.60
SID Trp	0.23	0.20	0.19	0.17

[1]Formulated using NRC (2012)

[2]Titanium dioxide added at 0.5% in diet, in place of corn

[3]Vitamin premix supplied per kg of diet: vitamin A (as retinyl acetate), 5500 IU; vitamin D (as cholecalciferol), 550 IU; vitamin E (as tocopheryl acetate), 30 IU; vitamin K (as menadione dimethylpyrimidinol bisulfate), 4.4 mg; riboflavin, 11.0 mg; d-pantothenic acid, 22.05 mg; niacin, 33.0 mg; vitamin B_{12} (as cyanocobalamin), 33.0 mg

[4]Trace mineral premix containing: copper (as $CuSO_4H_2O$), 10 mg/kg; iodine (as $Ca(IO_3) \cdot H_2O$), 0.25 mg/kg; iron (as $FeSO_4 \cdot 2H_2O$), 125 mg/kg; manganese (MnO), 15 mg/kg; selenium (Na_2SeO_3), 0.3 mg/kg; zinc ($ZnSO_4 \cdot H_2O$), 125 mg/kg

Table 3.3. Growth performance[1] data from Exp. 1 for pigs selected for genotype (*CC, CT,* and TT)[2] vaccinated for or inoculated with porcine circovirus type 2 (PCV2)

Item	Inoculated			Vaccinated		SE	*P*-value
	CC	CT	TT	CT	TT		
BW, kg							
d 0[3]	7.1	7.1	7.1	7.1	7.1		
d 7	8.5	8.7	8.9	8.8	8.8	0.21	0.65
d 14	11.7	11.6	12.0	11.8	11.7	0.39	0.87
d 21	15.6	15.4	15.7	16.0	15.4	0.62	0.89
d28	20.0	19.2	19.6	20.2	19.7	0.82	0.79
d 35	25.5	24.5	24.9	25.8	25.4	1.11	0.78
d 42	31.8	30.5	30.8	32.0	31.5	1.40	0.83
d 0-7							
ADFI, kg/d	0.48	0.51	0.52	0.55	0.53	0.067	0.67
ADG, kg/d	0.22	0.23	0.22	0.24	0.22	0.030	0.70
G:F	0.47	0.44	0.46	0.48	0.43	0.084	0.99
d 7-14							
ADFI, kg/d	0.80	0.73	0.68	0.72	0.73	0.047	0.82
ADG, kg/d	0.48	0.43	0.44	0.45	0.40	0.035	0.78
G:F	0.60	0.59	0.64	0.62	0.56	0.043	0.48
d 14-21							
ADFI, kg/d	0.83	0.85	0.87	0.91	0.89	0.073	0.91
ADG, kg/d	0.58	0.54	0.51	0.60	0.51	0.047	0.75
G:F	0.70	0.64	0.59	0.67	0.59	0.074	0.93
d 21-28							
ADFI, kg/d	1.15	1.11	1.06	1.07	1.10	0.093	0.97
ADG, kg/d	0.69	0.56	0.53	0.64	0.58	0.053	0.51
G:F	0.61	0.51	0.51	0.61	0.54	0.082	0.78
d 28-35							
ADFI, kg/d	1.54	1.25	1.43	1.30	1.48	0.151	0.30
ADG, kg/d	0.84	0.76	0.72	0.81	0.79	0.063	0.79
G:F	0.55	0.63	0.53	0.64	0.54	0.098	0.78
d 35-42							
ADFI, kg/d	1.58	1.60	1.56	1.58	1.64	0.097	0.86
ADG, kg/d	0.93	0.87	0.83	0.89	0.85	0.058	0.96
G:F	0.59	0.55	0.54	0.57	0.52	0.060	0.93

[1]BW = body weight; ADFI = average daily feed intake; ADG = average daily gain; G:F = gain to feed ratio

[2]CC = resistant to PCV2, CT = heterozygous, TT = susceptible to PCV2

[3]Group average body weight of 7.1 kg was used for statistical estimates

Table 3.4. Growth performance[1] data from 14-week grow-finish period (Experiment 2) of pigs selected for genotype (*CC, CT,* and *TT*) and vaccinated for or inoculated with porcine circovirus type 2 (PCV2)

Treatment	Inoculated			Vaccinated		SEM	*P*-value
Genotype	CC	CT	TT	CT	TT		
BW, kg							
wk 0	40.0	40.1	37.5	35.2	39.5	2.04	0.414
wk 1 to 2	53.0	54.0	51.3	48.1	52.9	2.39	0.473
wk 2 to 6	77.5	77.0	76.0	73.9	76.7	2.05	0.755
wk 6 to 10	106.9	108.6	105.5	104.5	106.9	2.60	0.826
wk 10 to 14	131.4	135.6	133.9	132.5	130.6	3.78	0.886
ADFI, kg/d							
wk 1 to 2**	2.16[a]	2.63[ab]	2.79[b]	2.46[ab]	2.46[ab]	0.122	0.029
wk 2 to 6	2.93	3.04	3.35	3.12	3.08	0.133	0.295
wk 6 to 10††	3.39[ab]	3.23[a]	4.08[b]	3.49[ab]	3.71[ab]	0.182	0.041
wk 10 to 14[#]	3.28	3.57	4.50	3.76	3.61	0.277	0.069
ADG, kg/d							
wk 1 to 2	0.93	0.99	0.98	0.93	0.96	0.04	0.689
wk 2 to 6	1.16	1.16	1.16	1.21	1.13	0.039	0.774
wk 6 to 10	1.03	1.05	1.04	1.08	1.03	0.075	0.987
wk 10 to 14	0.81	0.94	1.02	0.97	0.85	0.063	0.161
G:F							
wk 1 to 2	0.38	0.4	0.42	0.39	0.41	0.039	0.967
wk 2 to 6†	0.4	0.39	0.35	0.39	0.37	0.013	0.127
wk 6 to 10†	0.3	0.33	0.25	0.31	0.28	0.018	0.066
wk 10 to 14†	0.25	0.27	0.23	0.26	0.24	0.014	0.259

[1]BW = body weight; ADFI = average daily feed intake; ADG = average daily gain; G:F = gain to feed ratio

[a,b]Superscripts represent differences between treatments, $P < 0.05$

*Genotype, $P < 0.10$; **Genotype, $P < 0.05$

†PCV status, $P < 0.10$; ††PCV status, $P < 0.05$

[#]Genotype x PCV, $P < 0.10$; [##]Genotype x PCV, $P < 0.05$

Table 3.5. Carcass trait and digestibility coefficients during 14-week grow-finish (Experiment 2) period of pigs selected for genotype (CC, CT, and TT) and vaccinated for or inoculated with porcine circovirus type (PCV2)

Treatment	Inoculated			Vaccinated		SEM	*P*-value
Genotype	CC	CT	TT	CT	TT		
Backfat, cm	2.36	2.29	2.38	2.66	2.41	0.163	0.499
LEA[1], cm^2	54.4	53.9	55.6	55.7	52.4	1.83	0.644
Lean, kg	54.8	55.5	54.0	54.5	54.0	1.24	0.890
Lean, %	51.5	51.2	51.9	50.4	50.4	0.81	0.592
DM ATTD[1], %							
wk 2**	80.6	78.9	77.0	80.4	82.0	1.18	0.084
wk 6**	84.5^{b}	82.8^{ab}	81.3^{a}	83.0^{ab}	82.8^{ab}	0.61	0.035
wk 10**††$^{\#\#}$	85.1^{b}	84.4^{b}	80.8^{a}	83.9^{ab}	83.7^{ab}	0.73	0.023
wk 14$^{\#}$	83.8	84.8	81.7	83.9	84.1	0.84	0.165
GE ATTD[1], %							
wk 2**	80.4	78.3	76.5	80.3	82.0	1.27	0.065
wk 6**	84.9^{b}	82.8^{ab}	81.5^{a}	83.4^{ab}	83.0^{ab}	0.69	0.053
wk 10*††$^{\#}$	85.2^{b}	84.4^{ab}	80.6^{a}	83.9^{ab}	83.7^{ab}	0.84	0.037
wk 14$^{\#}$	83.3	84.7	81.1	83.3	83.9	0.91	0.123

[1]LEA = loin eye area; ATTD = apparent total tract digestibility; DM = dry matter; GE = gross energy

^{a,b}Superscripts represent differences between treatments, $P < 0.05$

*Genotype, $P < 0.10$; **Genotype, $P < 0.05$

†PCV status, $P < 0.10$; ††PCV status, $P < 0.05$

$^{\#}$Genotype x PCV, $P < 0.10$; $^{\#\#}$Genotype x PCV, $P < 0.05$

Table 3.6. Production costs for experiment 2 when sold at same live weight or age.

Treatment	PCV[1]			VAC[1]		SEM	*P*-value
Genotype	CC	CT	TT	CT	TT		
Market at same live weight							
Feed used, kg	263.7	312.28	278.93	255.93	277.63	10.74	0.020
Feed cost, $	87.17	102.90	92.10	84.70	91.54	3.47	0.020
Barn cost[2], $	16.93	16.87	16.98	16.6	16.93	0.34	0.939
Carcass value[3], $	154.70	154.70	154.70	154.70	154.70		
Net Profit, $	50.63	34.93	45.65	53.45	46.20	3.68	0.028
Market at same age							
Feed used, kg	263.5	314.15	227.9	267.5	277.73	10.01	0.021
Feed cost, $	87.10	103.55	91.80	88.42	91.65	3.29	0.023
Live weight, kg	127.99	129.12	127.46	131.35	128.03		
Carcass value[3], $	154.73	156.09	154.08	158.79	154.78	4.08	0.928
Net Profit, $	59.03	43.96	53.70	61.80	54.51	3.58	0.029

[1]PCV = inoculated with porcine circovirus 2; VAC = vaccinated for porcine circovirus 2

[2]Barn cost $0.10 per pig day

[3]Carcass value based on $54.95 per cwt, National Base Average price reported 12 Dec 2015 www.dailylivestockreport.com

Table 3.7. Growth performance[1] of high and low residual pigs selected for genotype (CT or TT) and previously vaccinated for or inoculated with PCV2

Treatment	Inoculated				Vaccinated				SEM	*P*-value		
Genotype	CT		TT		CT		TT			Trt	Genotype	Residual
Residual	H	L	H	L	H	L	H	L				
BW, kg												
wk 0	44.5	29.4	41.7	25.0	40.2	31.8	41.3	34.4	2.61	0.334	0.637	0.001
wk 1 to 2	52.1	52.1	52.2	51.3	51.5	51.5	52.6	52.7	1.6	0.827	0.666	0.902
wk 2 to 6	82.2	74.1	78.6	70.9	77.3	72.5	77.8	78.6	4.51	0.964	0.978	0.223
wk 6 to 10	109.2	109.1	107.4	106.4	106.8	103.4	105.0	112.0	4.71	0.628	0.816	0.881
wk 10 to 14	132.8	140.7	134.5	136.8	131.4	130.0	128.9	139.5	5.59	0.235	0.692	0.332
ADFI, kg/d												
wk 1 to 2	2.34	2.04	2.21	1.86	2.29	2.21	2.41	2.40	0.136	0.007	0.995	0.141
wk 2 to 6	3.04	3.07	3.00	2.90	2.82	2.87	2.93	3.21	0.146	0.594	0.419	0.618
wk 6 to 10	3.10	3.71	3.23	3.78	3.32	3.48	3.19	3.72	0.226	0.812	0.512	0.028
wk 10 to 14	3.16	3.87	3.62	3.81	3.32	3.67	3.19	3.70	0.221	0.243	0.530	0.033
ADG, kg/d												
wk 1 to 2	1.16	1.14	1.16	1.06	1.11	1.10	1.19	1.19	0.116	0.781	0.703	0.750
wk 2 to 6	1.17	1.23	1.12	1.24	1.13	1.11	1.04	1.26	0.065	0.123	0.881	0.102
wk 6 to 10	0.84	1.28	0.97	1.30	0.99	1.10	0.94	1.18	0.099	0.383	0.379	0.004
wk 10 to 14	0.89	1.10	0.95	0.99	0.87	0.92	0.82	0.91	0.103	0.091	0.618	0.290
G:F												
wk 1 to 2	0.49	0.55	0.52	0.59	0.49	0.50	0.49	0.50	0.049	0.102	0.499	0.419
wk 2 to 6	0.38	0.41	0.37	0.44	0.40	0.39	0.35	0.40	0.016	0.084	0.440	0.047
wk 6 to 10	0.26	0.35	0.30	0.35	0.30	0.32	0.29	0.32	0.018	0.406	0.385	0.008
wk 10 to 14	0.28	0.28	0.27	0.26	0.26	0.25	0.25	0.25	0.023	0.139	0.266	0.772

[1]BW = body weight; ADFI = average daily feed intake; ADG = average daily gain; G:F = gain to feed ratio; H = high; L = low

BW wk0: Trt*residual 0.035

ADFI G1: Trt*Genotype 0.049; Trt*residual 0.096; G2: Trt*Genotype 0.053

ADG G2: Genotype*residual 0.037; F1: Trt*residual 0.082

G:F G2: Genotype*residual 0.005; F1: Trt*Residual 0.037

Table 3.8. Carcass traits and digestibility of DM and GE of high and low residual pigs selected for genotype (CT or TT) and previously vaccinated for or inoculated with porcine circovirus type 2 (PCV2)

Treatment	Inoculated				Vaccinated				SEM	*P*-value		
Genotype	CT		TT		CT		TT			Trt	Genotype	Residual
Residual	H	L	H	L	H	L	H	L				
Backfat, cm	2.45	2.71	2.62	2.74	2.10	2.63	2.59	2.96	0.251	0.661	0.071	0.160
LEA, cm^2	51.9	57.8	50.2	54.3	54.2	49.6	48.0	50.9	3.35	0.124	0.169	0.484
Lean, kg	53.1	56.0	52.0	49.5	53.7	52.0	52.4	50.8	2.05	0.713	0.027	0.679
Lean, %	50.3	50.8	49.2	50.3	52.4	49.1	48.5	47.9	1.43	0.375	0.035	0.623
DM ATTD, %												
wk 1-2	83.9	84.2	84.3	85.2	84.6	83.9	85.3	84.7	1.54	0.798	0.388	0.994
wk 2 to 6	83.2	83.8	83.4	83.6	84.6	83.6	83.2	85.1	1.19	0.359	0.953	0.662
wk 6 to 10	84.1	84.6	84.3	85.1	85.2	83.8	82.3	85.3	1.17	0.550	0.771	0.488
wk 10 to 14	85.9	86.9	84.6	84.9	84.7	85.0	83.3	85.6	1.17	0.157	0.125	0.358
GE ATTD, %												
wk 1-2	83.3	84.1	83.8	85.4	84.3	83.4	85.0	84.6	1.79	0.818	0.354	0.856
wk 2 to 6	83.5	84.0	83.7	84.1	85.0	83.7	83.8	85.7	1.22	0.301	0.663	0.743
wk 6 to 10	84.3	84.5	84.4	85.2	85.2	83.5	82.5	85.5	1.25	0.552	0.990	0.598
wk 10 to 14	85.9	86.8	84.4	84.8	84.5	84.8	82.8	85.6	1.23	0.140	0.113	0.311

[1]LEA = loin eye area; ATTD = apparent total tract digestibility; DM = dry matter; GE = gross energy

Lean%: Trt*residual 0.098

DM F1: Genotype*Residual 0.072

GE F1: Genotype* residual 0.052

Table 3.9. Production costs for experiment 3 when sold at same live weight or age.

	PCV[1]		VAC[1]			Residual			*P*-value[4]
Item	CT	TT	CT	TT	SEM	High	Low	SEM	Residual
Market at same live weight									
Feed used, kg	249.88	262.91	266.34	260.57	6.79	244.86	274.99	4.8	0.0001
Feed cost, $	82.6	86.65	87.85	86.25	2.12	81.1	90.58	1.5	0.0001
Barn cost[2], $	16.43	16.88	17.04	16.54	0.31	16.33	17.1	0.22	0.0189
Carcass value[3], $	117.0	117.0	117.0	117.0		117.0	117.0		
Net Profit, $	17.98	13.44	12.09	14.21	2.38	19.54	9.31	1.69	0.0002
Market at same age									
Feed used, kg	268.59	263.39	259.99	274.9	7.09	266.44	266.99	5.02	0.938
Feed cost, $	88.61	86.83	85.85	90.79	2.34	88	88.04	1.65	0.987
Live weight, kg	131.48	126.06	124.93	130.52	3.44	132.56	123.94	2.43	0.0186
Carcass value[3], $	120.16	115.21	114.18	119.29	3.14	121.15	113.27	2.22	0.0186
Net Profit, $	22.94	19.79	19.74	19.9	1.68	24.55	16.63	1.19	0.0001

[1]PCV = inoculated with porcine circovirus 2; VAC = vaccinated for porcine circovirus 2

[2]Barn cost $0.10 per pig day

[3]Carcass value based on $54.95 per cwt, National Base Average price reported 12 Dec 2015 www.dailylivestockreport.com

[4]No significant differences were found for PCV-status or genotype

Table S3.1. Experimental design for experiment 1.

156 barrows

↓

Screened for genotype and PCV2-specific maternal antibodies[1]

↓

111 barrows

Genotype	CC	CT		TT	
PCV-status[2]	PCV	PCV	VAC	PCV	VAC
Pigs / treatment	11	32	20	28	20
Pens / treatment	3	7	4	6	4

[1]Sample to positive ratio < 1.26 (IgG) and 1.0 (IgM) were used
[2]PCV = inoculated with PCV2; VAC = vaccinated with PCV2

Table S3.2. Experimental design for experiment 2 and 3.

Experiment 1 pigs (n = 111)

Statistical model for expected body weight v. actual body to calculate residual

Pigs with residual near 0 → Experiment 2

Pigs with residual farther from 0 → Experiment 3

Genotype	CC	CT		TT		CT				TT				
PCV-status[1]	PCV	PCV	VAC	PCV	VAC	PCV		VAC		PCV		VAC		
Residual						H	L	H	L	H	L	H	L	
Pigs/treatment	8	8	8	8	8	4	4	4	4	4	4	4	4	

[1]PCV = inoculated with PCV2; VAC = vaccinated with PCV2

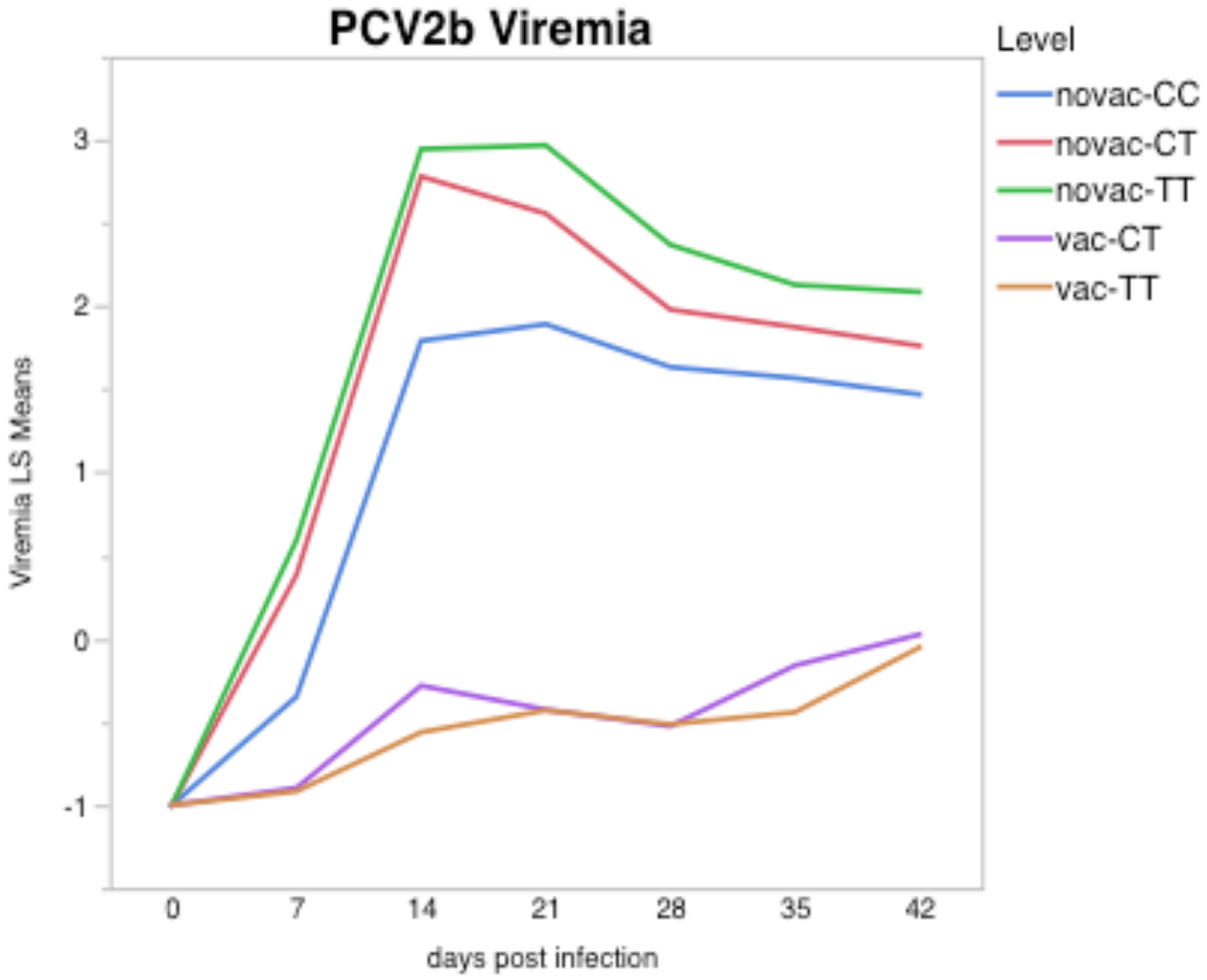
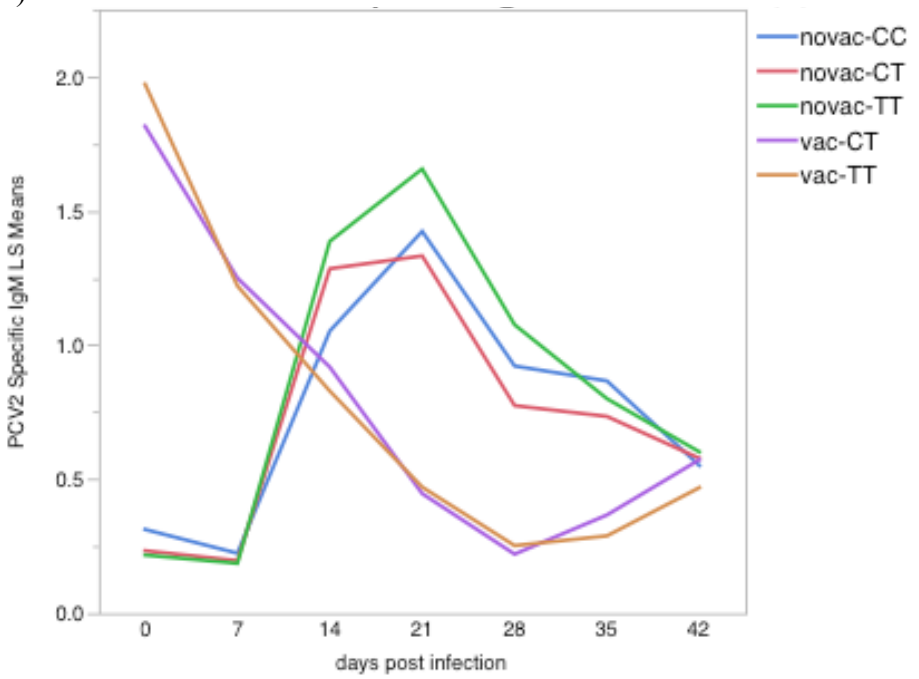


Figure 3.1. Weekly serum viremia of pigs vaccinated for or inoculated with porcine circovirus type 2 (PCV2) and genotyped during experiment 1.

A)



B)

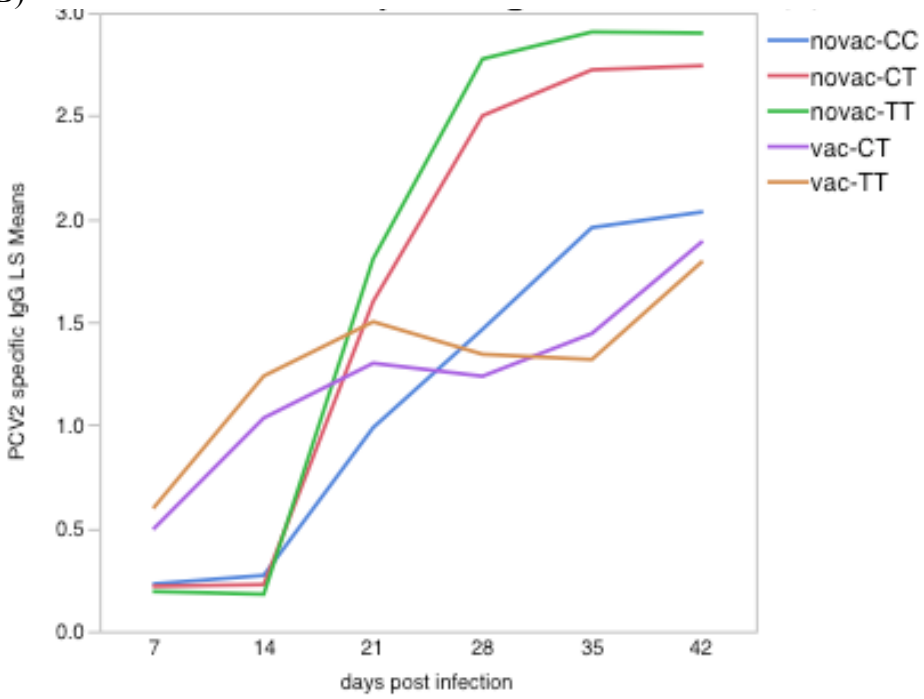


Figure 3.2. Serum titers of porcine circovirus type 2 (PCV2)-specific IgM (A) and IgG (B) in pigs vaccinated for or inoculated with PCV2 and genotyped during experiment 1.

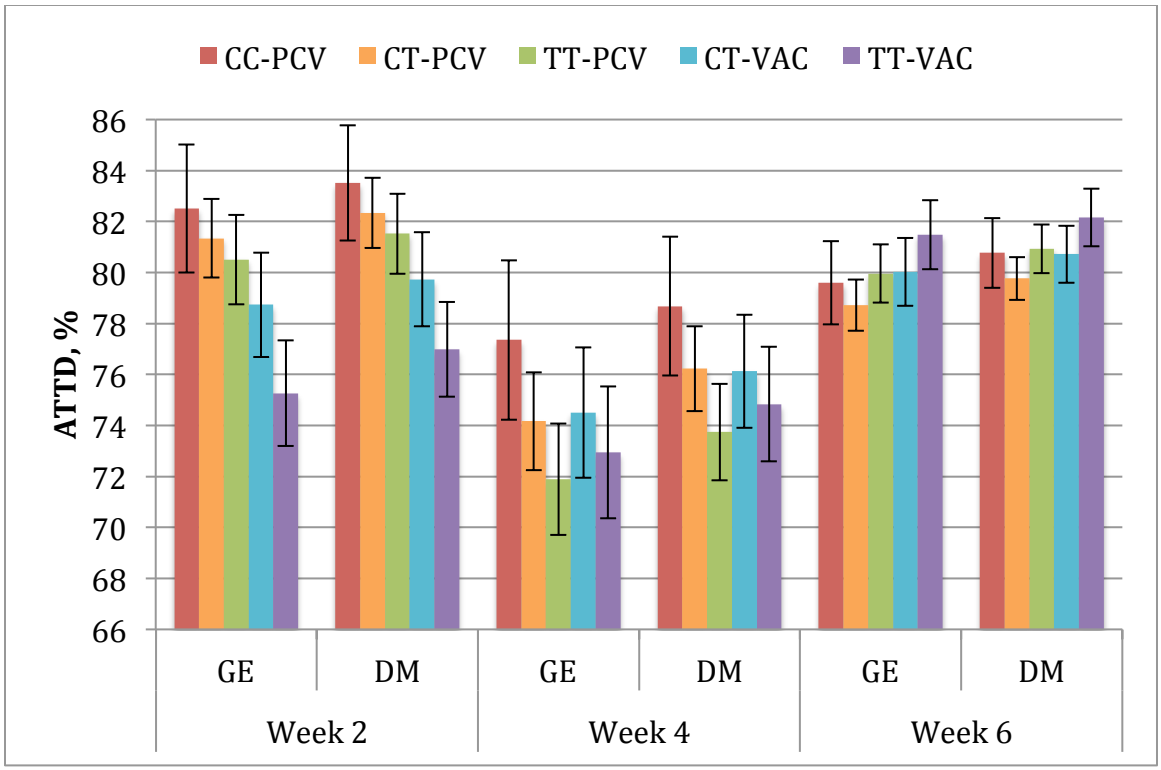


Figure 3.3. Apparent total tract digestibility coefficients for gross energy (GE) and dry matter (DM) at wk 2, 4, and 6 of Experiment 1. PCV = inoculated with porcine circovirus 2 (PCV2); VAC = vaccinated with PCV2. Bars represent least square means ± SEM.

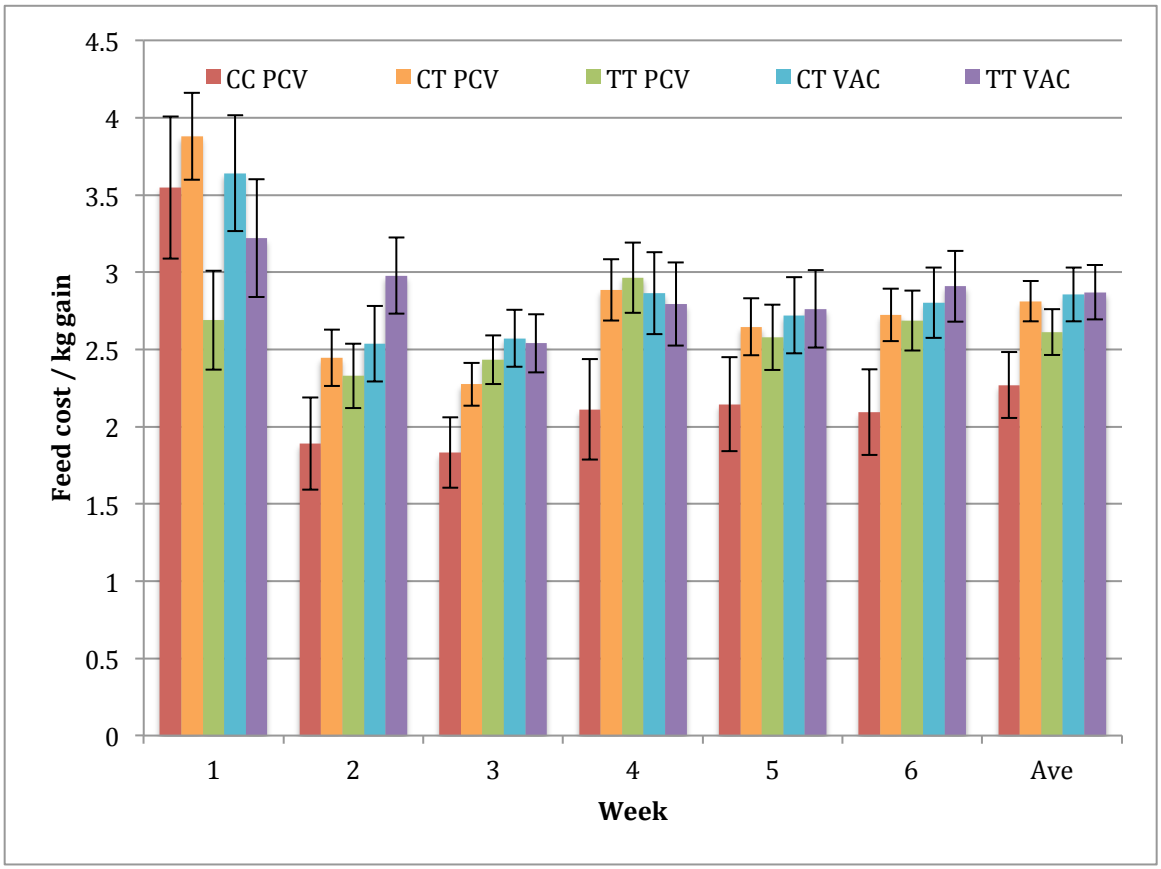


Figure 3.4. Feed cost per kg of body weight gain during experiment 1. PCV = inoculated with porcine circovirus 2 (PCV2); VAC = vaccinated with PCV2. Bars represent least square means ± SEM

CHAPTER 4. SUPPLEMENTATION OF TRYPTOPHAN ALTERS PIGLET GROWTH PERFORMANCE, DIGESTIBILITY, AND *LACTOBACILLUS* POPULATIONS

A manuscript prepared for submission to the *Journal of Animal Science*

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ABSTRACT: Weaning is a particularly important transition for the young pig due to a variety of factors. This is emphasized with current trends to restrict the use of antibiotics in feed. In an effort to improve weaning transition, two experiments were conducted to examine the effects of additional tryptophan (Trp) in nursery diets on weaned pig growth performance, digestibility, and *Lactobacillus* populations. In Exp. 1, 72 weanling pigs were split across two replications and randomly allocated across six pens, with a total of six pens per treatment. A two-phase nursery diet was utilized with a control diet (0.22 SID Trp:Lys) or control plus L-Trp (0.025%, Phase 1; 0.02%, Phase 2; 0.24 SID Trp:Lys). In Exp. 2, 72 weanling pigs were randomly allocated across 18 pens and assigned to one of three diets: control and Trp diets (as in Exp. 1), or Trp+ (0.05%, Phase 1; 0.04%, Phase 2; 0.26 SID Trp:Lys). Diets in Exp. 2 contained titanium dioxide as a marker of digestibility. In both experiments, phase 1 and 2 diets were fed for 2 and 3 wk, respectively. Growth performance was recorded weekly, and fecal samples were collected at the end of each dietary phase for microbiome analysis. Statistical analysis was performed utilizing Proc Mixed (SAS 9.2) with pen as the experimental unit and wk 0 BW as a covariate. In Exp. 1, no difference was observed in BW, ADG, or ADFI. Gain to feed ratio, however, was increased 9% in pigs on the Trp diet across both phases ($P < 0.01$). In Exp. 2, no differences in BW, ADG, ADFI, or G:F were observed. Phase 2 DM and GE digestibility was increased ($P < 0.01$) in Trp pigs, as compared with to control pigs ($P < 0.01$). *Lactobacillus* populations increased with Trp supplementation in Exp. 1 ($P > 0.10$), but not Exp. 2. Together, increased Trp supplementation may improve nursery pig performance.

Key words: Lactobacillus, pig, tryptophan

INTRODUCTION

Implementation of the Veterinary Feed Directive has resulted in the removal of antibiotics being used for growth promotion purposes in animal agriculture from the market. As a result, there is increased emphasis on using antibiotic alternatives to improve the weaning transition and promote growth. Despite the large number of antibiotic alternatives on the market, a recent literature review by Schweer et al. (2017) found that 66.5% of alternatives exhibit no impact on ADG compared to the 28.6% that have a positive response. Thus, there is a need to continue developing strategies for improving piglet performance.

Tryptophan is an essential amino acid that can be limiting in the swine diet. Because of its role in immune function, limiting tryptophan reduces N retention and protein deposition efficiency of tryptophan under immune stimulation (de Ridder et al., 2012). Supplementing tryptophan in the diet has been shown to be beneficial to animals under health challenge by supporting immune function and improving feed efficiency (Capozzalo et al., 2012; Messori et al., 2013; Xu et al., 2014; Capozzalo et al., 2015). Tryptophan is used for proteins synthesis and tissue deposition, but also serves as a precursor to serotonin, niacin, and indole derivatives. While tryptophan is important for host tissue growth and function, microbes in the gut can also utilize nutrients prior to their absorption by the host.

Lactobacillus species are prominent members of the microbiome in suckling and weanling piglets when lactose is consumed. However, *Lactobacillus* decreases in abundance, proportionally speaking, as the piglet ages and the diet changes (Frese et al., 2015). The *Lactobacillus* species are capable of metabolizing tryptophan under

carbohydrate starvation conditions (Gummalla and Broadbent, 1999), but may metabolize tryptophan into indole derivatives under normal gut nutrient conditions (Zelante et al., 2013). Tryptophan supplementation in conventional mice resulted in an increase in *Lactobacillus reuteri* in the stomach and *L. reuteri* mediated conversion of indole-3-aldehyde from tryptophan causing increased IL-22 production via the aryl hydrocarbon receptor (AhR) (Zelante et al., 2013). Interleukin-22 stimulates antimicrobial factors from Paneth cells in the intestinal crypts (Eyerich et al., 2017). It has been reported that Paneth cells are absent in pigs (Trautman and Fiebiger, 1952), however, it was shown that epithelial cells can respond to IL-22 stimulus resulting in production of antiviral factors and beta defensin 2 (Xue et al., 2017). Altogether, metabolism of tryptophan by *Lactobacillus* can stimulate the AhR-IL-22 pathway potentially benefitting host gut health.

Although tryptophan has been supplemented in pig diets and under disease challenges previously (Capozzalo et al., 2012; Messori et al., 2013; Xu et al., 2014; Capozzalo et al., 2015; Goncalves et al., 2015), it has not been shown if supplementing tryptophan benefits pigs through an increase in intestinal *Lactobacillus* populations or animal health through the AhR-IL-22 pathway.

MATERIALS AND METHODS

The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Nebraska, Lincoln.

Animals and Experimental Design

A total of 144 ((Landrace x Nebraska White) x Duroc) were used across two experiments. Piglets were weaned at the ENREC Swine Unit (Mead, NE) of the University of Nebraska. Pigs were transported to the Animal Science Complex for studies.

For experiment 1, 72 pigs across two replicates (36 pigs per replicate) were housed in six pens and assigned to one of two treatments (six pigs per pen; three pens per treatment): a control diet (**Con**) or the control with supplemental tryptophan (**Trp**). For experiment 2, 72 pigs were housed in 18 pens and assigned to one of three treatments (four pigs per pen; six pens per treatment): a control diet, control with supplemental Trp, and a control with two times the supplemental Trp. In all experiments, pigs were on trial for five weeks with body weight (**BW**) and feed disappearance monitored weekly to calculate average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain to feed ratio (**G:F**).

Diets. Pigs were fed a two-phase nursery diet and fed the nursery 1 diet for two weeks and nursery 2 for three weeks (**Table 4.1**). Tryptophan was supplemented at 0.025 and 0.020% for phase 1 and 2, respectively, to ensure the standardized ileal digestibility (**SID**) Trp : Lys remained the same during the trial. In Exp. 2, Trp was supplemented at 0.05 and 0.04% for the Trp+ diet. Diets contained no antibiotics or antibiotic alternatives and were formulated to meet or exceed NRC (2012) requirements. Titanium dioxide was fed at 0.5% in Exp. 2 to facilitate digestibility measurements.

Sample Collection. During Exp. 1, blood samples were obtained on wk 1 and 5 from two pigs per pen (5-9 mL each) via jugular venipuncture in serum tubes. Tubes were allowed to clot before harvesting serum by centrifugation (1,500 x g for 10 min at 4˚C). Serum samples were aliquoted and stored at -80˚C for subsequent analyses. On wk 0, 2, and 5, fecal samples were collected directly from the rectum and frozen at -80˚C for microbial analysis. At wk 1, two pigs per pen were euthanized using a lethal dose of sodium pentobarbital. Fresh ileum and cecum tissue were collected and frozen at -80˚C for later analysis.

Cytokine Analysis. Serum and tissue homogenates were used to measure interleukin-8 and -22 respectively. Interleukin-8 was measured using porcine specific ELISA kit (R & D Systems, Minneapolis, MN) following manufacturers instructions with inter- and intra-assay CV of 8.4 and 7.9%. Interleukin-22 was measured using a porcine specific ELISA kit (RayBiotech, Norcross, GA) following manufacturers instructions with inter- and intra-assay CV of 3.3 and 6.45%.

Digestibility Analysis. Proximate analysis was carried out on feed and pooled fecal samples as previously described (Patience et al., 2009; Jacobs et al., 2011). At the end of each phase fecal samples were collected from each pen for Exp. 2. All samples were frozen at -20˚C for later analysis. Samples were later dried in a 100˚C forced-air oven for 3 d and then ground afterwards. Samples were analyzed for dry matter (**DM**) and gross energy (**GE**) using bomb calorimetry (Parr, Moline, IL). Total tract digestibility coefficients were calculated using indigestible marker methodology (Kerr et al., 2010).

Microbial Extraction and Sequencing. Fecal samples were extracted using the Mag Bind Soil DNA kit (Omega Bio-tek, Carlsbad, CA) following instructions with the following modifications. Samples (0.25 g) were loaded in to 2-mL Safe-Lock tubes (Eppendorf) with 0.5 g of 0.1 mm zirconia beads (Biospec Products, Bartlesville, OK) and 700 uL of SLX-Mlus buffer. Samples were lysed using a Tissuelyser (Qiagen, Valencia, CA) for 10 m at frequency 20. Samples tubes were incubated at 90˚C for 7 m.

Prior to the KingFisher step, the nucleic acids were precipitated in the following steps. 70 uL of 10 mM sodium acetate was added to each tube, mixed, and placed on ice for 5 m. Tubes were centrifuged at 16,000 x g for 15 m at 4˚C. Supernatant was added to a new tube and 400 uL of ice-cold isopropanol was added and the tube vortexed. Tubes were incubated on ice for 30 m and centrifuged at 16,000 x g for 15 m at 4˚C. Supernatant was discarded and the nucleic acid pellet was washed with 500 uL ice cold 70% ethanol and vortexed. Tubes were centrifuged at room temperature for 2 m at 13,000 x g. The wash was decanted, and the pellet allowed to dry for 3 m. The pellet was then dissolved in 450 uL of Tris (10 mM pH 8) and transferred to a deep well plate. Following the Omega kit again, DNA was purified using a MagMax Express 96 (Applied Biosystems, Foster City, CA) and stored at -20˚C.

Amplicons for the V4 region of the 16S rRNA gene were prepared as described by Kozich et al. (2013). Amplification for each sample via PCR was done in 25 uL reactions of the following: 12.5 uL Terra buffer, 0.5 uL Terra Polymerase (CloneTech Laboratories, Mountainview, CA), 1 uL of indexed fusion primers, 9 uL of PCR water, and 2 uL of DNA. Conditions for amplification were as follows: 98˚C for 3 m; 25 cycles

of 98˚C for 30 s, 55˚C for 30 s and 68˚C for 45 s; and a single final extension step at 68˚C for 4 m. Purity of amplified samples was determined by running samples on a 1.5% agarose gel. Libraries were normalized using the PCR Purification and Normalization kit (Charm Biotech, San Diego, CA) and the library subsequently pooled. Pooled libraries were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Bethlehem, PA). To concentrate libraries, a DNA precipitation was performed as follows: 0.1X volume of Sodium acetate (3M, pH 5.2), 2.5X volume of 100% isopropanol, and 1 uL of carrier RNA (Qiagen, Valencia, CA) were added to each library and mixed. Libraries were stored at -80˚C overnight and centrifuged at 14,000 x g for 30 m at 4˚C. Supernatant was removed and pellets washed with 50 uL of ice cold 70% ethanol. Libraries were centrifuged at 14,000 x g for 15 m at 4˚C. Supernatant was discarded, tubes were allowed to dry for 3 m, and 6-10 uL of elution buffer (Qiagen, Valencia, CA) added to dissolve the pellet.

Libraries were checked for quality using a High Sensitivity DNA Chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and quantified using a High Sensitivity dsDNA kit (Denovix, Wilmington, DE). All libraries were pooled, and re-quantified to confirm concentrations. Pooled libraries were run on an Illumina MiSeq platform (Illumina, San Diego, CA) using the V2 500 cycles kit according to manufacturer's instructions.

Microbiome Sequence Processing

Protocol for processing of sequences can be found in Appendix B. In short, generated fastq files were processed in RStudio using the dada2 pipeline (https://benjjneb.github.io/dada2/tutorial.html). The quality profile for sequenced reads

fell below a quality score of 30 around read 250. When filtering and truncating the reads, a threshold of a 240 was used for the forward and reverse reads and max of 2 expected errors per read. Samples were dereplicated into unique amplicon sequence variants (**ASV**). The ASV is alternate method allowing for unique sequences to be resolved down to single nucleotide differences as opposed to using operational taxonomic units which clusters sequences with less than 3% dissimilarity threshold (Callahan et al., 2017). Chimeras were removed with and represented less than 1% of total sequence variant abundance. Taxonomy was assigned using the Silva reference database (v132) (https://mothur.org/wiki/Silva_reference_files). A phylogenetic tree was created using Mothur and the Silva v132 seed database. The phylogenetic tree, biom file and mapping file were then merged using phyloseq (https://joey711.github.io/phyloseq/index.html). The data was rarefied to an even depth using the minimum number of sequence reads in the dataset. Bray-Curtis, Unifrac, and Weighted Unifrac distance matrices were generated from the dataset to be used in a PERMANOVA analysis. Additionally, samples were processed through Quantitative Insights Into Microbial Ecology (QIIME) to generate taxa plots as shown in Appendix A.

Separately, using the ASV counts, relative abundance of each *Lactobacillus* ASV in every sample was calculated. Total *Lactobacillus* relative abundance was then calculated.

Economic Data. Pen feed disappearance by week was multiplied by cost per kg of feed, with respect to treatment and phase, to determine feed usage cost. The feed usage cost

was divided by weekly net BW gain to calculate the feed cost per kg of BW gain. The difference in cost between Trp or Trp+ and the control were calculated.

Statistics. All experiments were analyzed using the Proc MIXED procedure of SAS 9.2 (SAS Inst. Inc., Cary, NC) and all results were expressed as least square means ± SEM. Pen was the experimental unit and the model included fixed effect of treatment; with random effects of initial BW. If initial BW was found to be significant, it was added as a covariate to the model. Relative abundance of *Lactobacillus* ASV was analyzed with pig as the experimental unit. Additionally, data from experiment 1 was analyzed with replication as a covariate, where necessary. Statistical significance of differences was determined by Tukey's range test for pair wise comparisons. Differences were deemed significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

Pigs were supplemented with and without extra Trp in the diet for five weeks and replication being significant ($P < 0.05$). Individually, improvements for G:F in the Trp group were observed in replication 1 ($P < 0.10$) and replication 2 ($P < 0.05$). No difference was found in BW, ADFI, or ADG during the trial period (**Table 4.2**). Despite this, pigs supplemented with Trp were 9.3% more feed efficient through both phases and over the total experimental period ($P < 0.05$) (**Figure 4.1A**). Throughout, the trial the Trp pigs had a \$0.20-0.28 lower feed cost/kg gain ($P > 0.05$) (**Table 4.4**). Similar to the previous experiments, Exp. 2 resulted in no differences in BW, ADFI, and ADG. The control pigs had numerically lower G:F compared with to both Trp treatments and the Trp+ pigs had reduced G:F compared to the Trp group although no statistical difference

was found ($P > 0.10$) (**Figure 4.1B)**. The Trp treatments saw a $0.18-0.25 feed cost per kg gain difference with the control pigs in phase 1, but this benefit was lost in phase 2 as cost increased to $0.05-0.07 over the control ($P > 0.10$). Surprisingly, the Trp group showed a 1.5% decrease in ATTD during phase 1, but a 1.5% increase in ATTD during phase 2 compared with the control and Trp+ groups. ($P < 0.05$) (**Figure 4.2**). No differences were found for IL-8 in the serum or IL-22 in intestinal samples (**Figure 4.3**). Sequencing of 156 samples yielded 2.55 million sequences with a mean of 16347 sequences per sample. Overall, no differences were seen between treatments while age of pig had an effect ($P < 0.05$). Supplementing tryptophan numerically increased the relative abundance of the Lactobacillales order and *Lactobacillus*, across 30 *Lactobacillus* ASV identified in experiment 1 ($P > 0.10$), although this pattern was not seen in experiment 2 (**Figure 4.4; Figure 4.5**).

DISCUSSION

The goal of this experiment was to assess the benefit of supplemental Trp on nursery pig performance and changes in *Lactobacillus* populations. The SID Trp: Lys requirement for nursery pigs was estimated at 16.3% in the NRC (2012). The requirement was estimated to be higher than 19.5% by Guzik et al. (2005) and a meta-analysis by Simongiovanni et al. (2012) found increasing the requirement from 17% to 22% improved ADG by 8%. Goncalves et al. (2015) estimated the SID Trp:Lys requirement at 20.4% and 23.9% for G:F and ADG, respectively, in 6 to 11 kg BW pigs, and 16.6% and 21.2% for G:F and ADG, respectively, in 11 to 20 kg BW pigs. Nursery diets used for the Nebraska swine herd are currently formulated at 22% SID Trp:Lys. As

we wanted to ensure that supplemented Trp was in excess of requirement, usage of 22% was deemed appropriate for this study.

Supplementing pigs with Trp improved feed efficiency, although this was more pronounced in the first two experiments and not the third. The titration study by Goncalves et al. (2015), had similar SID Trp:Lys levels at the top end of curve, but whereas 24% level had a decrease in G:F, our study showed an increase. Considering the potential differences between location, genetics, and diet, these could account for the differences in performance seen. However, similar to the prior study, we did find that the 26% level (Trp+) in our third experiment did have reduced performance compared to the 24% (Trp) thus indicating that there was point where extra Trp was not as beneficial. We were surprised to find that Exp. 2 did not show the same results for G:F compared with Exp. 1. Week 1 growth performance and observed diarrhea appeared to indicate subclinical illness was present, but despite the inclusion of Trp and numerical increases in performance overall, no significant differences were seen in the performance. This is surprising as numerous studies (Capozzalo et al., 2012; Messori et al., 2013; Xu et al., 2014; Capozzalo et al., 2015) have shown supplementing Trp to be beneficial to pigs in poor sanitation conditions or exposed to a health challenge. This may have also impacted ATTD as the control and Trp+ pigs had increased digestibility at the end one phase 1 when the pigs were likely recovering. Phase 2 ATTD was increased in the Trp, however this gain was not reflected in ADG or G:F and no measurements were taken to assess backfat or loin eye area.

We also wanted to assess if tryptophan had an effect on *Lactobacillus* populations. Overall, we saw a numerical increase in *Lactobacillus* abundance in Exp. 1,

but not Exp. 2. Few studies with tryptophan have looked into *Lactobacillus* populations. Zelante et al. (2013) showed that proportions of individual *Lactobacillus* species changed in the murine stomach and feces but didn't quantify *Lactobacillus* abundance relative to other Firmicutes. Liang et al. (2018), showed a decrease in *Lactobacillus* abundance as Trp concentrations increased, however, Trp concentrations were 10-fold higher than our study and exceed other published literature values. The lack of differences in *Lactobacillus* abundance may explain why we didn't see any difference in IL-8 or IL-22, although our model was also a low stress model compared to prior work (Zelante et al., 2013; Capozzalo et al., 2012; Capozzalo et al., 2015).

Production cost is extremely important to producers. Supplementation of Trp did increase in G:F, but simply measuring this using feed intake did not account for ADG improvement in replication 2. As such, we compared feed consumption cost against the tissue deposited during the trial. Even though the Trp diet was more expensive due to increased Trp content, the $3.11 per pig savings in efficiency offset the cost in experiment 1. Experiment 3 saw a reduced benefit in the phase 1 compared to the prior experiment, but still positive. However, much like the growth performance from phase 2, supplementation of Trp provided no benefit. However, performing a follow-up study and assessing backfat and loin eye area would be prudent for elucidating additional economic factors.

Overall, increasing Trp in the diet appears to have positive effects on growth performance via feed efficiency, can potentially improve nutrient digestibility, and decreased cost of production. As feed cost is the most variable and generally costly component of production, reducing these costs can be beneficial to the producer's bottom

line. However, more work is needed to determine additional benefits of Trp supplementation across swine production as it evolves.

LITERATURE CITED

Callahan, B. J., P. J. McMurdie, and S. P. Holmes. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. The Isme Journal 11:2639.

Capozzalo, M. M., J. C. Kim, J. K. Htoo, C. F. de Lange, B. P. Mullan, C. F. Hansen, J.-W. Resink, P. A. Stumbles, D. J. Hampson, and J. R. Pluske. 2012. An increased ratio of dietary tryptophan to lysine improves feed efficiency and elevates plasma tryptophan and kynurenine in the absence of antimicrobials and regardless of infection with enterotoxigenic escherichia coli in weaned pigs. J Anim Sci 90:191-193.

Capozzalo, M. M., J. C. Kim, J. K. Htoo, C. F. M. de Lange, B. P. Mullan, C. F. Hansen, J.-W. Resink, P. A. Stumbles, and J. R. Pluske. 2015. Effect of increasing the dietary tryptophan to lysin ratio on plasma levels of tryptophan, kynurenine and urea and on production traits in weaner pigs experimentally infected with an enterotoxigenic strain of escherichia coli. Arch Anim Nutr 69:17-29.

de Ridder, K., C. L. Levesque, J. K. Htoo, and C. F. M. de Lange. 2012. Immune system stimulation reduces the efficiency of tryptophan utilization for body protein deposition in growing pigs. J Anim Sci 90:3485-3491.

Eyerich, K., V. Dimartino, and A. Cavani. 2017. Il-17 and il-22 in immunity: Driving protection and pathology. European Journal of Immunology 47:607-614.

Frese, S. A., K. Parker, C. C. Calvert, and D. A. Mills. 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome 3:28.

Goncalves, M. A. D., S. Nitikanchana, M. D. Tokach, S. S. Dritz, N. M. Bello, R. D. Goodband, K. J. Touchette, J. L. Ursy, J. M. DeRouchey, and J. C. Woodworth. 2015. Effects of standardized ileal digestible tryptophan:Lysine ratio of growth performance of nursery pigs. J Anim Sci 93:3909-3918.

Gummalla, S., and J. R. Broadbent. 1999. Tryptophan catabolism by lactobacillus casei and lactobacillus helveticus cheese flavor adjuncts. J Dairy Sci 82:2070-2077.

Guzik, A. C., M. J. Pettitt, E. Beltranena, L. L. Southern, and B. J. Kerr. 2005. Threonine and tryptophan ratios fed to nursery pigs*. Journal of Animal Physiology and Animal Nutrition 89:297-302.

Jacobs, B. M., J. F. Patience, W. A. Dozier 3rd, K. J. Stalder, and B. J. Kerr. 2011. Effects of drying methods on nitrogen and energy concentrations in pig feces and urine, and poultry excreta. J. Anim. Sci. 89:2624-2630.

Kerr, B. J., T. E. Weber, P. S. Miller, and L. L. Southern. 2010. Effect of phytase on apparent total tract dgestibility of phosphorus in corn-soybean meal diets fed to finishing pigs. J. Anim. Sci. 88:238-247.

Kozich, J. J., S. L. Westcott, N. T. Baxter, S. K. Highlander, and P. D. Schloss. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. Applied and Environmental Microbiology 79:5112.

Messori, S., P. Trevisi, A. Simongiovanni, D. Priori, and P. Bosi. 2013. Effect of susceptibility to enterotoxigenic escherichia coli f4 and of dietary tryptophan on gut microbiota diversity observed in healthy young pigs. Vet Microbiol 162:173-179.

Patience, J. F., A. D. Beaulieu, and N. H. Williams. 2009. Response to dietary digestible energy concentration in growing pigs fed cereal grain-based diets. Journal of Animal Science 87:965-976.

Schweer, W. P., J. F. Patience, K. Schwartz, D. Linhares, C. Rademacher, H. Allen, C. Loving, A. Ramirez, and N. K. Gabler. 2017. A review and evaulation of (growth promoting) antibiotic alternatives in the literature. In: Midwest Section ASAS, Omaha, NE

Simongiovanni, A., E. Corrent, N. Le Floc'h, and J. van Milgen. 2012. Estimation of the tryptophan requirement in piglets by meta-analysis. Animal 6:594-602.

Trautman, A., and J. Fiebiger. 1952. Fundamentals of the histology of domestic animals. Comstock Pub., Cornell Univ. Press, Ithaca, N. Y.

Xu, S., Y. Zhao, J. Shen, Y. Lin, Z. Fang, L. Che, and D. Wu. 2014. Threonine and tryptophan supplementation enhance porcine respiratory and reproductive syndrome (prrs) vaccine-induced immune responses of growing pigs. Anim Sci J 86:294-304.

Xue, M., J. Zhao, L. Ying, F. Fu, L. Li, Y. Ma, H. Shi, J. Zhang, L. Feng, and P. Liu. 2017. Il-22 suppresses the infection of porcine enteric coronaviruses and rotavirus by activating stat3 signal pathway. Antiviral Research 142:68-75.

Zelante, T., R. G. Iannitti, C. Cunha, A. De Luca, G. Giovannini, G. Pieraccini, R. Zecchi, C. D'Angelo, C. Massi-Benedetti, F. Fallarino, A. Carvalho, P. Puccetti, and L. Romani. 2013. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity 39:372-385.

Table 4.1. Experimental diet composition for Exp. 1 and 2[1]

	Phase 1			Phase 2		
Item	Control	Trp	Trp+	Control	Trp	Trp+
Ingredient, %						
Corn	43.63	43.60	43.57	60.21	60.18	60.16
SBM	32.00	32.00	32.00	33.75	33.75	33.75
Whey	15.00	15.00	15.00	0.00	0.00	0.00
Fishmeal	4.00	4.00	4.00	0.00	0.00	0.00
Corn oil	3.00	3.00	3.00	3.00	3.00	3.00
Dicalcium	1.00	1.00	1.00	1.65	1.65	1.65
Limestone	0.35	0.35	0.35	0.63	0.63	0.63
Sodium Chloride	0.30	0.30	0.30	0.30	0.30	0.30
Vitamin Mineral Premix[2,3]	0.40	0.40	0.40	0.40	0.40	0.40
DL-Met	0.03	0.03	0.03	0.03	0.03	0.03
Zn Oxide	0.30	0.30	0.30	0.00	0.00	0.00
L-Trp	0.00	0.025	0.05	0.00	0.02	0.04
L-Lys	0.00	0.00	0.00	0.04	0.04	0.04
Calculated analysis						
Net energy, kcal/kg	2561	2561	2562	2542	2542	2543
SID AA, %						
Lys	1.21	1.21	1.21	1.03	1.03	1.03
Met	0.37	0.37	0.37	0.32	0.32	0.32
Thr	0.78	0.78	0.78	0.66	0.66	0.66
Trp	0.26	0.29	0.31	0.23	0.25	0.27
Trp:Lys	0.215	0.240	0.256	0.223	0.243	0.262

[1]For experiment 2, 0.5% Titanium dioxide was added in place of corn

[2]Vitamin premix supplied per kg of diet: vitamin A (as retinyl acetate), 5500 IU; vitamin D (as cholecalciferol), 550 IU; vitamin E (as tocopheryl acetate), 30 IU; vitamin K (as menadione dimethylpyrimidinol bisulfate), 4.4 mg; riboflavin, 11.0 mg; d-pantothenic acid, 22.05 mg; niacin, 33.0 mg; vitamin B12 (as cyanocobalamin), 33.0 mg

[3]Trace mineral premix containing: copper (as CuSO4H2O), 10 mg/kg; iodine (as Ca(IO3) · H2O), 0.25 mg/kg; iron (as FeSO4 · 2H2O), 125 mg/kg; manganese (MnO), 15 mg/kg; selenium (Na2SeO3), 0.3 mg/kg; zinc (ZnSO4 · H2O), 125 mg/kg

Table 4.2. Growth performance[1] of pigs supplemented with or without tryptophan (Trp) for 5 wk (Exp. 1)[2]

Item	Con	Trp[3]	SEM	*P*-value
BW, kg				
Wk 0	5.9	5.9	0.02	0.548
Wk 1	6.9	6.8	0.14	0.947
Wk 2	9.3	9.4	0.23	0.837
Wk 3	12.0	12.2	0.36	0.590
Wk 4	15.6	15.9	0.50	0.657
Wk 5	19.8	20.3	0.65	0.604
ADFI, kg/d				
Wk 1	0.19	0.17	0.013	0.539
Wk 2	0.45	0.43	0.027	0.512
Wk 3	0.72	0.69	0.034	0.593
Wk 4	0.87	0.84	0.039	0.581
Wk 5	1.07	0.99	0.051	0.350
Phase 1	0.31	0.29	0.018	0.518
Phase 2	0.89	0.84	0.038	0.443
Total	0.64	0.61	0.027	0.430
ADG, kg/d				
Wk 1	0.14	0.14	0.014	0.748
Wk 2	0.35	0.36	0.017	0.627
Wk 3	0.38	0.41	0.023	0.362
Wk 4	0.52	0.53	0.026	0.885
Wk 5	0.60	0.62	0.029	0.576
Phase 1	0.23	0.24	0.013	0.627
Phase 2	0.50	0.52	0.022	0.538
Total	0.39	0.40	0.017	0.531

[1]BW = body weight; ADFI = average daily feed intake; ADG = average daily gain.

[2]Phase 1 diet was fed for wk 1 and 2; Phase 2 diet was fed for wk 3 to 5.

[3]Tryptophan added at 0.025 and 0.02% for phase 1 and 2, respectively.

Table 4.3. Growth performance of pigs supplemented with or without tryptophan (Trp) for 5 wk (Exp. 2)[1]

Item	Con	Trp	Trp+	SEM	*P*-value
BW, kg					
Wk 0	6.5	6.4	6.6	0.05	0.029
Wk 1	6.7	6.7	6.7	0.14	0.980
Wk 2	8.2	8.6	8.3	0.26	0.453
Wk 3	10.6	10.9	10.7	0.33	0.754
Wk 4	14.6	15.2	14.7	0.45	0.605
Wk 5	19.5	20.3	20.1	0.61	0.534
ADFI, kg/d					
Wk 1	0.10	0.11	0.10	0.012	0.806
Wk 2	0.25	0.30	0.26	0.020	0.211
Wk 3	0.53	0.52	0.53	0.031	0.988
Wk 4	0.88	0.88	0.84	0.046	0.802
Wk 5	1.12	1.19	1.20	0.033	0.174
Phase 1	0.18	0.21	0.18	0.015	0.316
Phase 2	0.84	0.86	0.86	0.033	0.880
Total	0.59	0.60	0.59	0.026	0.996
ADG, kg/d					
Wk 1	0.01	0.04	0.02	0.019	0.524
Wk 2	0.21	0.27	0.23	0.022	0.095
Wk 3	0.35	0.34	0.35	0.019	0.742
Wk 4	0.56	0.60	0.57	0.028	0.499
Wk 5	0.70	0.73	0.76	0.030	0.285
Phase 1	0.10	0.16	0.12	0.022	0.118
Phase 2	0.52	0.56	0.55	0.025	0.303
Total	0.35	0.40	0.38	0.022	0.246

[1]BW = body weight; ADFI = average daily feed intake; ADG = average daily gain.

[2]Phase 1 diet was fed for wk 1 and 2; Phase 2 diet was fed for wk 3 to 5.

[3]Tryptophan added at 0.025 and 0.05% for phase 1 and 0.02 and 0.04% for phase 2 for Trp and Trp+ diets, respectively.

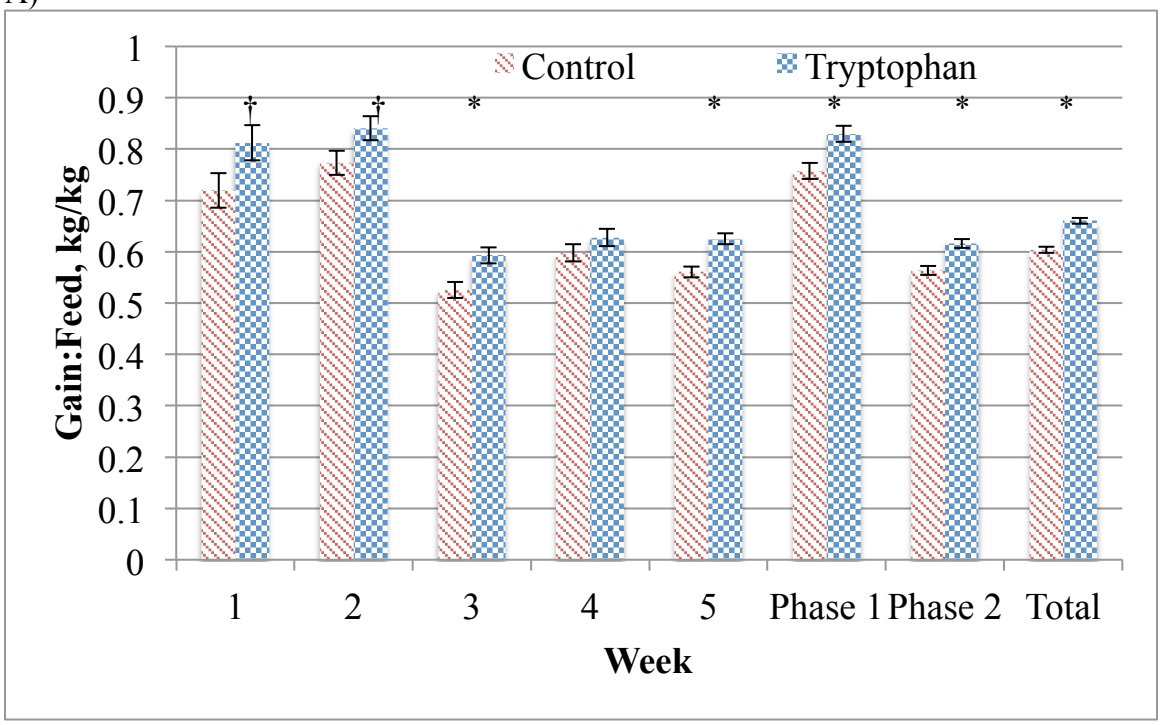
Table 4.4. Pig feed cost per kg gain, and savings by treatment for Exp. 1 and 2.

	Phase 1				Phase 2			
Exp. 1	Control	Trp	SEM	*P*-value	Control	Trp	SEM	*P*-value
Feed $/ kg gain	3.33	3.05	0.070	0.019	2.46	2.26	0.037	0.004
Difference, $		0.28				0.2		
Gain/pig, kg	3.5				10.7			
Savings/pig, $[1]		0.97				2.14		

Exp. 2	Control	Trp	Trp+	SEM	*P*-value	Control	Trp	Trp+	SEM	*P*-value
Feed $/ kg gain	2.93	2.68	2.76	0.202	0.661	1.67	1.75	1.72	0.05	0.569
Difference, $		0.25	0.18				-0.07	-0.05		
Gain/pig, kg		1.8					11.6			
Savings/pig, $[1]		0.46	0.33				-0.81	-0.58		

[1]Savings per pig is difference between Trp or Trp+ and Control treatments

A)



B)

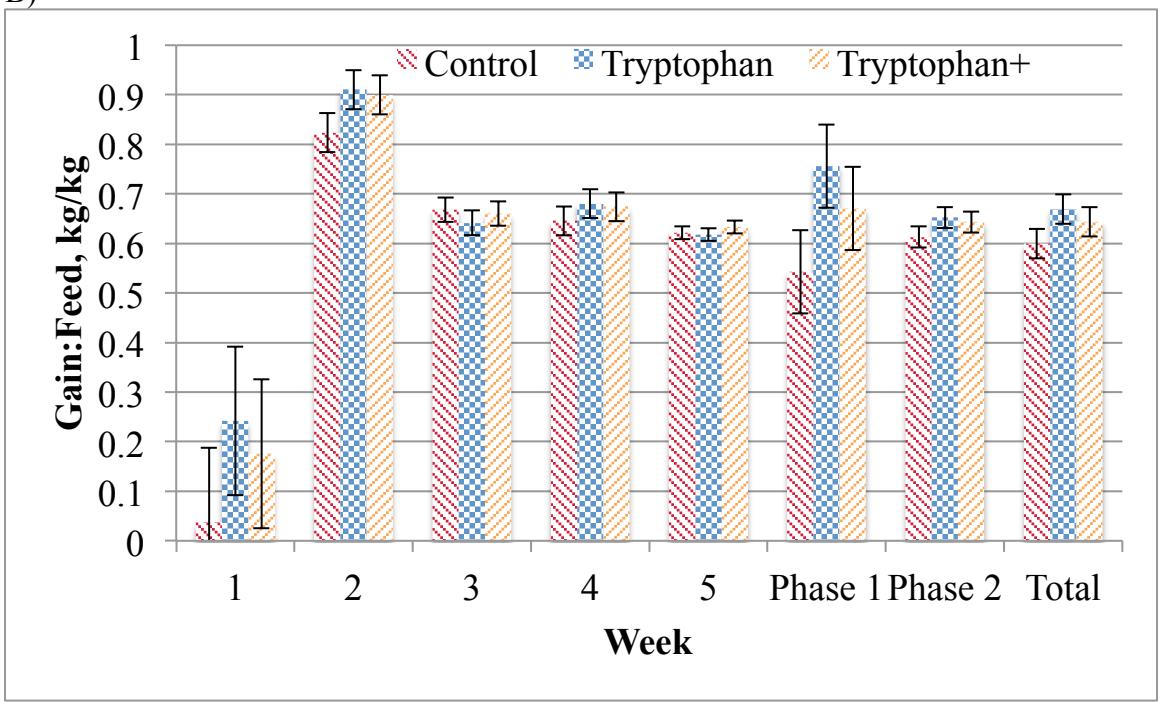


Figure 4.1. Feed efficiency for Exp. 1 (**A**) and Exp. 2 (**B**) by week, phase, and overall. Bars represent least square means ± SEM.†, $P < 0.10$; *, $P < 0.05$

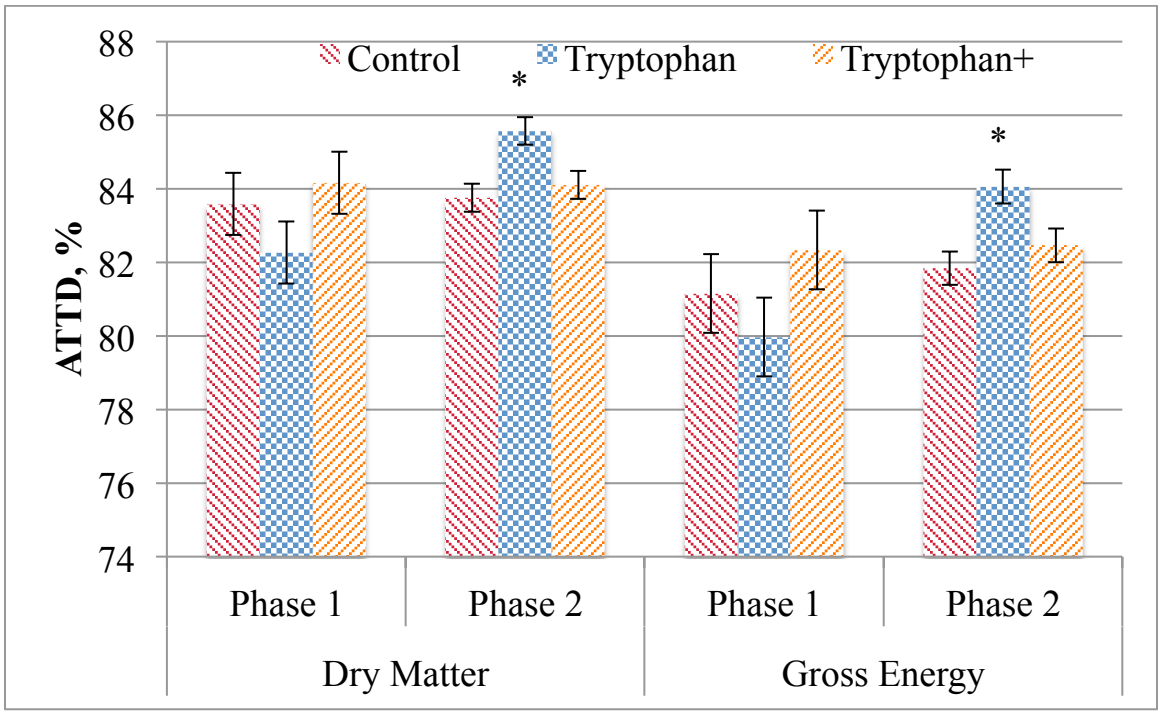
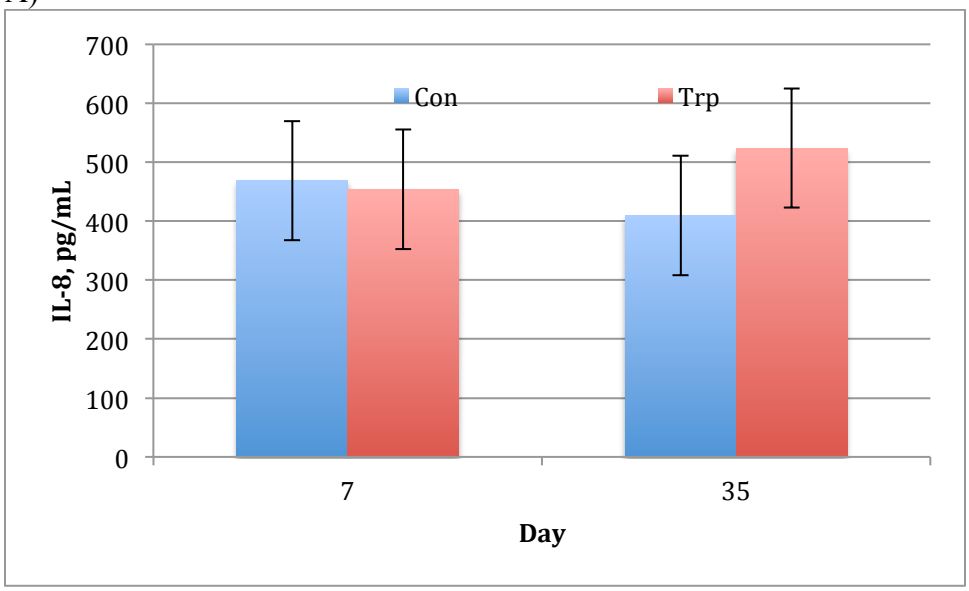


Figure 4.2. Apparent total tract digestibility (ATTD) coefficients of dry matter and gross energy for Exp. 2. Bars represent least square means ± SEM. *, $P < 0.05$

A)



B)

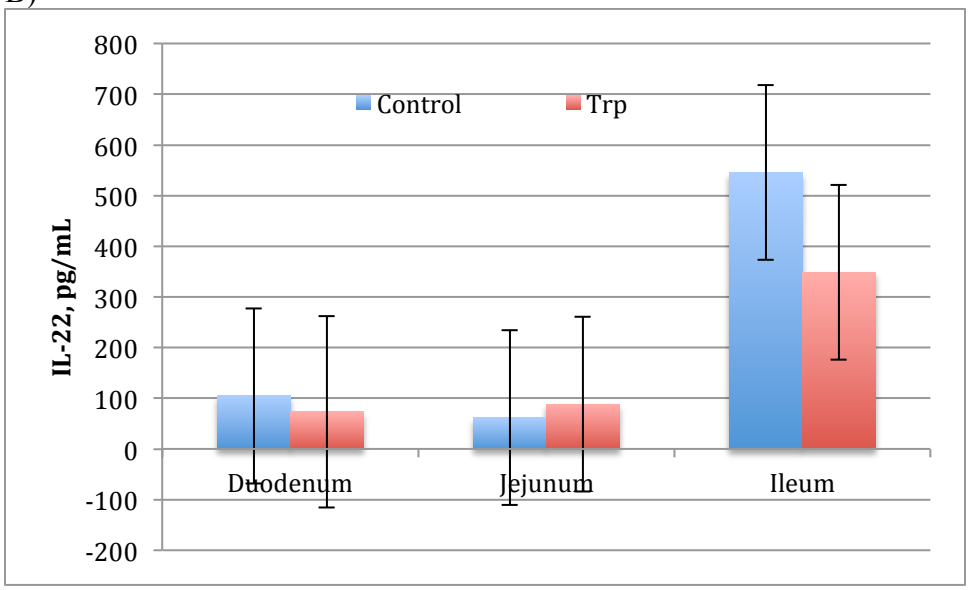
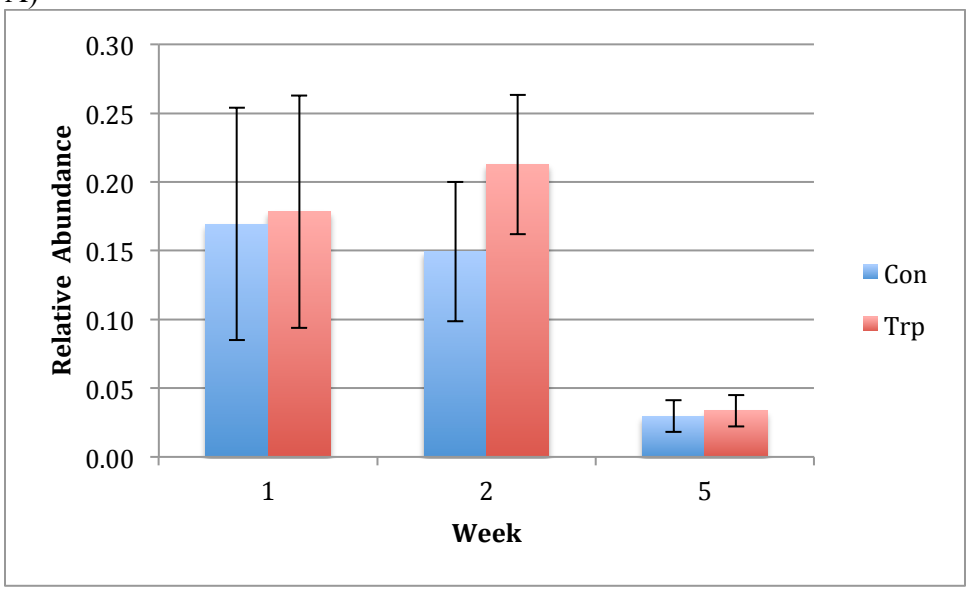


Figure 4.3. Cytokine concentrations from pigs supplemented with tryptophan in experiment 1. Interleukin-8 from d 7 and 35 serum samples (**A**). Interleukin-22 concentrations from intestinal tissue homogenates (**B**). Bars represent least square means ± SEM.

A)



B)

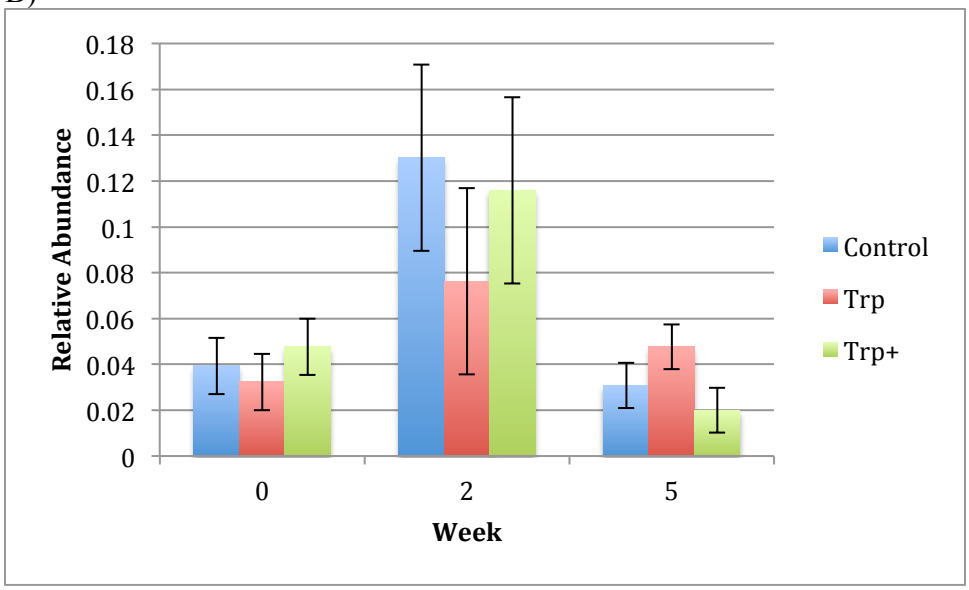


Figure 4.4. Relative abundance of *Lactobacillus* amplicon sequence variants in pigs supplemented with tryptophan in the diet. Experiment 1 (A) and 2 (B). Bars represent least square means ± SEM.

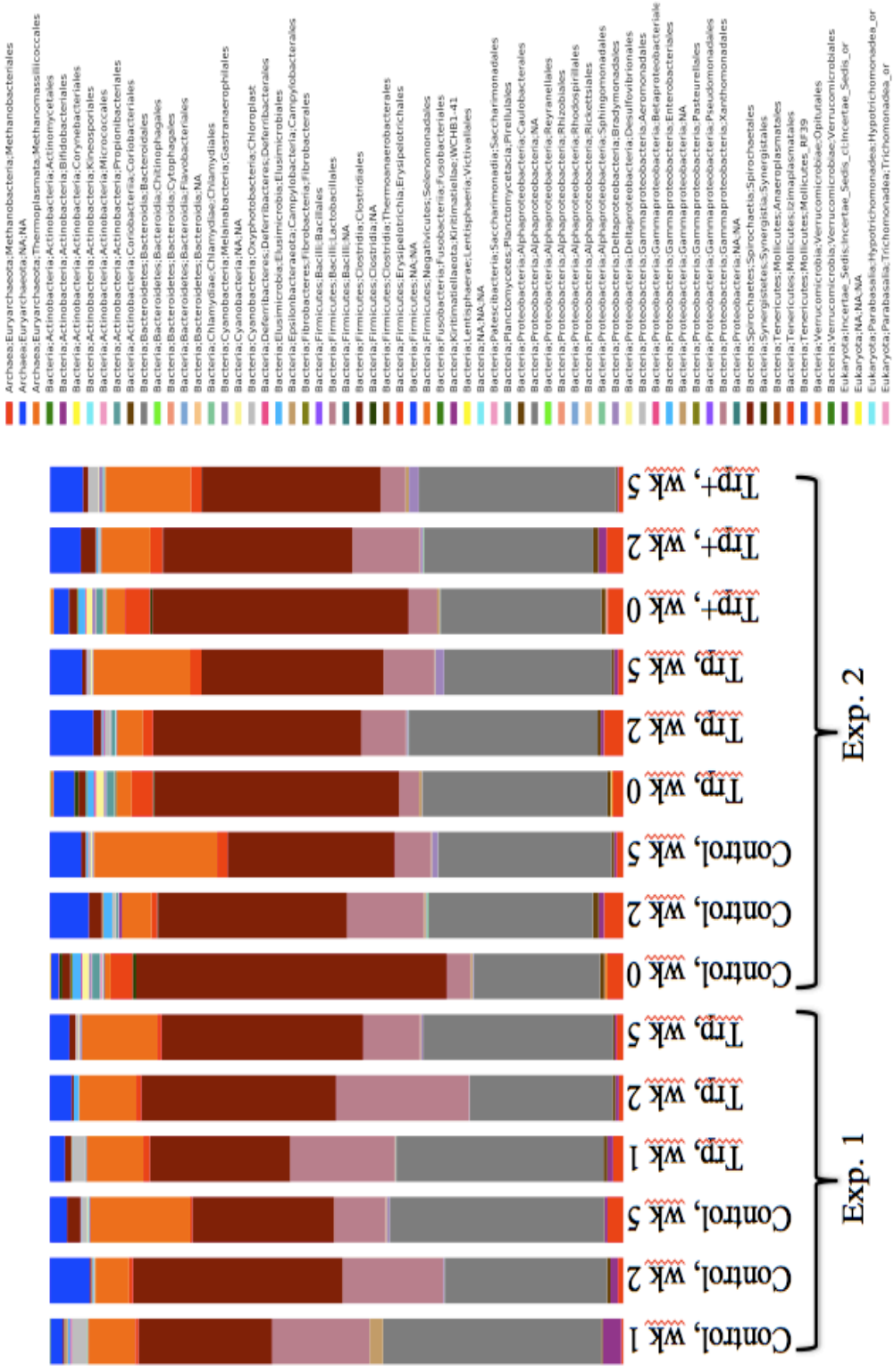


Figure 4.5. Relative abundance of the microbiome at the order level of pigs supplemented with tryptophan.

CHAPTER 5: RESPONSE OF IPEC-J2 CELLS AND JEJUNAL EXPLANTS TO TREATMENT WITH RHAMNOLIPIDS

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ABSTRACT: Rhamnolipids (RL) are glycolipids secreted by bacteria that aid in motility, biofilm formation, nutrient uptake, and have antimicrobial activity. The latter two may be of use to improve swine nutrition and health. Work conducted *in vitro* utilized IPEC-J2 cells to determine cellular response to treatment. In the first experiment, cells were treated with 0, 0.01, 0.05, 0.5, 1.0, 2.4, and 10% RL or 1 μg/mL lipopolysaccharide (LPS). In the second experiment, cells were treated with 0, 0.0005, 0.001, 0.005, and 0.01% RL or 1 μg/mL LPS. For both experiments, treated cells were incubated for 1 h and rinsed with PBS. Cells were further incubated in fresh non-treated media for 0, 1, 3, 6, 12 or 24 h in Exp. 1, or 6 h in Exp. 2. Transepithelial resistance (TER) measurements were collected immediately after addition of fresh media and after incubations. Media from both the apical and basolateral sides were collected for IL-8 analysis. Visual observation and TER analysis of Exp. 1 showed RL treatment decreased TER after initial incubation, with RL concentrations ≥ 0.05% exhibiting significant cell death and loss of cellular matrix adherence to the Trans-well membrane. Using lower dosages in Exp. 2, TER changed inversely with RL dosage, however, even 0.0005% RL reduced TER by at least 20% over the 6 h period ($P > 0.10$). Production of IL-8 was lower in RL treatments compared with either the control or LPS wells on the apical side ($P > 0.10$). Basolateral IL-8 was expressed in a dose dependent fashion, but only the 0.01% RL had higher concentration than the control ($P > 0.10$). A third experiment was conducted by culturing jejunal explants in media with 0, 0.0005, 0.005, 0.05, and 0.5% RL or 1 ug/mL LPS for 1 h followed by 3 h incubation in fresh media. Similar to Exp. 2, explants showed dose dependent IL-8 production in tissue obtained from 5 and 7 wk old pigs with 0.5% RL having higher IL-8 concentration compared to control or LPS samples

($P < 0.01$). The lower doses of RL had similar or lower IL-8 production to the control or LPS samples ($P > 0.10$). Together these data show that low doses of RL (≤ 0.005%) can significantly impact IPEC-J2 TER, but do not cause increases in IL-8 production. More data is needed to determine the effect of RL on nutrient absorption, gut health, and the microbiome in pigs.

Keywords: pig, rhamnolipid

INTRODUCTION

Rhamnolipids (**RL**) are well-known glycolipid microbial biosurfactants, which reduce surface tension, as anionic amphiphilic molecules, with (hydrophilic) mono- or di-rhamnose sugar heads and (lipophilic) beta-hydroxyalkanoic acid tails of varying lengths. As reviewed by Chong and Li (2017), rhamnolipids are predominately produced by bacteria in the genus *Pseudomonas* genus with *P. aeruginosa* being the most widely researched due to its consistent and high production of RL in fermentation. Secretion of RL provides numerous benefits to *Pseudomonas* by enhancing motility, biofilm formation, and nutrient uptake while also being able to inhibit growth of other bacteria (both Gram negative and positive) and fungi (Abdel-Mawgoud et al., 2010). Rhamnolipids also act as potential virulence factors by causing histamine release from mast cells, suppressing phagocytic action of macrophages, or increasing permeability by opening up tight junctions in epithelial cells (Abdel-Mawgoud et al., 2010). These factors certainly benefit *P. aeruginosa,* which is a known and common colonizer and pathogen in Cystic Fibrosis patients.

However, RL is commonly produced and used for various industrial purposes (Chong and Li, 2017). The amphipathic nature of RL makes it an ideal emulsifier and has been used in bioremediation of petroleum sites, food applications, and pharmaceuticals. As an antimicrobial, RL have been used in food applications against foodborne pathogens (e.g., *Listeria monocytogenes*) and used as biopesticides on crops to reduce phytopathogens. Additionally, RL has been utilized as an excipient in the pharmaceutical industry to aid in absorption of drugs by opening the tight junctions of intestinal epithelial cells. Outside of industrial applications, RL has been researched for its effects on lung epithelial cells. However, investigation of the effect of RL on intestinal tissue has been limited to drug studies or cell culture models. Usage of RL, especially at higher doses, has been shown to decrease TER, increase paracellular permeability, and decrease cell viability of normal and cancer cell lines (Jiang et al., 2013; Jiang et al., 2014; Wallace et al., 2014; Perinelli et al., 2017).

Considering the antimicrobial and emulsifying effects of RL, usage as an antibiotic alternative would be of interest to the U.S. swine industry. Anecdotally, RL has been used as a feed additive in other species with improvements in growth performance. However, the lack of published evidence requires the need for a study to evaluate the effects of RL for use in swine. Thus, the goal of this study was to test the viability of RL for use in the swine industry using a porcine epithelial cell model (IPEC-J2) and jejunal explants.

MATERIALS AND METHODS

Cells. The following experiments used the IPEC-J2 cell line. These cells are a non-transformed jejunal epithelial cells derived from neonatal pigs (Rhoads et al., 1994) and characterized by Schierack et al. (2006). Cells were cultured in Dulbecco's modified eagle medium (DMEM)/Ham's F12 (1:1) (Gibco) with 5% fetal bovine serum (Hyclone), 1% Streptomycin/pencillin (Gibco), 1% insulin-transferrin-sodium selenite solution (Sigma), and 5 ng/mL human epidermal growth factor (Sigma). Cells were grown in 5% CO_2 atmosphere at 37˚C. For experiments, cells were seeded into 12-well polyethylene terephthalate transwell inserts (Falcon). Cells were cultured for 7 d to reach confluency and were used when transepithelial resistance was greater than 4000-Ω cm^2. Transepithelial resistance (TER) was measured using an EVOM^2 voltohmmeter (World Precision Instruments, Sarasota, FL).

Experiment 1. Two replications were conducted. Cells were treated with antibiotic free media 24 h prior to experiment. Cells were then treated for 1 h with the following treatments: 0, 0.01, 0.05, 0.5, 1.0, 2.4, and 10% rhamnolipid (Jeneil Biotech Inc, Saukville, WI), or 1 μg/mL lipopolysaccharide (LPS; Sigma). After incubation with RL, cells were washed with PBS and given fresh media and then incubated for 0, 1, 3, 6, 12, and 24 h. Transepithelial resistance measurements were collected after the media change and at the end of respective incubation times. Media was collected from both apical and basolateral sides of transwells and frozen at -20˚C. Cells were fixed using formalin.

Experiment 2. Cells were treated with 0, 0.0005, 0.001, 0.005, and 0.01% RL or 1 μg/mL LPS for 1 h. Cells were subsequently washed with PBS and were incubated for 6 h in

fresh media. Transepithelial resistance measurements were taken at 0, 2, 4, and 6 h after media change. After media harvest, inserts were bathed in formalin to fix cells for histological staining. To identify mucin content, inserts were stained using Alcian Blue-Period Acid Schiff stain kit (Thermo Scientific) and counterstained with hematoxylin following manufacturer's instructions. Images of stained inserts were obtained using a microscope camera (Olympus, Center Valley, PA).

Experiment 3. Approximately 0.5 m of fresh jejunum tissue, taken 3 m from the terminal ileum was obtained from 5 and 7-week old pigs euthanized via overdose using sodium pentobarbitol. Tissue was rinsed with cold PBS and opened longitudinally. Tissue explants were taken using an 8 mm biopsy punch (Integra York PA, York, PA). Explants were stored in warm IPEC-J2 cell culture media until all explants were collected. Four explants were placed in each well of a 12-well plate and treated with 0, 0.0005, 0.005, 0.05, 0.5% RL, or 1 μg/mL LPS for 1 h. Afterwards, explants were washed, and fresh media added. Explants were further incubated for 3 h. Media was collected and frozen back for later use.

Cytokine analysis. Interleukin-8 was measured using porcine specific ELISA kit (R & D Systems, Minneapolis, MN) following manufacturer's instructions with inter- and intra-assay CV of 8.4 and 7.9%.

Image analysis. Images were analyzed using Fiji and processed using a custom-made macro (**Appendix C**). In short, images were deconvoluted using the hematoxylin-PAS

option. Nucleated cells were counted and outlined by using the "Analyze Particles" function to identify nuclei with a size of 40-500 pixels and a circularity of 0.50-1.00. Partial nuclei were excluded. Outlined nuclei were compared against the original image to ensure only nuclei were being counted. Cell and nuclei area fractions were determined through additional processing and results outputted (**Appendix C**). Eight images were analyzed for each well with three wells per treatment.

Statistics. Data were analyzed using the Proc MIXED procedure from SAS 9.2 (SAS Inst. Inc., Cary, NC) and all results were expressed as least square means ± SEM. Well insert served as the experimental unit with treatment as a fixed effect and plate as a random effect. Statistical significance of differences was determined by Tukey's range test for pair wise comparisons. Differences were deemed significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

Experiment 1. IPEC-J2 cells were treated with rhamnolipids and LPS. Regardless of concentration, low TER was observed for cells treated with RL both after initial 1 h incubation and over the 24 h afterwards (**Figure 5.1A**). Further observation under a microscope revealed that wells treated with RL were mostly devoid of IPEC-J2 cells, supporting the previous observation that cells were being aspirated off the wells during washing and media changing steps. Due to lack of cells after the initial incubation, media was collected from 0 h plate during the second repetition of plates right after the 1 h incubation. Production of IL-8 (**Figure 5.1B**) was inversely related to concentration of

RL. This was likely due to the cytotoxicity at higher concentrations of RL lysing cells sooner in the incubation period.

Experiment 2. Due to the cytotoxicity issues in Exp. 1, RL concentrations were reduced for experiment 2. Lower concentrations appeared to reduce cell cytotoxicity to RL allowing for cell monolayers to be retained on the well membrane upon visual inspection via microscopy. Likewise, TER measurements were obtained over the 6 h incubation period (**Figure 5.2**). As was expected, RL treated cells had reduced TER in dose response relative to the control, with the exception of the 0.005% RL. Cells treated with 0.001 and 0.01% RL had >60% reduction in TER compared with control and LPS treatments ($P < 0.001$). Interleukin-8 was measured in the media as a marker for inflammation. After the initial hour of incubation with RL (**Figure 5.3A)**, the 0.01% RL showed the highest level of IL-8, about two times compared with all other treatments ($P < 0.001$). At 6 hours post RL treatment, apical secretion (**Figure 5.3B)** of IL-8 from RL-treated cells was lower than either the control or LPS ($P < 0.076$). Oddly, the 0.01% and 0.0005% RL treatments had the lowest and highest IL-8 responses, respectively, which was not expected. On the basolateral side, IL-8 secretion appeared to numerically decrease as RL concentration decreased ($P = 0.69$) and only the 0.01% RL exceeded the control.

Cell inserts were stained for mucin content (**Figure 5.4**). Overall, the lowest concentrations of RL have similar or slightly lower mucin content compared with the control and LPS samples. The 0.01% RL appears to have less mucin content, as more white space is apparent, likely due to cell death. Using Fiji, images were quantified to

determine nuclei count and nuclei and cell area, the latter including mucin secretions. As expected, nuclei count (**Figure 5.5A**) for all RL treatments were decreased compared to the control and LPS treatments ($P < 0.003$). The 0.01% RL concentration lowest counts numerically but was not statistically different than the other RL treatments. The nuclei and cell area fractions (both, $P < 0.001$) (**Figure 5.5B**) were reduced by 19-22% in the three lowest RL treatments, and by 45-50% for the 0.01% RL treatment.

Experiment 3. To determine the effect of RL on the jejunum, fresh explants were taken at two different time points and incubated with RL. Secretion of IL-8 (**Figure 5.6**) occurred in a dose-dependent fashion and the 0.01% RL treatment had a 73% increase compared to the control and LPS ($P < 0.001$).

DISCUSSION

The goal of this experiment was to assess the response of IPEC-J2 cells and jejunal explants when exposed to rhamnolipids. To our knowledge this is the first study performed using a small intestinal cell line or a porcine cell line in conjunction with RL. This study shows that rhamnolipids can reduce TER, stimulate IL-8 secretion, and can cause cytotoxic cell loss in IPEC-J2 cells when used at concentrations ≥ 0.01%. However, when used at lower concentrations, inflammation and TER are similar to the control and LPS, with some potential for cell loss.

In our study, exposure to RL decreased TER relative to dosage. This agrees with similar TER and paracellular permeability values found in Caco-2 cells (Jiang et al., 2013; Wallace et al., 2014; Perinelli et al., 2017) and Calu-3 cells (Perinelli et al., 2017).

This decrease in barrier function appears to be mediated by tight junction regulation, not degradation (Halldorsson et al., 2010). While paracellular transport was increased with RL exposure, Jiang et al. (2013) reported an increase in transcellular transport using the marker, propranolol. Jiang also reported an increase in transport of rhodamine 123 from the basolateral to apical side with an increase in RL, likely due to increase paracellular permeability.

Although permeability was not measured in our data, another potential reason for an increase in permeability would be to cell loss due to cytotoxicity of RL at high concentrations. We found a decrease in nuclei counts and nuclei and cell area indicating the both less loss and decrease in cell size. Lactate dehydrogenase assays showed a minor non-dose dependent increase in leakage with RL treatment (Wallace et al., 2014), however, a dose dependent response was seen in other studies (Jiang et al., 2014; Perinelli et al., 2017). Cell viability was also shown to decrease as dose increased (Jiang et al., 2014; Perinelli et al., 2017) although earlier work by Jiang et al. (2013) showed no difference regardless on dosage.

Most studies have used rhamnolipid mixtures containing both mono- and di-rhamnolipids. Jiang et al. (2014) conducted a study evaluating the effects of mono- and di-rhamnolipids alone with normal and cancer cells lines. Their work found that as rhamnolipid concentration increases, mono-rhamnolipids have greater impact on reducing cell viability and increasing lactate dehydrogenase leakage compared with the di-rhamnolipids. The ability of rhamnolipid to decrease surface tension is a hallmark of surfactants and as rhamnolipid concentration increases, surface tension decreases and enables increased formation of micelles (Jiang et al., 2014; Perinelli et al., 2017). In

addition, decreasing surface tension increases cell cytotoxicity rapidly from 41 to 30 mN/m (Jiang et al., 2014). This is important as decreasing surface tension increases likelihood for cell death, but it also necessary for emulsification. Interestingly, addition of FBS in the media increases surface tension, although this effect is lost at higher rhamnolipid concentrations (Jiang et al., 2014). Future studies aim to use rhamnolipid in the diet to increase in nutrient uptake in pigs. The interplay between rhamnolipid concentrations, the feed matrix containing RL, and the interaction with the intestinal epithelium will be of interest for determining impact on gut barrier and nutrient transport responses.

Due to the diverse environment in the gut, homeostasis of the immune system is important for regulating barrier function and whole host animal health. We determined that the initial incubation of IPEC-J2 cells with rhamnolipids promoted an increased IL-8 response in the 0.01% RL treatment, but once the stimulus was removed IL-8 production was reduced below the control and LPS treatments. Jejunum explants similarly exposed to rhamnolipids resulted in an increase in IL-8 secretion above control and LPS at a level of 0.5%. Gerstel et al. (2009) found rhamnolipids only stimulated IL-8 production in human keratinocytes by inducing flagellin removal from *P. aeruginosa.* Rhamnolipids derived from *Burkholderia* (*Pseudomonas*) *plantarii* were shown to have endotoxin activity by stimulating TNF-a production, however, this stimulation was far below LPS from *E. coli* (Andrä et al., 2006) and came at the additional cost of poor antimicrobial activity compared to other rhamnolipids (Benincasa et al., 2004).

Presence of rhamnolipids can alter intestinal permeability, inflammation, and cell death in IPEC-J2 cells. Our work demonstrates that lower concentrations of rhamnolipids

may be less detrimental to cells under direct exposure. At present, there are no published studies using rhamnolipids in live animal models. We speculate that addition of rhamnolipids into animal feed may have a positive effect on nutrient uptake without causing negative effects on intestinal health when used at less than 0.5%. Additionally, the antimicrobial activity of rhamnolipids may induce changes to the microbiome. However, these effects will remain unclear until future animal studies are conducted.

LITERATURE CITED

Abdel-Mawgoud, A. M., F. Lépine, and E. Déziel. 2010. Rhamnolipids: Diversity of structures, microbial origins and roles. Appl Microbiol Biotechnol 86:1323-1336.

Andrä, J., J. Rademann, J. Howe, H. J. Koch Michel, H. Heine, U. Zähringer, and K. Brandenburg. 2006. Endotoxin-like properties of a rhamnolipid exotoxin from burkholderia (pseudomonas) plantarii: Immune cell stimulation and biophysical characterization Biological Chemistry No. 387. p 301.

Benincasa, M., A. Abalos, I. Oliveira, and A. Manresa. 2004. Chemical structure, surface properties and biological activities of the biosurfactant produced by pseudomonas aeruginosa lbi from soapstock. Antonie van Leeuwenhoek 85:1-8.

Chong, H., and Q. Li. 2017. Microbial production of rhamnolipids: Opportunities, challenges and strategies. Microbial Cell Factories 16:137.

Gerstel, U., M. Czapp, J. Bartels, and J.-M. Schröder. 2009. Rhamnolipid-induced shedding of flagellin from pseudomonas aeruginosa provokes hbd-2 and il-8 response in human keratinocytes. Cellular Microbiology 11:842-853.

Halldorsson, S., T. Gudjonsson, M. Gottfredsson, P. K. Singh, G. H. Gudmundsson, and O. Baldursson. 2010. Azithromycin maintains airway epithelial integrity during pseudomonas aeruginosa infection. American Journal of Respiratory Cell and Molecular Biology 42:62-68.

Jiang, L., X. Long, and Q. Meng. 2013. Rhamnolipids enhance epithelial permeability in caco-2 monolayers. International Journal of Pharmaceutics 446:130-135.

Jiang, L., C. Shen, X. Long, G. Zhang, and Q. Meng. 2014. Rhamnolipids elicit the same cytotoxic sensitivity between cancer cell and normal cell by reducing surface tension of culture medium. Appl Microbiol Biotechnol 98:10187-10196.

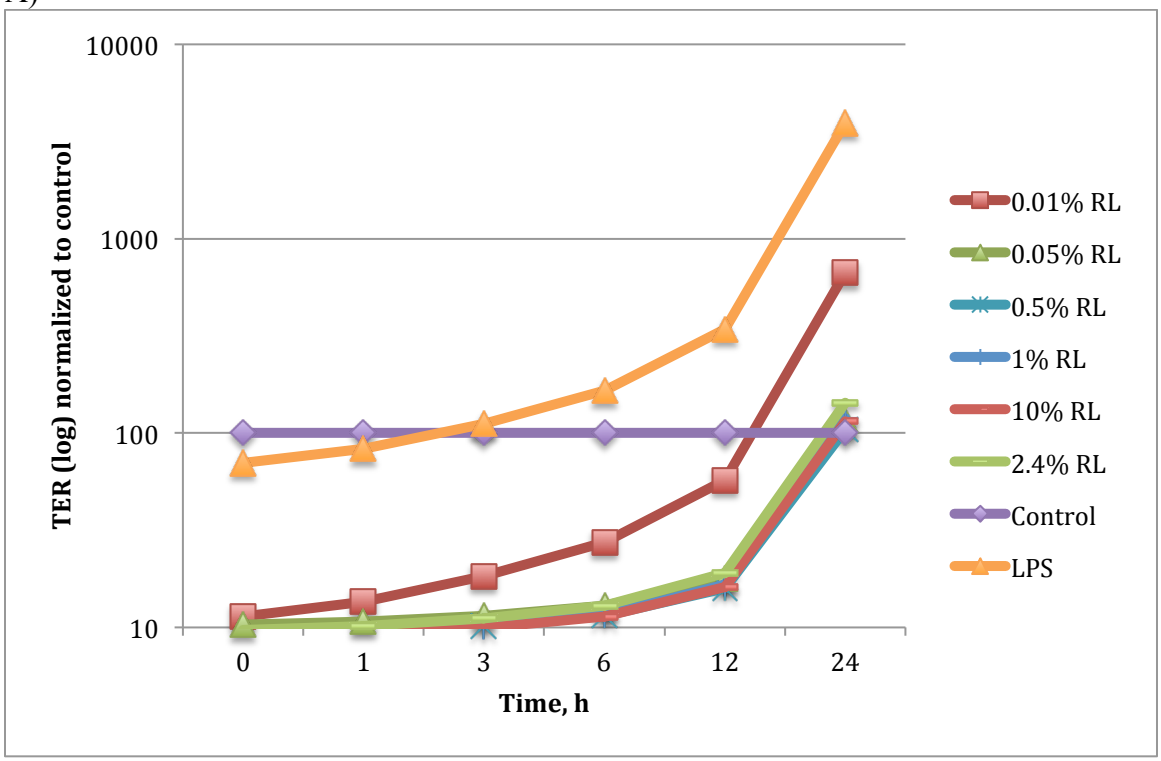
Perinelli, D. R., D. Vllasaliu, G. Bonacucina, B. Come, S. Pucciarelli, M. Ricciutelli, M. Cespi, R. Itri, F. Spinozzi, G. F. Palmieri, and L. Casettari. 2017. Rhamnolipids as epithelial permeability enhancers for macromolecular therapeutics. European Journal of Pharmaceutics and Biopharmaceutics 119:419-425.

Rhoads, J. M., W. Chen, P. Chu, H. M. Berschneider, R. A. Argenzio, and A. M. Paradiso. 1994. L-glutamine and l-asparagine stimulate na+ -h+ exchange in porcine jejunal enterocytes. American Journal of Physiology-Gastrointestinal and Liver Physiology 266:G828-G838.

Schierack, P., M. Nordhoff, M. Pollmann, K. D. Weyrauch, S. Amasheh, U. Lodemann, J. Jores, B. Tachu, S. Kleta, A. Blikslager, K. Tedin, and L. H. Wieler. 2006. Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochemistry and Cell Biology 125:293-305.

Wallace, C. J., S. H. Medina, and M. E. H. ElSayed. 2014. Effect of rhamnolipids on permeability across caco-2 cell monolayers. Pharmaceutical Research 31:887-894.

A)



B)

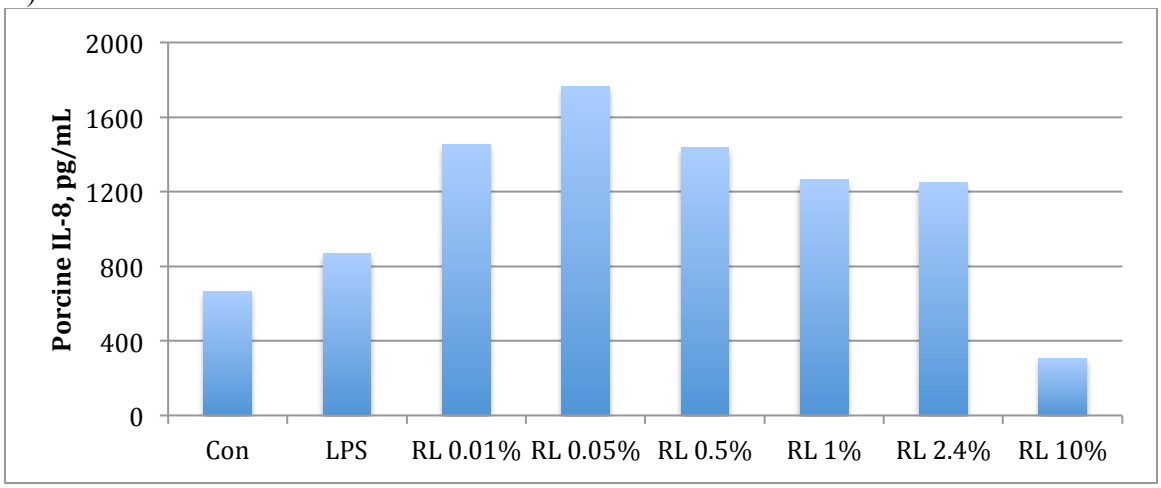


Figure 5.1. Transepithelial resistance of IPEC-J2 cells initially treated for 1 h and incubated in fresh media for 1 to 24 h. Data presented in logarithmic form (**A**). Interleukin-8 concentrations from cells incubated with treatments for 1 h (**B**).

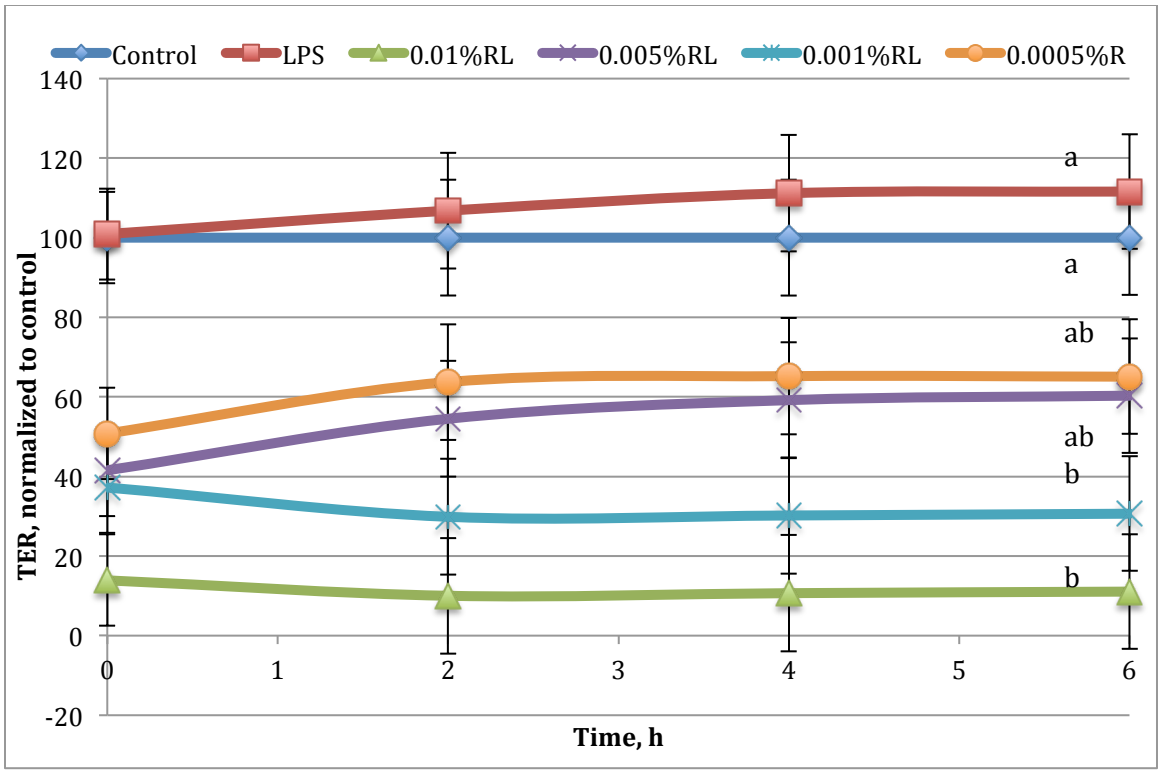


Figure 5.2. Transepithelial resistance of IPEC-J2 cells initially treated for 1 h and incubated in fresh media for 6 h. Points represent least square means ± SEM. Letters indicate statisitcal difference, $P < 0.001$.

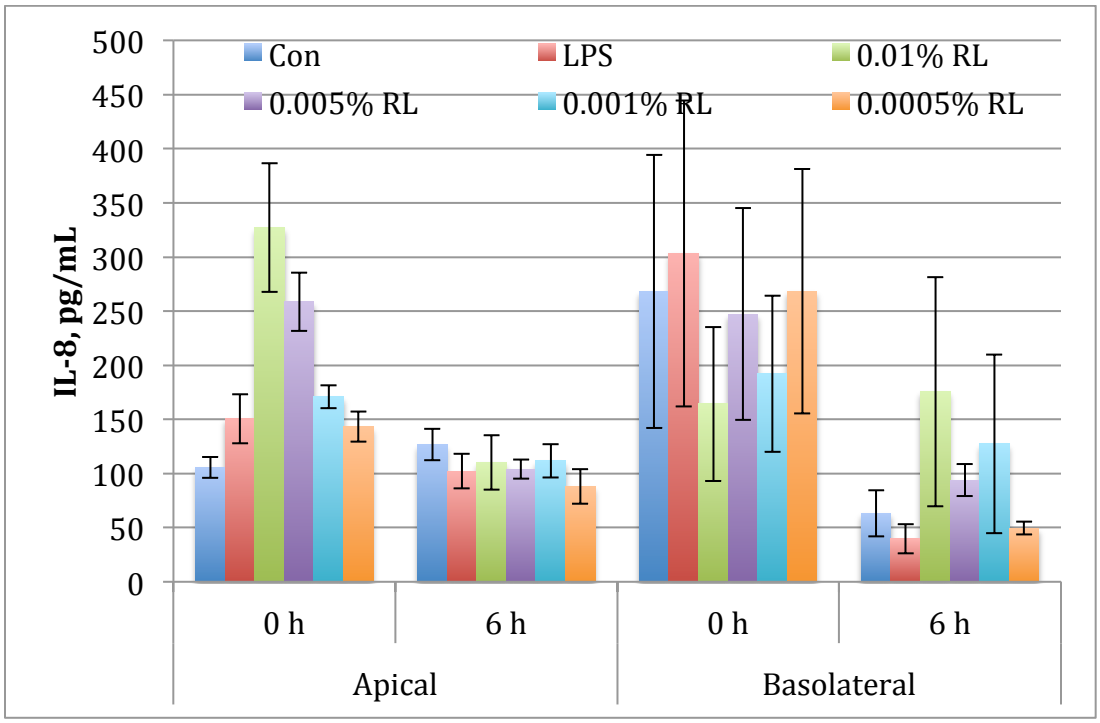


Figure 5.3. Interleukin-8 secretion from IPEC-J2 cells treated with rhamnolipids. Concentrations of IL-8 after 1 h rhamnolipid incubation (0 h) or 6 h incubation in fresh media. Bars represent least square means ± SEM. Statistical difference indicated by *, $P < 0.0001$.

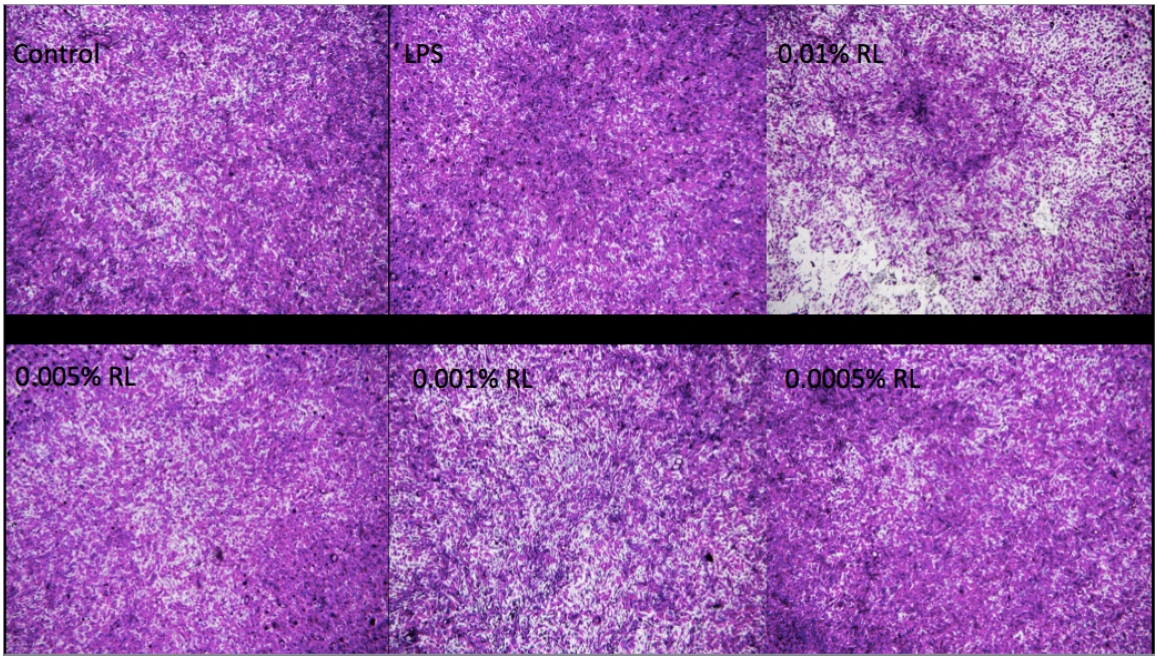


Figure 5.4. Cell inserts of IPEC-J2 cells treated with rhamnolipids for 1 h and incubated in fresh media for 6 h. Cells stained with Alcian Blue, Periodic Acid Schiff, and counterstained with hematoxylin (4x magnification). Mucins appear magenta to violet in color depending on composition of substance.

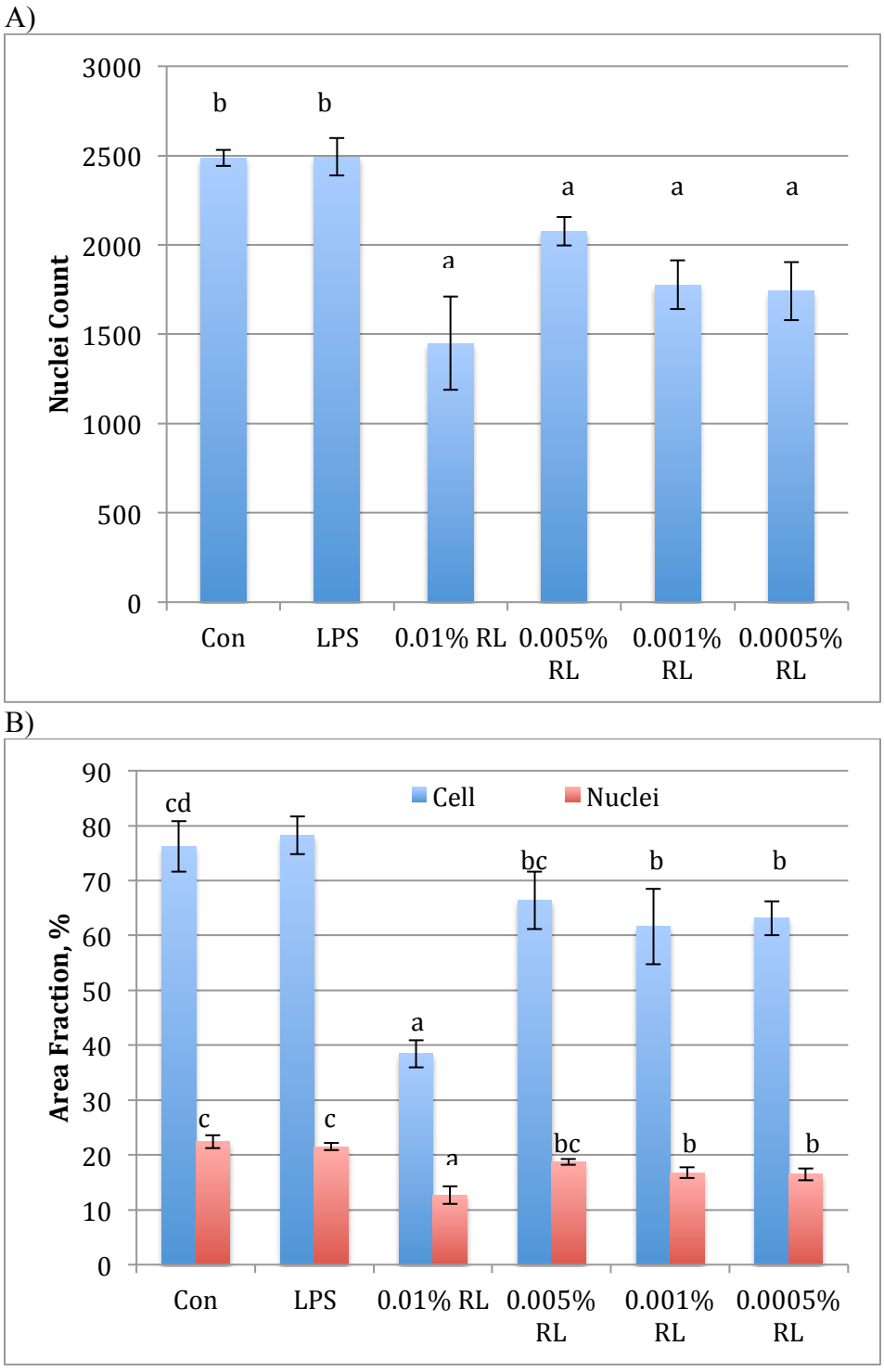


Figure 5.5. Image analysis of IPEC-J2 cells treated with rhamnolipids for 1 h and incubated in fresh media for 6 h. Nuclei counts (**A**) and cell and nuclei area fractions (**B**) measured using Fiji. Bars represent least square means ± SEM. Statistical differences denoted by superscripts, $P < 0.05$.

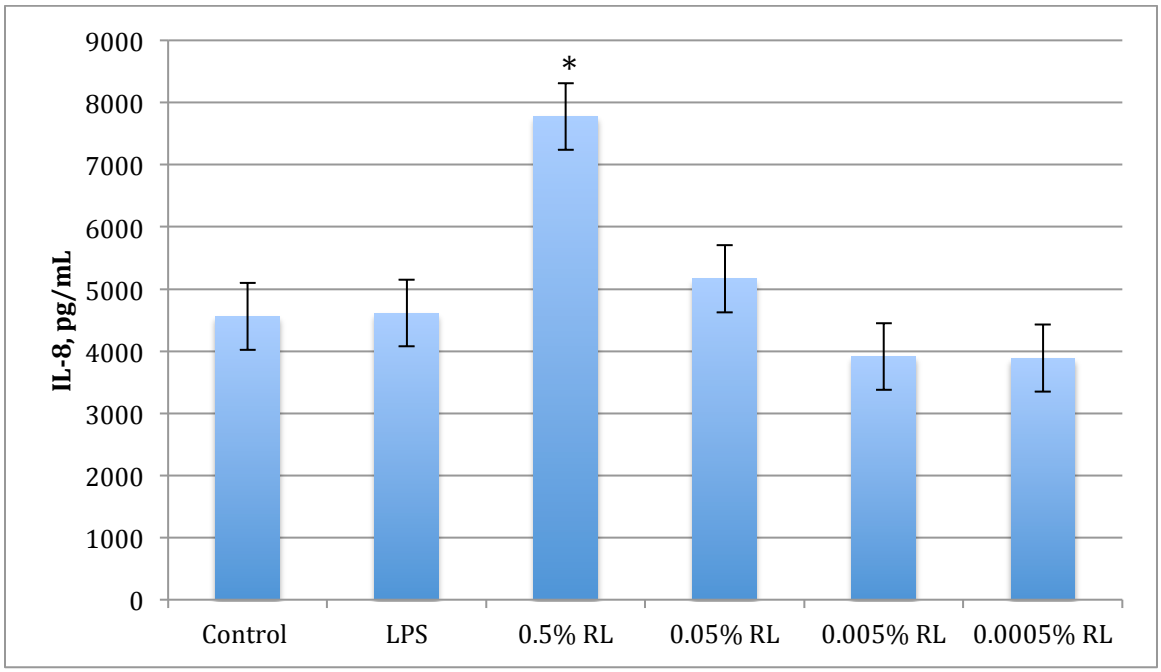


Figure 5.6. Interleukin-8 secretion from jejunum explants incubated with rhamnolipids for 1 h and fresh media for 3 h. Bars represent least square means ± SEM. Statistical difference indicated by *, $P < 0.001$.

CHAPTER 6: GENERAL DISCUSSION

The swine industry is always looking for new ways to innovate and improve herd health, nutrition, and reproduction while also decreasing costs. Since the early 20th century, this has been achieved through genetic selection, expanded knowledge of nutrition, changes in housing conditions, and disease mitigation strategies. These strategies will continue to be employed in the future as dietary ingredients change or new ones become available, new diseases may emerge, and we improve our understanding of animal housing and welfare.

The growing pig requires nutrients for tissue growth, so maximizing nutrient uptake is critical for pig growth and health. However, the nursery pig represents a challenge as it must overcome the numerous stresses it experiences during the weaning transition (Campbell et al., 2013) and adapt to solid feed. The weaning transition is especially impactful on the gut as it induces short and long-term changes to intestinal absorption, secretion, and barrier function (Boudry et al., 2004). Disruptions to barrier function such as increased permeability may further enable translocation of bacteria or toxins that lead to further illness or tissue damage (Moeser et al., 2017). The use of in feed antibiotics has been useful in suppressing pathogenic bacteria and improving growth performance (Cromwell, 2002), although implementation of the Veterinary Feed Directive has put a halt to using antibiotics for growth promotion purposes (FDA, 2017). However, with this change there will be a greater focus on other types of antimicrobials and antibiotic alternatives for use in the swine industry (Liu et al., 2018). Viruses like porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome

(PRRS), and *Mycoplasma hyopneumoniae* are prevalent challenges in the swine industry, especially for younger pigs (Chae, 2016). However, the use of vaccines, particularly for PCV2 and *Mycoplasma*, has been successful in reducing clinical signs of illness and improving ADG (Witvliet et al., 2015; Park et al., 2016). Nevertheless, a poor weaning transition and disease can reduce pig growth performance and increase chances of morbidity and mortality leading to an increase production costs. Thus the overall theme of this dissertation was to assess different strategies for improving pig health and growth performance.

In chapter 2, we assessed changes to the microbiome in pigs either vaccinated (VAC) for or inoculated (PCV) with PCV2. Changes in the microbiome were primarily age driven similar to other published data (Pajarillo et al., 2014; Frese et al., 2015). Overall, changes in abundance of Prevotellaceae, Bacteroidales, and Ruminococcaceae occurred between d 14 and d 28 for PCV pigs, although these changes were inversed when compared to VAC pigs. Changes in the PCV pigs may have been driven by an active PCV2 infection compared to the VAC pigs that had already been vaccinated for PCV2. Using multivariate analysis by linear associations (MaAsLin), we found *Oscillibacter valericigenes* (-0.094) and *Intestimonas butyriciproducens* (-0.073) were negatively associated with blood IgM whereas *Prevotella stercorea* (0.214) and *Solitalea koreensis* (0.407) were positively associated with blood IgG. However, it is not clear how these bacteria are directly involved with immunoglobulin production.

Utilization of MaAsLin represents an opportunity to look for relationships of bacterial populations against matching phenotypic metadata (Morgan et al., 2015). This method has been shown to be useful in selecting for probiotic bacteria during a prebiotic

nursery trial (Li, 2017). This may be useful during and after a disease challenge to how bacteria respond. In addition, use of MaAsLin is being explored for use in human studies (Lim et al., 2016; Kim et al., 2018).

Using the same PCV2 infection model from chapter 2, we assessed genetic selection for resistance to PCV2 infection and how that impacted pig performance from nursery to finishing in chapter 3. No differences were seen in growth performance across experiments, regardless of treatment or genotype. In the nursery phase (Exp. 1), PCV2 viremia and IgG titers decreased with increased presence of the favorable C allele in the SNP site in infected pigs. Additionally, genotype appeared to have a positive impact on nutrient digestibility through the first four weeks as pigs with favorable genotypes had increased digestibility coefficients. Likewise, genotype had a beneficial impact on digestibility during the grow-finish phase of Exp. 2; however, this effect was not seen in the VAC pigs. In terms of production costs, PCV2 infected pigs with the CC genotype had a decreased feed cost per BW gain compared with the TT genotype. This trend carried through the grow-finish phase as favorable genotypes had a reduced feed costs compared with the susceptible pigs, regardless of PCV2 status. Surprisingly, in the PCV pigs, the CT genotype had higher feed costs in both the nursery and grow-finish phases than either the CC or TT pigs. Overall, the phenotypic effects of the favorable genotypes appeared to play a more prominent role during an active infection than vaccination, however, it is important to note that this experiment lacked sufficient CC genotype pigs to facilitate having a vaccinated CC group. Furthermore, the study design did not account for viremia and antibody titers of the vaccinated animals as the vaccination protocol occurred prior to the study.

Considering the prominence of PCV2 across North America, genetic selection for PCV2 resistance should be considered. Previous work identified three SNPs, including the SNP of interest in our study, associated with PCV2 viral load (McKnite et al., 2014). McKnite's findings demonstrate that possession of at least one favorable allele across the three SNP sites conferred a 5% reduction in viral load and a 30% increase in ADG; however, the majority of pigs in the study possessed two to three favorable alleles. Further work by Engle et al. (2014) would also suggest that presence of some of the favorable alleles is common across pig breeds. There are also a number of effective vaccines for PCV2 on the market (da Silva et al., 2014) and our data would suggest that a PCV2 resistant genotype may be beneficial in reducing feed cost by $5-10 per head over the life of a market pig even when vaccinated.

One of the limitations of this experiment was the lack of vaccinated CC group. Based on our data, we would speculate that the CC genotype would have improved feed efficiency, although size of the difference is difficult to ascertain due to the differential response we had between CT and TT groups when vaccinated and infected with PCV2. In addition to replicating the study, we would also like to collect further viremia and immunoglobulin data during the vaccination phase and afterwards to potentially assess the phenotypic response to vaccine efficacy and subsequent infection with live PCV2. In our study we did not specifically infect the VAC pigs, only enabling them to potentially contract the virus through nose-to-nose contact. This might shed some light on the differential response between PCV and VAC status while also seeing the underlying immune response. Finally, utilizing this model in metabolism crates would be useful for determining how genotype not only affects nutrient digestibility but also nitrogen

retention. This is important as immune system stimulation can decrease nutrient digestibility (Rakhshandeh and de Lange, 2012; Rakhshandeh et al., 2012) and nitrogen retention (de Ridder et al., 2012), especially for immunologically relevant amino acids like tryptophan (Reeds et al., 1994).

For chapter 4, we wanted to see how supplementing additional tryptophan (Trp) in the diet would alter growth performance, nutrient digestibility, and the microbiome of nursery pigs. In our first experiment we found that feed efficiency was improved for pigs receiving Trp. This difference was driven by reduced feed intake in our parity 1 pigs (replication 1) and increased ADG in our parity 2 pigs (replication 2). Although we figured that supplemental Trp may benefit gut health through increased secretion of IL-22 through the AhR-IL-22 axis due to metabolism of Trp by *Lactobacillus*. We saw numerical increases in relative abundance of *Lactobacillus* but found no difference in either IL-22 or pro-inflammatory IL-8. This could be indicative of high health status and low stress conditions of which these animals were born and raised into. Our second experiment aimed to build on the prior by adding an additional Trp+, with twice the amount of Trp, and assessing nutrient digestibility to determine if that was the cause of the difference in feed efficiency. Overall, we saw numerically higher feed efficiency in the Trp and Trp+ groups, but the effect was subdued compared with Exp. 1. This may have been the result of subclinical illness that impacted the first week of data and may have been a confounding factor throughout the rest of the experiment. Indeed we saw an increase in nutrient digestibility of the Trp pigs compared to the control and Trp+ pigs in phase 2. This was not the case with phase 1 as the Trp group had the lowest digestibility coefficients, but it is unclear if this is a diet effect or a result of illness. Although it is

interesting that the relative abundance of *Lactobacillus* follows the same trend as the digestibility, but it is unclear if it means anything.

Tryptophan is seen as important to the swine industry as it can be a limiting amino acid (NRC, 2012) and is important in the acute phase response of the immune system (Reeds et al., 1994). It has been noted that the Trp requirement may be low relative to current NRC guidelines (Goncalves et al., 2015), especially under immune challenge conditions (de Ridder et al., 2012). Studies have demonstrated the benefit of supplemental Trp for low sanitation conditions or during health challenges in pigs (Capozzalo et al., 2012; Messori et al., 2013; Capozzalo et al., 2015) and mice (Zelante et al., 2013). Our study did not seek to directly challenge pigs, but to look at pig response post weaning. Additionally, our conditions were quite hygienic which is atypical for commercial nurseries; however, we did see an improvement in performance for Exp. 1, but not Exp. 2. Furthermore, Trp supplementation has been shown to decrease pro-inflammatory cytokines and increase tight junctions proteins (Liang et al., 2018). Although, we did not measure barrier function, it should be considered for future trials as the concentration utilized by Liang, exceeded the values reported by (Goncalves et al., 2015) for optimal growth. In fact, while diets are typically formulated to ensure adequate concentrations and ratios of essential amino acids, there has been a growing body of work looking at supplementing methionine, threonine, and tryptophan in the diet, particularly in conditions where health challenges may be more prevalent (Xu et al., 2014; van der Meer et al., 2016; van der Meer et al., 2017). While feeding extra amino acids would increase diet costs and reduce profits, the ability for disease to manifest and propagate through densely stocked finishers may result in a reduction of mortality, morbidity, and

overall production losses through supplementation. Although not the specific focus of this body of work, research and usage of antibiotic alternatives continues to important for understanding their impact on animal health and performance.

Finally, chapter 5 focused on using IPEC-J2 cells and tissue explants treated with rhamnolipids to determine how treatment altered barrier function and pro-inflammatory cytokine production. Similar to work done in Caco-2 cells (Jiang et al., 2013; Wallace et al., 2014), we found that rhamnolipids do reduce TEER with increasing concentrations. We have shown that concentrations greater than 0.01% can have cytotoxic effects on the IPEC-J2s, although lower concentrations of rhamnolipids may still facilitate some cell loss as we saw through histology analysis. The apical IL-8 response after an hour of incubation appeared to be dose dependent with the highest concentrations exceeding both our control and LPS treatments although no differences were observed on the basolateral side. A similar result was seen on the basolateral side after six hours without continued exposure to rhamnolipid, but we did not see any difference on the apical side. The jejunal explants were more robust (0.50%) in their tolerance to rhamnolipids. Contrary to our initial experiments with rhamnolipids, the higher doses induced an increasing IL-8 response, but did not appear to be blunted by cell death as we had seen in the IPEC-J2s. Overall, we determined that rhamnolipids have cytotoxic effects and induce IL-8 production, especially at higher concentrations.

Rhamnolipids have been considered for use as excipients due to ability to relax tight junctions, facilitating paracellular uptake of drugs (Jiang et al., 2013; Wallace et al., 2014). In the case of the swine industry, the emulsifying and antimicrobial properties may be of interest. Pigs, like other animals, produce bile to facilitate absorption of dietary

lipid, but the addition of an exogenous emulsifier like rhamnolipid may increase nutrient uptake, allowing for improved nutrient digestibility. However, Gerstel et al. (2009) reported rhamnolipids stimulating IL-8 production by inducing flagellin removal from *P. aeruginosa.* This implies rhamnolipids may facilitate the removal and transport of bacterial surface markers to or across the epithelium that would stimulate the immune system. Although to our best knowledge, there are no published articles with regards to rhamnolipids utilizing whole gut epithelium or animal models.

The antimicrobial properties could be beneficial in animal feed to determine if rhamnolipids are capable of interfering with any microbial contamination that may occur. Mold and mycotoxins are often a possible contaminate in feed. In the years following the 2013-2014 porcine epidemic diarrhea virus (PEDV) outbreaks, there was concern regarding transmission of the virus in feed ingredients (Bowman et al., 2015). Work by the Jones group at Kansas State University has looked at using feed additives such as medium chain fatty acids to mitigate viral survivability and infectivity in feed (Cochrane et al., 2016; Cochrane et al., 2017). Rhamnolipids have been shown to have a wide array of antimicrobial activity including antiviral properties (Remichkova et al., 2008; Abdel-Mawgoud et al., 2010). Additionally, the antibacterial properties have been shown to be effective against both Gram positive and negative bacteria including a number of pathogenic and opportunistic pathogens (Abdel-Mawgoud et al., 2010). Thus these properties may beneficial for suppressing pathogenic bacteria in the pig gut and improving growth performance in lieu of in feed antibiotics no longer allowed by the VFD. To our knowledge, no published experiments have been conducted in feed or

animal models to determine the effect of rhamnolipids; however, we are planning to conduct a nursery study in the near future to determine some of these effects.

In summary, these strategies represent opportunities to continue improving upon pig performance. With that said, there is a continued need to further research areas that improve swine health and performance not only in the nursery and grow-finish barns but even with gilts and sows. Continued emphasis on researching intestinal physiology, especially in conjunction with growing interest in the microbiome and the interplay with nutrition, will be necessary to help reduce intestinal perturbations that can impact feed intake, tissue deposition, and health.

LITERATURE CITED

Abdel-Mawgoud, A. M., F. Lépine, and E. Déziel. 2010. Rhamnolipids: Diversity of structures, microbial origins and roles. Appl Microbiol Biotechnol 86:1323-1336.

Boudry, G., V. Peron, I. L. Hueron-Luron, J. P. Lalles, and B. Seve. 2004. Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. J. Nutr. 134:2256-2262.

Bowman, A. S., R. A. Krogwold, T. Price, M. Davis, and S. J. Moeller. 2015. Investigating the introduction of porcine epidemic diarrhea virus into an ohio swine operation. BMC Veterinary Research 11:38.

Campbell, J. M., J. D. Crenshaw, and J. Polo. 2013. The biological stress of early weaned piglets. Journal of Animal Science and Biotechnology 4:19-19.

Capozzalo, M. M., J. C. Kim, J. K. Htoo, C. F. de Lange, B. P. Mullan, C. F. Hansen, J.-W. Resink, P. A. Stumbles, D. J. Hampson, and J. R. Pluske. 2012. An increased ratio of dietary tryptophan to lysine improves feed efficiency and elevates plasma tryptophan and kynurenine in the absence of antimicrobials and regardless of infection with enterotoxigenic escherichia coli in weaned pigs. J Anim Sci 90:191-193.

Capozzalo, M. M., J. C. Kim, J. K. Htoo, C. F. M. de Lange, B. P. Mullan, C. F. Hansen, J.-W. Resink, P. A. Stumbles, and J. R. Pluske. 2015. Effect of increasing the dietary tryptophan to lysin ratio on plasma levels of tryptophan, kynurenine and urea and on production traits in weaner pigs experimentally infected with an enterotoxigenic strain of escherichia coli. Arch Anim Nutr 69:17-29.

Chae, C. 2016. Porcine respiratory disease complex: Interaction of vaccination and porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, and mycoplasma hyopneumoniae. The Veterinary Journal 212:1-6.

Cochrane, R. A., S. S. Dritz, J. C. Woodworth, A. R. Huss, C. R. Stark, M. Saensukjaroenphon, J. M. DeRouchey, M. D. Tokach, R. D. Goodband, J. F. Bai, Q. Chen, J. Zhang, P. C. Gauger, R. J. Derscheid, R. G. Main, and C. K. Jones. 2017. 196 assessing the effects of medium chain fatty acids and fat sources on pedv rna stability and infectivity. Journal of Animal Science 95:94-94.

Cochrane, R. A., M. Saensukjaroenphon, S. S. Dritz, J. C. Woodworth, A. R. Huss, C. R. Stark, J. M. DeRouchey, M. D. Tokach, R. D. Goodband, J. F. Bai, Q. Chen, J. Zhang, P. C. Gauger, R. Main, and C. K. Jones. 2016. 107 evaluating the inclusion level of medium chain fatty acids to reduce the risk of pedv in feed and spray-dried animal plasma. Journal of Animal Science 94:50-50.

Cromwell, G. L. 2002. Why and how antibiotics are used in swine production. Animal Biotechnology 13:7-27.

da Silva, N., A. Carriquiry, K. O'Neill, T. Opriessnig, and A. M. O'Connor. 2014. Mixed treatment comparison meta-analysis of porcine circovirus type 2 (pcv2) vaccines used in piglets. Preventive Veterinary Medicine 117:413-424.

de Ridder, K., C. L. Levesque, J. K. Htoo, and C. F. M. de Lange. 2012. Immune system stimulation reduces the efficiency of tryptophan utilization for body protein deposition in growing pigs. J Anim Sci 90:3485-3491.

Engle, T., E. Jobman, T. Moural, A. McKnite, S. Barnes, E. Davis, J. Qiu, J. Bundy, J. Tart, T. Johnson, M. F. Rothschild, J. Galeota, R. K. Johnson, G. Plastow, S. Kachman, and D. C. Ciobanu. 2014. Genome-wide analysis of the differential response to experimental challenges with porcine circovirus 2b, 10th World Congress on Genetics Applied to Livestock Production.

FDA. 2017. Fact sheet: Veterinary feed directive final rule and next steps. Unites States Food and Drug Administration.

Frese, S. A., K. Parker, C. C. Calvert, and D. A. Mills. 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome 3:28.

Gerstel, U., M. Czapp, J. Bartels, and J.-M. Schröder. 2009. Rhamnolipid-induced shedding of flagellin from pseudomonas aeruginosa provokes hbd-2 and il-8 response in human keratinocytes. Cellular Microbiology 11:842-853.

Goncalves, M. A. D., S. Nitikanchana, M. D. Tokach, S. S. Dritz, N. M. Bello, R. D. Goodband, K. J. Touchette, J. L. Ursy, J. M. DeRouchey, and J. C. Woodworth.

2015. Effects of standardized ileal digestible tryptophan:Lysine ratio of growth performance of nursery pigs. J Anim Sci 93:3909-3918.

Jiang, L., X. Long, and Q. Meng. 2013. Rhamnolipids enhance epithelial permeability in caco-2 monolayers. International Journal of Pharmaceutics 446:130-135.

Kim, H.-N., Y. Yun, S. Ryu, Y. Chang, M.-J. Kwon, J. Cho, H. Shin, and H.-L. Kim. 2018. Correlation between gut microbiota and personality in adults: A cross-sectional study. Brain, Behavior, and Immunity 69:374-385.

Li, Y. 2017. Effects of prebiotics and probiotics on gut health, immunity, and growth of weanling pigs, University of Nebraska-Lincoln, ETD Collection.

Liang, H., Z. Dai, N. Liu, Y. Ji, J. Chen, Y. Zhang, Y. Yang, J. Li, Z. Wu, and G. Wu. 2018. Dietary l-tryptophan modulates the structural and functional composition of the intestinal microbiome in weaned piglets. Frontiers in Microbiology 9:1736.

Lim, M. Y., H. S. Yoon, M. Rho, J. Sung, Y.-M. Song, K. Lee, and G. Ko. 2016. Analysis of the association between host genetics, smoking, and sputum microbiota in healthy humans. Scientific reports 6:23745-23745.

Liu, Y., C. D. Espinosa, J. J. Abelilla, G. A. Casas, L. V. Lagos, S. A. Lee, W. B. Kwon, J. K. Mathai, D. M. D. L. Navarro, N. W. Jaworski, and H. H. Stein. 2018. Non-antibiotic feed additives in diets for pigs: A review. Animal Nutrition 4:113-125.

McKnite, A. M., J. W. Bundy, T. W. Moural, J. K. Tart, T. P. Johnson, E. E. Jobman, S. Y. Barnes, J. K. Qiu, D. A. Peterson, S. P. Harris, M. F. Rothschild, J. A. Galeota, R. K. Johnson, S. D. Kachman, and D. C. Ciobanu. 2014. Genomic analysis of the differential response to experimental infection with porcine circovirus 2b. Animal Genetics 45:205-214.

Messori, S., P. Trevisi, A. Simongiovanni, D. Priori, and P. Bosi. 2013. Effect of susceptibility to enterotoxigenic escherichia coli f4 and of dietary tryptophan on gut microbiota diversity observed in healthy young pigs. Vet Microbiol 162:173-179.

Moeser, A. J., C. S. Pohl, and M. Rajput. 2017. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. Animal Nutrition 3:313-321.

Morgan, X. C., B. Kabakchiev, L. Waldron, A. D. Tyler, T. L. Tickle, R. Milgrom, J. M. Stempak, D. Gevers, R. J. Xavier, M. S. Silverberg, and C. Huttenhower. 2015. Associations between host gene expression, the mucosal microbiome, and clinical outcome in the pelvic pouch of patients with inflammatory bowel disease. Genome Biology 16:67.

NRC. 2012. Nutrient requirements of swine. 11th Ed. ed. Natl. Acad. Press, Washington, DC.

Pajarillo, E. A., J. Chae, M. P. Balolong, H. B. Kim, and D. Kang. 2014. Assessment of fecal bacterial diversity among healthy piglets during the weaning transition. J Gen Appl Microbiol 60:140-146.

Park, C., J. Jeong, K. Choi, and C. Chae. 2016. Efficacy of a new bivalent vaccine of porcine circovirus type 2 and mycoplasma hyopneumoniae (fostera™ pcv mh) under experimental conditions. Vaccine 34:270-275.

Rakhshandeh, A., and C. F. de Lange. 2012. Evaluation of chronic immune system stimulation models in growing pigs. Animal 6:305-310.

Rakhshandeh, A., J. C. M. Dekkers, B. J. Kerr, T. E. Weber, J. English, and N. K. Gabler. 2012. Effect of immune system stimulation and divergent selection for residual feed intake on digestive capacity of the small intestine in growing pigs1. Journal of Animal Science 90:233-235.

Reeds, P. J., C. R. Fjeld, and F. Jahoor. 1994. Do the differences between the amino acid compositions of acute-phase and muscle proteins have a bearing on nitrogen loss in traumatic states? The Journal of nutrition 124:906-910.

Remichkova, M., D. Galabova, I. Roeva, E. Karpenko, A. Shulga, and A. S. Galabov. 2008. Anti-herpesvirus activities of pseudomonas sp. S-17 rhamnolipid and its complex with alginate. Z. Naturforsch C 63:75-81.

van der Meer, Y., W. J. J. Gerrits, A. J. M. Jansman, B. Kemp, and J. E. Bolhuis. 2017. A link between damaging behaviour in pigs, sanitary conditions, and dietary protein and amino acid supply. PLOS ONE 12:e0174688.

van der Meer, Y., A. Lammers, A. J. M. Jansman, M. M. J. A. Rijnen, W. H. Hendriks, and W. J. J. Gerrits. 2016. Performance of pigs kept under different sanitary conditions affected by protein intake and amino acid supplementation1,2. Journal of Animal Science 94:4704-4719.

Wallace, C. J., S. H. Medina, and M. E. H. ElSayed. 2014. Effect of rhamnolipids on permeability across caco-2 cell monolayers. Pharmaceutical Research 31:887-894.

Witvliet, M., H. Holtslag, T. Nell, R. Segers, and V. Fachinger. 2015. Efficacy and safety of a combined porcine circovirus and mycoplasma hyopneumoniae vaccine in finishing pigs. Trials in Vaccinology 4:43-49.

Xu, S., Y. Zhao, J. Shen, Y. Lin, Z. Fang, L. Che, and D. Wu. 2014. Threonine and tryptophan supplementation enhance porcine respiratory and reproductive

syndrome (prrs) vaccine-induced immune responses of growing pigs. Anim Sci J 86:294-304.

Zelante, T., R. G. Iannitti, C. Cunha, A. De Luca, G. Giovannini, G. Pieraccini, R. Zecchi, C. D'Angelo, C. Massi-Benedetti, F. Fallarino, A. Carvalho, P. Puccetti, and L. Romani. 2013. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity 39:372-385.

APPENDIX A: Bioinformatics Chapter 2

Bioinformatics Protocol

Adapted from protocol provided by Chris Anderson, Samodha Lab Group Fall 2014

Mothur to QIIME OTU table

./mothur_to_qiime_otu_map.pl -file=exp13404.16S.binseq.fasta

Number of input sequences: 65547
Number of OTUs: 2107

./mothur_to_qiime_rep_set.pl -file=exp13404.16S.repset.fasta

Total sequences (equal to OTUs): 2107

Make the OTU table, create a phylogenetic tree and remove samples with low sequencing numbers

Assign Taxonomy to your new qiime repset created above (the location of files for the –t and –r parameters may be different than my computer):

assign_taxonomy.py -i exp13404.qiime_repset.txt –t /macqiime/greengenes/gg_12_10_otus/taxonomy/97_otu_taxonomy.txt -r /macqiime/greengenes/gg_12_10_otus/rep_set/97_otus.fasta -o exp13404.assign_gg_taxa

Construct the OTU table in QIIME using the otu mapping file and assigned taxonomy:

make_otu_table.py -i exp13404.mapping.txt -o exp13404.biom -t exp13404.assign_gg_taxa/exp13404.qiime_repset_tax_assignments.txt

Convert the biom formatted OTU table to a txt file and ensure the taxonomy was added to the OTU table:
convert_biom.py -i exp13404.biom -o exp13404.txt -b --header_key taxonomy --output_metadata_id "Consensus Lineage"

#biom convert replaced convert_biom.py
biom convert –i exp13404.txt –o exp13404test.biom --table-type=”OTU table” --to-json –process-obs-metadata taxonomy

biom summarize-table –i exp13404.biom –o exp13404.summary.txt

You may want to remove any chloroplast sequences from your dataset:

filter_taxa_from_otu_table.py -i exp13404.biom -o exp13404.taxa_filter.biom -n p_Cyanobacteria

You can sort your OTU table by a certain treatment or category in your mapping file so that your results appear in that order:
sort_otu_table.py -i exp13404.taxa_filter.biom -m exp13404.mapping.txt -s Diet -o exp13404.sort.biom

-s could be trt, phase, or trtxphase

Get a sample distribution of your OTU table:
biom summarize-table –i exp13404.sort.biom –o exp13404.sort.summary.txt

Num samples: 35
Num observations: 893
Total count: 320235
Table density (fraction of non-zero values): 0.449
Table md5 (unzipped): 5529513b58e377bbc5c133a3d4e7e20a

Counts/sample summary:
 Min: 2137.0
 Max: 20051.0
 Median: 8479.000
 Mean: 9149.571
 Std. dev.: 4989.870
 Sample Metadata Categories: None provided
 Observation Metadata Categories: Taxonomy

Counts/sample detail:
 G24: 2137.0
 G31: 2714.0
 G10: 2787.0
 G16: 3217.0
 G29: 3240.0
 G33: 3682.0
 G34: 4529.0
 G32: 4816.0
 G12: 4865.0
 G1: 5212.0
 G28: 5435.0
 G36: 5728.0
 G27: 5774.0
 G22: 6003.0
 G14: 6202.0
 G9: 7268.0
 G15: 7672.0

G35: 8479.0
G7: 8755.0
G3: 9188.0
G25: 10464.0
G30: 11504.0
G8: 11544.0
G2: 11635.0
G13: 11964.0
G23: 12348.0
G4: 12689.0
G21: 13291.0
G26: 13492.0
G20: 14406.0
G6: 15074.0
G5: 17366.0
G19: 18004.0
G18: 18700.0
G17: 20051.0

Need to filter out the control samples and any samples that are due to low sequencing numbers:
filter_samples_from_otu_table.py -i exp13404.sort.biom -m exp13404.mapping.txt --sample_id_fp keep_samples.txt -o exp13404.sort.keep.biom

Align your qiime repset file in RDP and replace dots with dashes in the resulting file
Add AAAAAAAAAA
Remove the last line from RDP aligned (weird info on it)

Create a distance matrix in mothur using your aligned repset file
mothur > dist.seqs(fasta=repset.qiime.aligned.fasta, output =phylip, processors=5)
mothur > clearcut (phylip=phylip.dist)
For QIIME, we do three levels of analysis generally: Total, No Singletons (singletons are generally biologically insignificant to us and may actually be sequencing errors), and Core defined by a treatment of interest. For this analysis, I have created a mapping file already when I demulitplexed and quality controlled your data.

Note: remove AAAAAAAAAA from .tre file. Check alignment text file for OTUs that had incorrect alignment start and stops. Save a list of chimeras in chimera.txt

filter_otus_from_otu_table.py –i exp13404.sort.keep.biom –o exp13404.no_chimera.keep.biom –e chimera

Total Analysis
Create a rarefraction curve for looking at alpha diversity (this will take a while but you can open another terminal window and proceed to the next commands while it runs)

alpha_rarefaction.py -i exp13404.sort.keep.biom -m exp13404.mapping.txt -p qiime_parameters_working.txt -n 10 -o analysis_singletons/total.alpha_rarefaction

#qiime_working_parameters.txt not actively kept up anymore, may need to transfer old copy

Look at the some alpha diversity measures:
alpha_diversity.py -i exp13404.sort.keep.biom -m chao1,shannon,observed_species,ace,simpson -o analysis_singletons/total.alpha_diversity.txt

Make an OTU network – can create cytoscape images from the resulting files later if you wish

make_otu_network.py -i exp13404.sort.keep.biom -m exp13404.mapping.txt -o analysis_singletons/total.network

Create a summary of the taxonomy information that be used to plot in the next command:

summarize_taxa.py -i exp13404.sort.keep.biom -L 2,3,4,5,6,7 -o analysis_singletons/total.summarize_taxa

Plot the taxonomy:
plot_taxa_summary.py -i analysis_singletons/total.summarize_taxa/rumen.sort.keep_L2.txt,analysis_singletons/total.summarize_taxa/rumen.sort.keep_L3.txt,analysis_singletons/total.summarize_taxa/rumen.sort.keep_L4.txt,analysis_singletons/total.summarize_taxa/rumen.sort.keep_L5.txt,analysis_singletons/total.summarize_taxa/rumen.sort.keep_L6.txt,analysis_singletons/total.summarize_taxa/rumen.sort.keep_L7.txt -l Phylum,Class,Order,Family,Genus,Species -c bar,area,pie -o analysis_singletons/total.taxa_plots

File names for the next two commands may be different but provides a contxt for the commands:

The –t parameter is the phylogenetic tre that you created in mothur
The –e parameter is the sample with the lowest number of reads (subsample the remaining samples to this amount)

Note: Need to run code further down to generate “no_singletons.biom”

beta_diversity_through_plots.py -i exp13404.no_singletons.biom -e 2511 -m exp13404.mapping.txt -p qiime_parameters_working.txt -t exp13404.align_pynast.tre -c Diet -o analysis_no_singletons/total.no_singletons.beta_diversity

“-e 8147” for Kelly, no –c option in this version of script

jackknifed_beta_diversity.py -i exp13404.no_singletons.biom -e 2511 -m exp13404.mapping.txt -p qiime_parameters_working.txt -t exp13404.align_pynast.tre -o analysis_no_singletons/total.no_singletons.jk.beta_diversity

Remove Singletons

Remove any OTUs with just 1 sequence:

filter_otus_from_otu_table.py -i exp13404.sort.keep.biom -n 2 -o exp13404.no_singletons.biom

Look at the OTU table breakdown now:
biom summarize-table –i exp13404.sort.biom –o exp13404.sort.summary.txt

Num samples: 35
Num observations: 893
Total count: 320235
Table density (fraction of non-zero values): 0.449
Table md5 (unzipped): 16b2b32e3480c505ab9cddaab0fe1997

Counts/sample summary:
Min: 2137.0
Max: 20051.0
Median: 8479.000
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G10: 2787.0
G16: 3217.0
G29: 3240.0
G33: 3682.0
G34: 4529.0
G32: 4816.0
G12: 4865.0
G1: 5212.0
G28: 5435.0
G36: 5728.0
G27: 5774.0
G22: 6003.0
G14: 6202.0

G9: 7268.0
G15: 7672.0
G35: 8479.0
G7: 8755.0
G3: 9188.0
G25: 10464.0
G30: 11504.0
G8: 11544.0
G2: 11635.0
G13: 11964.0
G23: 12348.0
G4: 12689.0
G21: 13291.0
G26: 13492.0
G20: 14406.0
G6: 15074.0
G5: 17366.0
G19: 18004.0
G18: 18700.0
G17: 20051.0

make_otu_heatmap.py -i exp13404.no_singletons.biom -o
analysis_no_singletons/total.no_singletons.otu_heatmap

make_otu_network.py -i exp13404.no_singletons.biom -o
analysis_no_singletons/total.no_singletons.network -m exp13404.mapping.txt

summarize_taxa.py -i exp13404.no_singletons.biom -L 2,3,4,5,6,7 -o
analysis_no_singletons/total.no_singletons.summarize_taxa

plot_taxa_summary.py -i
analysis_no_singletons/total.no_singletons.summarize_taxa/exp13404.no_singletons_L2.
txt,analysis_no_singletons/total.no_singletons.summarize_taxa/exp13404.no_singletons_
L3.txt,analysis_no_singletons/total.no_singletons.summarize_taxa/exp13404.no_singleto
ns_L4.txt,analysis_no_singletons/total.no_singletons.summarize_taxa/exp13404.no_singl
etons_L5.txt,analysis_no_singletons/total.no_singletons.summarize_taxa/exp13404.no_si
ngletons_L6.txt,analysis_no_singletons/total.no_singletons.summarize_taxa/exp13404.no
_singletons_L7.txt -l Phylum,Class,Order,Family,Genus,Species -c bar,area,pie -o
analysis_no_singletons/total.no_singletons.plot_taxa

alpha_diversity.py -i exp13404.no_singletons.biom -m
chao1,shannon,observed_species,ace,simpson -o
analysis_no_singletons/total.no_singletons.alpha_diversity.txt

alpha_rarefaction.py -i exp13404.no_singletons.biom -m exp13404.mapping.txt -p qiime_parameters_working.txt -n 10 -o analysis_no_singletons/total.no_singletons.alpha_rarefaction

Use the same –t file as you have been but the –e value changes (lowest number of sequences in a sample – see above OTU breakdown to understand where 2511 used below come from)

beta_diversity_through_plots.py -i exp13404.no_singletons.biom -e 2511 -m exp13404.mapping.txt -p qiime_parameters_working.txt -t exp13404.align_pynast.tre -c Diet -o analysis_no_singletons/total.no_singletons.beta_diversity
"-e8147" no –c command

jackknifed_beta_diversity.py -i exp13404.no_singletons.biom -e 2511 -m exp13404.mapping.txt -p qiime_parameters_working.txt -t exp13404.align_pynast.tre -o analysis_no_singletons/total.no_singletons.jk.beta_diversity

Core

Split the OTU table (with singletons removed) based on the category in the mapping file by which you wish to define the core:
split_otu_table.py -i exp13404.no_singletons.biom -m exp13404.keep.mapping.txt -f trtxphase -o exp13404.no_singletons.split

Filter the split OTU tables based on how many samples should have a given OTU to be considered a part of the core. For the example below I was doing 2 out of 2 samples, 2/3 (s=2), or 3/4 (s=3)

For exp13404 it was 5/6 (s=5)

filter_otus_from_otu_table.py -i exp13404.no_singletons.split/exp13404.no_singletons_d0_I.biom -s 5 -o exp13404.core.split/d0_I.core.biom

filter_otus_from_otu_table.py -i exp13404.no_singletons.split/exp13404.no_singletons_d0_V.biom -s 5 -o exp13404.core.split/d0_Vcore.biom

filter_otus_from_otu_table.py -i exp13404.no_singletons.split/exp13404.no_singletons_d14_I.biom -s 5 -o exp13404.core.split/d14_I.core.biom

filter_otus_from_otu_table.py -i exp13404.no_singletons.split/exp13404.no_singletons_d14_V.biom -s 5 -o exp13404.core.split/d14_Vcore.biom

filter_otus_from_otu_table.py -i exp13404.no_singletons.split/exp13404.no_singletons_d28_I.biom -s 5 -o exp13404.core.split/d28_I.core.biom

filter_otus_from_otu_table.py -i exp13404.no_singletons.split/exp13404.no_singletons_d28_V.biom -s 5 -o exp13404.core.split/d28_Vcore.biom

Merge the resulting OTU tables together:
merge_otu_tables.py -i exp13404.core.split/d0_I.core.biom,exp13404.core.split/d0_V.core.biom,exp13404.core.split/d14_I.core.biom,exp13404.core.split/d14_V.core.biom,exp13404.core.split/d28_I.core.biom,exp13404.core.split/d28_V.core.biom -o exp13404.core.biom

Now you have all the OTU identifiers that would be a part of the core. However, the abundances for those OTUs are just for the samples we defined. We need the abundances for all samples. So you need to take the OTU identifiers for the core and filter the OTU table that has singletons removed:

Convert the .biom formatted OTU table to a .txt OTU file. Now you copy the first column of the table (open in excel) into a word document and save the identifiers as a separate file

biom convert -i merged.core.biom -o merged.core.txt -b

Copy the OTU ids from the OTU .txt file into a separate file and save it (next command that file is core_keep.txt)
Filter the OTU table that has singletons removed:

filter_otus_from_otu_table.py -i exp13404.no_singletons.biom --negate_ids_to_exclude -e core_keep.txt -o exp13404.core.biom

Take a look at the OTU table breakdown now. Take note of the number of sequences that are in the core compared to the total and table with singletons removed. The core generally has 80-90% of the sequences of the total with singletons removed

biom summarize-table -i exp13404.core.biom
Num samples: 35
Num observations: 383
Total count: 249888
Table density (fraction of non-zero values): 0.519
Table md5 (unzipped): 0594eed876b94a648c5f476cf82c16f8

Counts/sample summary:
Min: 1657.0

Max: 17497.0
Median: 6298.000
Mean: 7139.657
Std. dev.: 4059.211
Sample Metadata Categories: None provided
Observation Metadata Categories: taxonomy

Counts/sample detail:
G24: 1657.0
G31: 1925.0
G33: 2414.0
G10: 2463.0
G16: 2646.0
G29: 2741.0
G32: 3238.0
G12: 3853.0
G34: 3864.0
G28: 4392.0
G35: 4436.0
G14: 4484.0
G1: 4593.0
G27: 4736.0
G22: 4908.0
G36: 5404.0
G3: 6045.0
G7: 6298.0
G9: 6532.0
G15: 6659.0
G21: 7756.0
G30: 8272.0
G8: 8650.0
G25: 8653.0
G23: 9009.0
G13: 10027.0
G2: 10030.0
G26: 10250.0
G6: 10766.0
G19: 10865.0
G4: 11680.0
G20: 12884.0
G5: 13999.0
G17: 16262.0
G18: 17497.0

Run the same commands for the core OTU table as you did for the total and no singletons analysis

make_otu_heatmap_html.py -i exp13404.core.biom -o analysis_core/core.heatmap

make_otu_network.py -i exp13404.core.biom -m exp13404.keep.mapping.txt -o analysis_core/core.network

summarize_taxa.py -i exp13404.core.biom -L 2,3,4,5,6,7 -o analysis_core/core.summarize_taxa

plot_taxa_summary.py -i analysis_core/core.summarize_taxa/exp13404.core_L2.txt,analysis_core/core.summarize_taxa/exp13404.core_L3.txt,analysis_core/core.summarize_taxa/exp13404.core_L4.txt,analysis_core/core.summarize_taxa/exp13404.core_L5.txt,analysis_core/core.summarize_taxa/exp13404.core_L6.txt,analysis_core/core.summarize_taxa/exp13404.core_L7.txt -l Phylum,Class,Order,Family,Genus,Species -c bar,area,pie -o analysis_core/core.plot_taxa

alpha_rarefaction.py -i exp13404.core.biom -m exp13404.keep.mapping.txt -p qiime_parameters_working.txt -n 10 -o analysis_core/core.alpha_rarefaction

Use the same –t file as you have been but the –e value changes (lowest number of sequences in a sample)

beta_diversity_through_plots.py -e 2369 -i exp13404.core.biom -m exp13404.keep.mapping.txt -c Diet -p qiime_parameters_working.txt -t exp13404.align_pynast.tre -o analysis_core/core.beta_diversity

jackknifed_beta_diversity.py -e 2369 -i exp13404.core.biom -m exp13404.keep.mapping.txt -p qiime_parameters_working.txt -t exp13404.align_pynast.tre -o analysis_core/core.jk.beta_diversity

APPENDIX B: Bioinformatics Chapter 4

Protocol adapted from dada2 pipeline and phyloseq tutorials by Wesley Tom, Samodha Lab group

R and RStudio will need to be installed on the computer. This was done on a Mac with OS X 10.11.6

Refer to the installation guides for the dada2 pipeline (https://benjjneb.github.io/dada2/tutorial.html) and phyloseq (https://joey711.github.io/phyloseq/index.html)

The Silva reference database (v132) used to generate amplicon sequence variants (ASV) was retrieved from https://mothur.org/wiki/Silva_reference_files

```
#Make sure that all required packages are installed via the following:
source("https://bioconductor.org/biocLite.R")
biocLite("BiocStyle")
#Updated all packages that are needed updated

#Once BiocStyle is installed you can run the following commands to install relevant packages ggplot, gridExtra, dada2, DECIPHER, and phangorn, as well as activate all of the relevant packages:

library("knitr")
library("BiocStyle")
.cran_packages <- c("ggplot2", "gridExtra")
.bioc_packages <- c("dada2", "phyloseq", "DECIPHER", "phangorn")
.inst <- .cran_packages %in% installed.packages()
if(any(!.inst)) {
 install.packages(.cran_packages[!.inst])
}
.inst <- .bioc_packages %in% installed.packages()
if(any(!.inst)) {
 source("http://bioconductor.org/biocLite.R")
biocLite(.bioc_packages[!.inst], ask = F)
}

#Load packages into session, and print package version
sapply(c(.cran_packages, .bioc_packages), require, character.only = TRUE)

#Set up the directory your fastq files are in to the main directory where output files will be stored and set the working directory to where fastq files are stored
path <-
("/Users/danavansambeek/Desktop/PhD/Tryptophan_Study/dada2_analysis/fastq_files")
```

```
list.files(path)
setwd("/Users/danavansambeek/Desktop/PhD/Tryptophan_Study/dada2_analysis/fastq_files /")

#Sort ensures forward/reverse reads are in the same order:
fnFs <- sort(list.files(path, pattern="_R1_001.fastq", full.names = TRUE))
fnRs <- sort(list.files(path, pattern="_R2_001.fastq", full.names = TRUE))

# Extract sample names, assuming filenames have format: SAMPLENAME_XXX.fastq
sample.names <- sapply(strsplit(basename(fnFs), "_"), `[`, 1)

#Visualizes the quality profiles of the forward and reverse reads
#fnFs[1:2] 2 profiles will be shown, 10 as shown below
plotQualityProfile(fnFs[1:10])
plotQualityProfile(fnRs[1:10])
#Will give you an idea where the quality score begins to drop. Keep this in mind for the filterAndTrim step
#My data fell below a QS threshold of 30 around read 250 for both forward and reverse. So Nirosh suggested truncating around 240 for both

#Before filtering we have to define the file name for the filtered reads.
filt_path <- file.path(path, "filtered")#places filtered files in filtered subdirectory
if(!file_test("-d", filt_path)) dir.create(filt_path)
filtFs <- file.path(filt_path, paste0(sample.names, "_F_filt.fastq"))
filtRs <- file.path(filt_path, paste0(sample.names, "_R_filt.fastq"))

#Now we filter and use a max of 2 expected errors per read (Edgar and Flyvberg 2015):
#Modify your truncation length for your reads here, forward and reverse respectively.
out <- filterAndTrim(fnFs, filtFs, fnRs, filtRs, truncLen = c(240,240), maxN = 0, maxEE = c(2,2),truncQ = 2, rm.phix = TRUE, multithread = TRUE )
head(out)

#Uses an algorithm to learn error rates
errF <- learnErrors(filtFs, multithread=TRUE)
errR <- learnErrors(filtRs, multithread=TRUE)

#Plots estimates of error rates
#Ideally your black trend line should fit the data well
plotErrors(errF, nominalQ=TRUE)
plotErrors(errR, nominalQ=TRUE)

#Next we want to dereplicate all redundant reads into unique sequences, all with a corresponding abundance.
derepFs <- derepFastq(filtFs, verbose = T)
derepRs <- derepFastq(filtRs, verbose = T)
#Name derep class objects by sample names
```

```
names(derepFs) <- sample.names
names(derepRs) <- sample.names

#Runs the dada2 algorithm for the forward and reverse reads
dadaFs <- dada(derepFs, err = errF, multithread = T)
dadaRs <- dada(derepRs, err = errR, multithread = T)

#Inspects the following object about sequence variants in your reads
dadaFs[[1]]
dadaRs[[1]]

#Merge froward and reverse reads processed by the dada2 algorithm
mergers <- mergePairs(dadaFs, derepFs, dadaRs, derepRs)

#Inspect the merger data.frame from the first sample
head(mergers[[1]])

#Constructs sequence table
seqtabAll <- makeSequenceTable(mergers[!grepl("1502_bact", names(mergers))])
dim(seqtabAll)

#Inspect distribution of sequence lengths
table(nchar(getSequences(seqtabAll)))

#Remove chimeras
seqtabNoC <- removeBimeraDenovo(seqtabAll)
#Shows how many chimeras make up merged sequence variants
dim(seqtabNoC)

#Shows non-chimeric sequence variants while also accounting for abundance. Mine was
about 0.99
sum(seqtabNoC)/sum(seqtabAll)

#Now we assign taxonomy, needs to know where your reference file location is
fastaRef <-
"/Users/danavansambeek/Desktop/PhD/Tryptophan_Study/dada2_analysis/fastq_files/sil
va_nr_v132_train_set.fa.gz"
taxTab <- assignTaxonomy(seqtabNoC, refFasta = fastaRef, multithread = T)
unname(head(taxTab))

#Try and summarize the filtration steps as follows
getN <- function(x) sum(getUniques(x))
track <- cbind(out, sapply(dadaFs, getN), sapply(mergers, getN), rowSums(seqtabAll),
rowSums(seqtabNoC))
colnames(track) <- c("input", "filtered", "denoised", "merged", "tabled", "nonchim")
rownames(track) <- sample.names
```

```
head(track)

#Now we want to attempt to construct a phylogenetic tree, showing the relationship between all ASVs
#which is used to calculate distances between samples.
#Use DECIPHER R package for alignment
seqs <- getSequences(seqtabNoC)
names(seqs) <- seqs #this spreads the sample name to the tips of the tree
alignment <- AlignSeqs(DNAStringSet(seqs), anchor = NA, verbose = F)

phangAlign <- phyDat(as(alignment, "matrix"), type = "DNA")
write.phyDat(phangAlign, file = "trp_alignment.fasta", format = "fasta")


#Now that we have our table made and we are happy with the quality control, we need to align our sequences. So to begin we want to create a directory and write tables of the results from the dada algorithm (dada_outputs_for_mothurQIIME/)
write.table(seqtabNoC, file = "trp_table.txt", col.names = TRUE, row.names = TRUE, sep = "\t")
write.table(track, file = "trp_track.txt", col.names = TRUE, row.names = TRUE, sep = "\t")
write.table(taxTab, file = "trp_taxa.txt", col.names = TRUE, row.names = TRUE, sep = "\t")

#Generate a table that will work with QIIME
transpose_seqtab <- t(seqtabNoC)
write.table(transpose_seqtab, file = "trp_table_for_qiime.txt", col.names = TRUE, row.names = TRUE, sep = "\t")

#Create a tab delimited text file to be used for creating a fasta file eventually to use for a phylogenetic tree.
asv_vector <- as.data.frame(row.names(transpose_seqtab), stringsAsFactors = default.stringsAsFactors())
rownames(asv_vector) <- row.names(transpose_seqtab)
colnames(asv_vector) <- NULL
write.table(asv_vector, file = "tab_sep_asvs_for_fasta.txt",sep = "\t")

#Note from Wes
#I haven't figured a nice way in code to do this, but the next thing we want to do is convert the tab delimited asv file into fasta format.
#I used
http://sequenceconversion.bugaco.com/converter/biology/sequences/tab_to_fasta.php to do this. It is fast and works well...
```

#Next we are going to use Mothur to align our ASVs, and use Phylip and Clearcut to generate distances and a phylogenetic tree within the mothur environment using the following steps

#Activate mothur using a terminal (note, you should be in the directory in which you just generated all of the ASV ouputs, specifically the fasta file of all ASV species)
#Alternatively, you could upload the fasta file onto Crane and use Mothur out there if it is set up.
#danavansambeeek$ ./mothur
#Mothur will load up
#The Silva reference can be retrieved from the Mothur website

#########
Wes' Example
'mothur > align.seqs(fasta=1502_bactASV.fasta,
reference=/Volumes/SBPD/Silva.seed_v132/silva.seed_v132.align, processors=8)'

Using 8 processors.

Reading in the /Volumes/SBPD/Silva.seed_v132/silva.seed_v132.align template sequences... DONE.
It took 15 to read 11180 sequences.
Aligning sequences from 1502_bactASV.fasta ...

It took 16 secs to align 4795 sequences.

Output File Names:
1502_bactASV.align
1502_bactASV.align.report

'mothur > dist.seqs(fasta=1502_bactASV.align, output=lt)""

Output File Names:
1502_bactASV.phylip.dist

It took 150 seconds to calculate the distances for 4795 sequences.

mothur > clearcut(phylip=1502_bactASV.phylip.dist)

Output File Names:
1502_bactASV.phylip.tre
##########

#Switch to phyloseq.R analysis

```
setwd("/Users/danavansambeek/Desktop/PhD/Tryptophan_Study/dada2_analysis/bacteria/")

bacteria_all_samples<- import_biom("trptaxa_noplates.otutable.biom", treefilename = "trp.phylip.tre", parseFunction = parse_taxonomy_default)
bacteria_all_samples_mapping<- read.delim2("mapping_noplates.txt", header = T, sep = "\t")
row.names(bacteria_all_samples_mapping)<- bacteria_all_samples_mapping[,1]
bacteria_all_samples_mapping<- sample_data(bacteria_all_samples_mapping, errorIfNULL = T)
bacteria_all_samples_ps<- merge_phyloseq(bacteria_all_samples, bacteria_all_samples_mapping)

#Rarefy phyloseqs to even depth:
bact_even_depth_ps<- rarefy_even_depth(bacteria_all_samples_ps, sample.size = min(sample_sums(bacteria_all_samples_ps)), rngseed = T, replace = TRUE, trimOTUs = F, verbose = TRUE)
#Notification
#`set.seed(TRUE)` was used to initialize repeatable random subsampling.
#Please record this for your records so others can reproduce.
#Try `set.seed(TRUE); .Random.seed` for the full vector

#create merged distance matrices based on even depth data:
bacteria_even_bray_dm <- phyloseq::distance(bact_even_depth_ps, method = "bray")
bacteria_even_bray_dm <- as.matrix(bacteria_even_bray_dm)

bacteria_even_unifrac_dm <- phyloseq::distance(bact_even_depth_ps, method = "unifrac")
bacteria_even_unifrac_dm <- as.matrix(bacteria_even_unifrac_dm)

bacteria_even_wunifrac_dm <- phyloseq::distance(bact_even_depth_ps, method = "wunifrac")
bacteria_even_wunifrac_dm <- as.matrix(bacteria_even_wunifrac_dm)

#convert mapping files to adonis compatible data.frames:
bacteria_all_metadata <- as(sample_data(bacteria_all_samples_ps), "data.frame")

#perform adonis PERMANOVA analysis on each of the datasets for each breed (6 permanovas in total):
#Now I want to run the PERMANOVA analysis for the bacteria for all samples and treatment samples:
adonis(bacteria_even_bray_dm ~ Experiment + Sex + Treatment + Week + Trtxweek + ExpxTrt + ExpxTrtxWk, data = bacteria_all_metadata, permutations = 999)
adonis(bacteria_even_unifrac_dm ~ Experiment + Sex + Treatment + Week + Trtxweek + ExpxTrt + ExpxTrtxWk, data = bacteria_all_metadata, permutations = 999)
```

```
adonis(bacteria_even_wunifrac_dm ~ Experiment + Sex + Treatment + Week +
Trtxweek + ExpxTrt + ExpxTrtxWk, data = bacteria_all_metadata, permutations = 999)
```

APPENDIX C: Fiji Code Chapter 5

Program: Fiji (Fiji Is Just ImageJ)

"Read and Write Excel" plugin required.
This plugin creates an Excel file on the desktop called "Rename me....."
Running a large number of images may result in system lag with Excel as this plugin will open the file, append the new data, and close the file. Eventually the file size will get quite large causing the potential lag. Consider running in batches.

Macro should be run with a few test images to ensure parameters are properly capturing nuclei and area. Adjustments can be made prior to running full batches.

Be careful when running as files in the output directory can be overwritten. This includes images although the summary table and Excel file are more likely to be overwritten. Consider using different directories for each run or change the name of the summary table and Excel files prior to running.

Macro file for Fiji: Cell_count.ijm
Procedure by: Elizabeth Cody, Department of Food Science, University of Nebraska
Marco by: Dana Van Sambeek, Department of Animal Science, University of Nebraska

```
/*
 * Macro template to process multiple images in a folder
 */

#@ File (label = "Input directory", style = "directory") input
#@ File (label = "Output directory", style = "directory") output
#@ String (label = "File suffix", value = ".tif") suffix

// See also Process_Folder.py for a version of this code
// in the Python scripting language.

processFolder(input);

// function to scan folders/subfolders/files to find files with correct suffix ".tif"
function processFolder(input) {
	list = getFileList(input);
	list = Array.sort(list);
	for (i = 0; i < list.length; i++) {
		if(File.isDirectory(input + File.separator + list[i]))
			processFolder(input + File.separator + list[i]);
		if(endsWith(list[i], suffix))
			processFile(input, output, list[i]);
	}
}
```

```
function processFile(input, output, file) {
        // Do the processing here by adding your own code.
        // Leave the print statements until things work, then remove them.
        // This code will process the images one by one.
        print("Processing: " + input + File.separator + file);
        open(input + File.separator + file);

        //Subtracts background noise from image
        run("Subtract Background...", "rolling=50 light");

        //Splits the image using the filter type. This create one image with
        hematoxylin stain (Colour_1), one with PAS stain (Colour_2), and the third
        will be the remainder (Colour_3).
        run("Colour Deconvolution", "vectors=[H PAS]");

        //Selects the hematoxylin window
        selectWindow(file + "-(Colour_1)");

        //Threshold will look at pixel darkness (0-255, higher is darker). Anything
        lower than 140 will be considered background and left white. Anything
        above 140 will be black and left for analysis, this should be the outline of
        your nuclei. Additional processing and cleanup occurs with “Convert to
        Mask”, “Despeckle”, and “Watershed”.
        setAutoThreshold("Default");
        run("Threshold...");
        setThreshold(0, 140);
        setOption("BlackBackground", false);
        run("Convert to Mask");
        run("Despeckle");
        run("Watershed");

        //Nuclei and nuclei area will be counted here. If the pixel quantity is too low,
        you will pick up noise that might not be nuclei. Likewise nuclei are somewhat
        round so circularity should be higher (0.00 would be a straight line, 1.00 is a
        perfect circle). Data will be summarized in a table.
        //A total count, average size, total area, and %area will be generated.
        run("Analyze Particles...", "size=40-500 circularity=0.50-1.00 show=Outlines
        display exclude clear include summarize");

        //The a new particle analysis image “Drawing of “filename” (Colour_1)” will
        have outlines for all nuclei counted. The next steps will modify the outlines to
        be red and then overlay this with Colour_1 and save so the user can
        determine if the parameters are correct for capturing the nuclei.
        run("Invert LUT");
        run("Red");
```

```
        run("Invert LUT");
        run("RGB Color");
        selectWindow(file);
        run("RGB Color");
        imageCalculator("Add create", file,"Drawing of " + file+  "-(Colour_1)");
        selectWindow("Result of " + file);
        saveAs("tiff", output +File.separator + "Overlay" + file);

        //This will add the nuclei back into the “Colour_2” for calculating total cell
        coverage area which includes mucin coverage of the PAS stain.
        imageCalculator("Subtract create", file + "-(Colour_2)", file +  "-(Colour_1)");
        setAutoThreshold("Otsu");
        run("Threshold...");
        setThreshold(0, 150);
        setOption("BlackBackground", false);
        run("Convert to Mask");
        run("Measure");

        //File will contain analysis of each picture include individual nuclei sizes, and
        the total area measurement.
        run("Read and Write Excel", file);

        //save Summarytable, and results table, this will save the summary data from
        each picture, count, total area, average size, and %area.
        print("Saving to: " + output);
        run("Close All");
        call("java.lang.System.gc");
}
selectWindow("Summary");
saveAs("text", output + File.separator + "SummaryTable");
```

APPENDIX D: Abstracts Authored

147 Effects of energy restriction during gilt development on characterization and quantification of milk oligo saccharides.

S. M. Barrett, M. D. Trenhaile-Grannemann, D. M. van Sambeek, P. S. Miller, J. Salcedo, D. Barile, T. E. Burkey

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Current research at the University of Nebraska-Lincoln (including 14 batches of gilts) has shown that energy restriction during gilt development will increase sow longevity and may also have beneficial impacts with respect to litter performance. Milk oligosaccharides (OS) are structurally diverse carbohydrates comprised of monomers of glucose, galactose, *N*-Acetylglucosamine, *N*-Acetyl galactosamine, fucose, and sialic acid, which may support gut health and development in suckling neonates via prebiotic mechanisms (e.g., modulation of gut microbes and mucosal immunity). The objective of this work was to evaluate the effects of energy restriction during gilt development on oligosaccharides production in milk. During the development period (d 123 to 240 of age), gilts (n = 128, 8 gilts/pen) were fed dietary treatments including: 1) Control diet formulated to NRC (2012) specifications (CTL); 2) Restricted (20% energy restriction via addition of 40% soy hulls; RESTR). Diets were fed ad libitum and applied in a 3 phase feeding regimen. At 240 d of age gilts were bred and fed a common diet. For this analysis, milk samples were collected (n = 7/treatment) on d 0 and 14 post-farrowing (i.e., early and mid-lactation). Milk OS were characterized by Nano LC Chip QTOF MS and quantified by High Performance Anion Exchange Chromatography–Pulsed Amperometric Detection. Across the two diets (RESTR and CTL), 63 OS were identified (58.7, 25.4 and 15.9%, neutral, acidic OS and fucosyl, respectively). On d 0, CTL had greater neutral and less acidic OS ($P < 0.05$) compared to RESTR. Of the neutral OS quantified, RESTR had greater LNnT (a neutral OS) than CTL ($P < 0.05$). Also, both RESTR and CTL had an increase in fucosyl OS and decrease in acidic OS from d 0 to d 14 ($P<0.05$). Of the fucosyl OS quantified, samples from CTL had greater LNDFH-I (a fucosyl OS) than RESTR ($P < 0.05$) at d 0. Lastly, only the RESTR showed an increase in neutral OS over time. Total OS quantification was lower in the RESTR when compared to CTL ($P < 0.05$). Quantification of OS also decreased in both dietary treatments over time ($P < 0.0001$). In conclusion, nutritional management of the developing gilt may impact OS profile during lactation; however, more research is warranted to further OS understanding and to evaluate the effects of OS on gut health and litter performance.

399 Response of IPEC-J2 cells and jejunal explants to treatment with rhamnolipids.

D. M. van Sambeek, T. E. Burkey, L. Smalley

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Rhamnolipids (RL) are glycolipids secreted by bacteria that aid in motility, biofilm formation, nutrient uptake, and have antimicrobial activity. The latter two may be of use to improve swine nutrition and health. Work conducted *in vitro* utilized IPEC-J2 cells to determine cellular response to treatment. In the first experiment, cells were treated with 0, 0.01, 0.05, 0.5, 1.0, 2.4, and 10% RL or 1 ug/mL lipopolysaccharide (LPS). In the second experiment, cells were treated with 0, 0.0005, 0.001, 0.005, and 0.01% RL or 1 ug/mL LPS. For both experiments, treated cells were incubated for 1 h and rinsed with PBS. Cells were further incubated in fresh non-treated media for 0, 1, 3, 6, 12 or 24 h in Exp. 1, or 6 h in Exp. 2. Transepithelial resistance (TER) measurements were collected immediately after addition of fresh media and final incubation. Media was collected from both sides of Trans-well inserts for IL-8 analysis. Visual observation and TER analysis of Exp. 1 showed RL treatment decreased TER after initial incubation, with RL concentrations ≥ 0.05% exhibiting significant cell death and loss of cellular matrix adherence to the Trans-well membrane. Using lower dosages in Exp. 2, TER changed inversely with RL dosage, however, even 0.0005% RL reduced TER by at least 20% over the 6 h period ($P > 0.10$). Production of IL-8 was lower in RL treatments compared with either the control or LPS wells on the apical side ($P > 0.10$). Basolateral IL-8 was expressed in a dose dependent fashion, but only the 0.01% RL had higher expression than the control ($P > 0.10$). A third experiment was conducted by culturing jejunal explants in media with 0, 0.0005, 0.005, 0.05, and 0.5% RL or 1 ug/mL LPS for 1 h followed by 3 h incubation in fresh media. Similarly to Exp. 2, explants showed dose dependent IL-8 production in 5 and 7 wk old tissue with 0.5% RL having higher IL-8 concentration compared to control or LPS samples ($P < 0.01$). The lower doses of RL had similar or lower IL-8 production to the control or LPS samples ($P > 0.10$). Together these data show that low doses of RL (≤ 0.005%) can significantly impact IPEC-J2 TER, but do not cause increases in IL-8 production. More data is needed to determine the effect of RL on nutrient absorption, gut health, and the microbiome in pigs.

166 Effects of energy restriction during gilt development on milk nutrient profile and progeny biomarkers

S. M. Barrett, M. D. Trenhaile-Grannemann, P. S. Miller, T. E. Burkey, D. M. van Sambeek

Journal of Animal Science, Volume 95, Issue suppl_2, 1 March 2017, Pages 78–79,https://doi.org/10.2527/asasmw.2017.12.166

Research at the University of Nebraska investigating the effects of energy restriction on gilt development (including 14 batches with data collected over 4 parities per batch) has lead to the observation that this approach increases sow longevity but may also provide beneficial effects to first parity progeny with respect to health and growth. Specifically, parity 1 progeny may have increased weaning weight compared to progeny derived from gilts fed an ad libitum control diet. Thus, our objective was to evaluate the effects of energy restriction during gilt development on milk nutrient profile and post-natal progeny biomarkers. During the development period, gilts (n = 128, 8 gilts/pen) were fed three dietary treatments including: 1) Control diet formulated to NRC (2012) specifications (CTL); 2) Restricted (20% energy restriction via addition of 40% soy hulls; RES); and 3) Control diet plus addition of crystalline amino acids equivalent to the SID Lys:Met of the RES diet (CTL+). All diets were fed ad libitum and applied in a 3 phase feeding regimen during gilt development (d 120 to 240). Average daily feed intake was used to estimate daily ME intake (Mcal/d) during each phase (Phase 1: 10.13, 6.97, 9.95; Phase 2: 11.25, 8.05, 10.94; and Phase 3: 9.47, 7.95,11.07) for CTL, RES, and CTL+, respectively. At 240 d of age gilts were bred and fed a common diet. For this preliminary analysis, milk samples were collected from batch 14 gilts (n = 7/treatment) on d 0 and 14 post-farrowing for analysis of N, CP, DM, GE and milk insulin, and piglet blood samples (n = 6 piglets/sow) were obtained on d 1 and 15 for quantification of glucagon-like peptide-2 (GLP-2) and insulin. No effects of diet were observed for milk N, CP, or insulin; however, N, CP, and insulin were increased ($P < 0.05$) on d 1 compared to d 14. When evaluating DM and GE, no diet or time effects were observed. For piglet GLP-2, a treatment by time interaction was observed ($P < 0.009$); specifically, GLP concentrations were greater ($P < 0.001$) in CTL+ compared to RES (6.73 vs. 1.21 ng/mL). For serum insulin, a treatment by time interaction was observed ($P < 0.01$); specifically, insulin in RES was greater ($P < 0.03$) than CTL on d 1. In conclusion, nutritional management of the developing gilt may impact piglet serum biomarkers during lactation.

212 Effects of mannan oligosaccharides and *Lactobacillus mucosae* on the intestinal morphology of weanling pigs challenged with *Escherichia coli* lipopolysaccharides

Y. S. Li, M. D. Trenhaile, D. M. van Sambeek, K. C. Moore, S. M. Barrett, S. C. Fernando, P. S. Miller, T. E. Burkey

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To determine the effects of feeding mannan oligosaccharides (MOS) and *Lactobacillus mucosae* (LM) on intestinal morphology of weanling pigs under immune challenge, 96 pigs (BW = 5.88 kg; d 23 post-farrowing) were randomly allotted to 16 experimental pens with a 2 × 2 factorial arrangement of treatments (4 pens per treatment; mixed gender). Diets with or without 0.1% MOS were randomly assigned to pens and 10^9 cfu/pig LM broth or an equal volume of control broth were top-dressed daily. Pigs were given 1 of the 4 dietary treatments (control, MOS, LM, and MOS+LM) during phase-1 and phase-2 (d 0 to 7 and d 7 to 21 post-weaning, respectively). On d 14, all pigs were challenged with 100 µg/kg BW of *Escherichia coli* lipopolysaccharides (LPS). On d 15 and 21, 1 pig per pen was euthanized for collection of intestinal tissue samples. Villus height (VH), crypt depth (CD), and villus surface area of duodenal and ileal samples were measured. Data were analyzed using PROC GLIMMIX of SAS. On d 15, feeding LM tended ($P < 0.10$) to decrease ileal CD; pigs fed LM had decreased ($P < 0.05$) ileal CD compared to pigs fed the control diet, but were not different ($P > 0.10$) from the pigs fed MOS or MOS+LM diets. However, all other ileal and duodenal measurements on d 15 and 21 were not different ($P > 0.10$) among treatments. In conclusion, feeding LM may have beneficial effects on gut health of weanling pigs under LPS-challenge.

289 Effects of mannan oligosaccharides and *Lactobacillus mucosae* on the growth performance and immune response of weanling pigs challenged with *Escherichia coli* lipopolysaccharides

Y. S. Li, M. D. Trenhaile, D. M. van Sambeek, K. C. Moore, S. M. Barrett, S. C. Fernando, T. E. Burkey, P. S. Miller

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Previously, dietary mannan oligosaccharides (MOS) increased fecal abundances of *Lactobacillus mucosae*, which was positively correlated with circulating IgA concentration. To determine the effects of feeding MOS and *L. mucosae* as prebiotic and probiotic sources in weanling pigs under immune challenge, 96 pigs (5.88 kg BW; d 23 after farrowing) were randomly allotted to 16 experimental pens with a 2 × 2 factorial arrangement of treatments (4 pens per treatment; mixed gender). Corn–soybean meal–based diets (without plasma or antibiotics) with or without 0.1% yeast-derived MOS (*Saccharomyes cerevisiae*) were randomly assigned to pens, and 10^9 cfu/pig *L. mucosae* broth or a control broth were top dressed daily. Pigs were given 1 of the 4 dietary treatments (control, MOS, *L. mucosae*, and MOS + *L. mucosae*) in phase 1 and phase 2 (d 0 to 7 and d 7 to 21 after weaning, respectively) and a common diet during phase 3 (d 21 to 35 after weaning). On d 14, all pigs were challenged with 100 μg/kg BW of *Escherichia coli* lipopolysaccharides (LPS) via intraperitoneal injection. Feed disappearances and pig BW were measured weekly. Blood samples were collected weekly and on d 1 and 3 after LPS challenge. Data were analyzed using PROC GLIMMIX of SAS (SAS Inst. Inc., Cary, NC). From d 0 to 14, feeding *L. mucosae*decreased ($P < 0.05$) G:F. From d 14 to 21, G:F (g/kg) in pigs fed *L. mucosae* (715) was greater compared with pigs fed MOS + *L. mucosae* ($P < 0.05$; 600) and the control ($P < 0.10$; 615) but was not different ($P > 0.10$) from pigs fed MOS (674). After removal of treatments (d 28 to 35), G:F was decreased ($P < 0.05$) in the *L. mucosae* treatment group. Feeding MOS- vs. non-MOS-treated diets increased IgG (mg/mL) on d 1 and 3 after LPS challenge ($P < 0.05$; 3.15 and 4.39 vs. 2.58 and 3.34, respectively) and on d 14 and 21 after weaning ($P < 0.10$; 3.68 and 4.56 vs. 2.94 and 3.72, respectively). On d 21, serum IgA concentrations (mg/mL) were greater ($P < 0.05$) in pigs fed *L. mucosae* (0.416) compared with pigs fed MOS (0.341) and MOS + *L. mucosae* (0.342) and tended to be greater ($P < 0.10$) in pigs fed *L. mucosae* vs. pigs fed control (0.347). Using d 0 ($P < 0.05$) IL-1β concentration as a covariate, circulating IL-1β in control and MOS + *L. mucosae* pigs increased ($P < 0.05$) on d 1 after LPS challenge but did not change ($P > 0.10$) in MOS and *L. mucosae* groups. In conclusion, feeding *L. mucosae* alone improved feed efficiency during the first week of LPS challenge; additionally, feeding *L. mucosae* and MOS may have beneficial effects relative to immune biomarkers.

326 Evaluation of a quantitative trait loci for porcine circovirus type 2b viral load on long-term growth performance and nutrient digestibility in inoculated or vaccinated pigs for porcine circovirus type 2b

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A previous experiment evaluated pigs selected for a porcine circovirus type 2b (PCV2)-susceptibility marker QTL (C, resistant, and T, susceptible) and were inoculated (PCV) with or vaccinated (VAC) against PCV2 in the nursery phase. Pigs genotyped for the C allele had reduced viremia and IgG production compared with the T allele when infected with PCV. Two experiments were conducted to evaluate growth performance through the finishing phase. A model was used to assess the residual between final and predicted BW on pigs from the previous trial. Pigs with a low or high net residual were used for Exp. 1 or Exp. 2, respectively. In Exp. 1, a total of 40 pigs (38.5 kg; 8 pigs/treatment) were selected from (genotype-PCV status) CC-PCV, CT-PCV, TT-PCV, CT-VAC, and TT-VAC. Pigs were housed by treatment, with 2 pigs per pen and 4 pens per treatment. In Exp. 2, 4 pigs from each residual, high (41.9 kg BW) and low (30.2 kg BW), were selected from the following (genotype-PCV status): CT-PCV, TT-PCV, CT-VAC, and TT-VAC, for a total of 32 pigs, individually housed. All pigs had ad libitum access to a 4-phase grow–finish corn–soybean meal diet that met or exceeded the NRC (2012) requirements, with titanium dioxide as an indigestible marker. Growth performance was measured every 14 d, whereas blood and fecal samples were collected at the end of each phase. Loin eye area and backfat were determined via ultrasound at the end of phase 4, and HCW was determined at harvest. For Exp. 2, data was analyzed using initial BW as a covariate. In Exp. 1, ADFI in phase 1 was reduced in the CC-PCV group compared with the TT-PCV group ($P < 0.05$). In phase 3, ADFI was reduced in the VAC group compared with the PCV group ($P < 0.05$). In phase 1 thru 3, digestibility of GE and DM were greater in the CC-PCV group compared with the TT-PCV group ($P < 0.05$). In Exp. 2, low residual pigs had greater G:F during the phases 2 and 3 ($P < 0.05$). No differences were found for GE and DM digestibility between groups ($P > 0.10$). Pigs with the CT genotype were found to have less backfat ($P < 0.10$) and greater lean ($P < 0.05$) and percent lean ($P < 0.05$) than those with TT genotype with no difference in BW. Together, these data suggest that the PCV2-susceptibility marker genotype may affect ADFI, nutrient digestibility, and carcass traits during the growing–finishing period.

155 Growth performance and serum IgA concentrations in weanling pigs fed dietary prebiotics

Y. S. Li, M. D. Trenhaile, M. M. Lima, K. C. Moore, D. M. van Sambeek, K. C. Moore, T. E. Burkey, P. S. Miller

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To determine the effects of dietary prebiotics on growth performance and immune status of weanling pigs, 64 crossbred pigs (initial BW = 6.6 kg; d 20 to 22 post-farrowing) were selected and randomly allotted to 16 experimental pens with 4 dietary treatments (2 barrows and 2 gilts per pen, 4 pens per treatment). The treatments were maintained during Phase I (d 0 to 14 postweaning) and Phase II (d 14 to 28 postweaning). The control diet was a conventional nursery diet formulated with corn, soybean meal, dried whey, fish meal, bovine plasma, and supplements to meet or exceed the 2012 NRC requirements. The additional 3 diets were formulated to contain 0.1% chicory, 0.1% mannan oligosaccharides (MOS), and 0.02% chitosan, respectively. Pigs were given ad libitum access to feed and water in an environmentally-controlled room. From d 0 to 28 postweaning, feed disappearance and individual BW were measured weekly for determination of ADG, ADFI, and G:F. Blood samples were collected weekly for measurements of serum IgA concentrations. Data were analyzed as a completely randomized design using the MIXED procedure of SAS. Overall, dietary prebiotics did not affect ($P > 0.10$) BW (average 16.7 kg; d 28), ADG (361 g), or ADFI (513 g). However, from d 7 to 14, pigs fed MOS had lower (671 g/kg; $P < 0.05$) G:F compared to pigs fed control (830 g/kg), chicory (851 g/kg), and chitosan (871 g/kg) diets. For Phase I, G:F of pigs fed MOS tended to be lower (656 g/kg; $P < 0.10$) than pigs fed control (791 g/kg) and chitosan (783 g/kg), but was not different from chicory (755 g/kg; $P > 0.10$). For Phase II, G:F of pigs fed control (673 g/kg), chicory (686 g/kg), MOS (695 g/kg), and chitosan (695 g/kg) were not different ($P > 0.10$). There were no time × treatment interactions ($P > 0.10$) affecting circulating IgA concentrations. Serum IgA increased ($P < 0.05$) over time from 0.148 to 0.438 mg/mL (d 0 to 28 postweaning), but was not affected by dietary prebiotic. In conclusion, with the exception of subtle decreases in feed efficiency, prebiotic supplementation had no effect on growth performance or serum IgA concentrations in weanling pigs.

195 Effect of energy restriction on feed efficiency, nutrient digestibility, and immune biomarkers of growing/finishing pigs

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Many factors affect control of feed intake and regulation of energy balance including external (e.g., environment) and internal (e.g., hormones) factors. The objective was to evaluate effects of nutrient restriction on feed efficiency, apparent total tract digestibility (ATTD), and an immune biomarker in growing-finishing pigs. Crossbred barrows (n = 36; initial BW = 52.3 kg) were randomly allotted to 36 individual pens with 2 dietary treatments in an 8 wk experiment. Treatments included a control (ADLIB; n = 16 pigs) diet formulated to meet or exceed 2012 NRC requirements and an energy restricted (RESTR; n = 16) diet. Pigs maintained on RESTR were provided feed representing a 50% (wk 1) or 25% (wk 2 to 8) reduction in amount of feed relative to amount of feed provided to ADLIB pigs. All diets were corn-soybean meal-based, fed in 2 phases (phase 1, wk 1 to 4; phase 2, wk 5 to 8) and contained 0.5% TiO_2 (digestibility marker). Feed disappearance and individual BW were measured weekly for determination of ADG, ADFI, G:F. At the end of each phase, fecal samples were collected from each pig twice daily for 3 consecutive days and pooled by phase. Feces were analyzed for DM, TiO_2, and GE. Blood samples were collected from each pig (wk 0, 1, 2, 4, 6, and 8) and serum analyzed for C-reactive protein (CRP). No differences in BW (P = 0.79) were observed on d 0 and RESTR pigs had lower (P < 0.001) BW compared to ADLIB at subsequent time points. Final mean BW was 100.5 and 112.0 kg, respectively for RESTR and ADLIB pigs. Overall, ADG (0.86 vs. 1.05 kg) and ADFI (2.65 vs. 3.44 kg) decreased (P < 0.001) and G:F (0.37 vs. 0.34 kg/kg) increased in RESTR compared to ADLIB pigs, respectively. With respect to ATTD, no differences were detected in phase 1; however, in phase 2, DM (83.45 vs. 81.62%) and GE digestibility (82.88 vs. 80.87%) was increased (P < 0.008) in RESTR compared to ADLIB pigs, respectively. For CRP, no overall differences were observed; however, CRP tended to decrease (P = 0.06) in RESTR compared to ADLIB pigs in wk 1. Pigs may compensate for nutrient restriction by becoming more efficient with respect to nutrient assimilation.

309 Evaluation of a QTL for porcine circovirus type 2b (PCV2) viral load on growth performance in inoculated and vaccinated pigs for PCV2

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Data from a previous PCV2b experimental challenge revealed the presence of a QTL associated with viral load, a measure of susceptibility. A study was conducted to evaluate the effects of this QTL on growth in PCV2b-experimentally infected and vaccinated pigs. Before challenge the pigs were genotyped for the QTL (*CC*, resistant; *TT*, susceptible; and *CT*) and profiled for PCV2-specific maternal antibodies. Experimental pigs were either vaccinated (VAC) at 3 wk of age, or inoculated (NOV) with PCV2b at 5 wk of age. Pigs (n = 111) were allocated to 5 groups; VAC included pigs with *CT* and *TT* genotypes while NOV included pigs with all genotypes. Pigs were allocated to pens by genotype-PCV2b treatment, and were ad libitum fed an antibiotic free, corn-soybean meal based nursery diet that met or exceeded NRC (2012) requirements. Titanium dioxide was added to the diet as an indigestible marker. Feed disappearance and individual BW were obtained weekly for determination of ADG and feed disappearance. Blood (for viremia analysis) and fecal samples (for microbiome analysis; data not shown) were collected weekly. Pen fecal samples were collected at wk 2, 4, and 6 for digestibility analyses (data not available). Overall, ADG, feed disappearance, and BW were not different between treatment, genotype, or treatment × genotype ($P > 0.10$). In the NOV group, the *CC* genotype had numerically greater BW than *CT* and *TT* genotypes throughout the 6 wk trial (33.9, 30.9, and 29.5 kg at wk 6, respectively). This trend, with the *C* allele being considered favorable, was consistent in the VAC pigs (*CT* = 32.8 kg; *TT* = 30.4 kg). Although d 7–28 viremia data showed an increased viral titer in NOV pigs as expected, the *CC* genotype had a reduced titer compared with the *CT* and *TT* genotypes ($P < 0.05$). These preliminary data suggest that selection for the *CC* genotype may reduce the need for PCV2 vaccination by providing greater resistance to PCV2 challenge compared to the other genotypes. However, additional research is needed to delineate the long term and cellular effects of this QTL.

Alteration of the pig intestinal microbiome when vaccinated against or inoculated with porcine circovirus 2 using a multivariate analysis model[1]

D. M van Sambeek, H. Tran, S. C. Fernando, D. C. Ciobanu, P. S. Miller, T. E. Burkey

Poster Submission for The 13th Meeting Digestive Physiology of Pigs, Kliczkow, Poland, May 2015

Abstract and publishing data in Chapter 2.