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A BIOINFOMATIC ANALYSIS OF LUMINAL AND MUCOSAL ILEAL BACTERIA COLONIZING NURSERY PIGS FED DIFFERENT PROTEIN SOURCES

BY

JAMIE L. ORTMAN

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Animal Science

South Dakota State University

2019

THESIS ACCEPTANCE PAGE

Jamie L. Ortman

This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

°C	Degrees Celsius
µg	micrograms
AA	Amino acid
ADG	Average daily gain
ANF	Anti-nutritional factor
bp	Base pairs
BW	Body weight
CD	Crypt depth
cfu	Colony forming unit
Crypts	Crypts of Lieberkühn
D / (d)	day(s)
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acids
ESBM	Enzymatically converted soybean meal
ETEC	Enterotoxigenic E. coli
FDA	Food and Drug Administration
FM	Fishmeal
FSBM	Fermented soybean meal
GALT	Gut-associated lymphoid tissue
gDNA	Genomic DNA
GIT	Gastrointestinal tract
h	Hours

H&E	Hematoxylin and eosin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kDa	kiloDaltons
kg	kilogram
LPS	Lipopolysaccharide
LT	Heat labile
mg	milligram
MSBM	Microbially-enhanced soybean meal
nt	Nucleotide
OTU	Operational taxonomic unit
PAS	Periodic acid Schiff
PCA	Principle component analysis
PCR	Polymerase chain reaction
PEDV	Porcine epidemic diarrhea virus
POS	Positive control
PWD	Post-weaning diarrhea
s	Seconds
SAS	Statistical analysis system
SBM	Soybean meal
SDP	Spray-dried plasma
SDSU	South Dakota State University

SID	Standard ileal digestibility
SRA	Sequence read archive
ST	Heat stable
VFD	Veterinary Feed Directive
VH	Villus height

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ABSTRACT

A BIOINFOMATIC ANALYSIS OF LUMINAL AND MUCOSAL ILEAL BACTERIA COLONIZING NURSERY PIGS FED DIFFERENT PROTEIN SOURCES

JAMIE L. ORTMAN

2019

Weaning is a critical time in a young pig's life that will greatly impact its adult growth and development. The objective of this research was to evaluate the effects of various protein sources on the bacterial composition within luminal and mucosal populations in simple and complex diets to gain a better understanding of factors influencing gut health. Weaned pigs were fed 1 of 3 simple experimental diets: 1) basic diet containing corn and soybean meal (Negative Control - NEG), 2) basic diet + fishmeal (FM; Positive Control - POS), and 3) basic diet + microbially-enhanced soybean meal (MSBM). Phase I POS and MSBM diets (d0 – d7 post-wean) included FM or MSBM at 7.5%, while Phase II POS and MSBM diets (d8 – d21) included FM or MSBM at 5.0%, respectively. Host tissue and ileal digesta were collected from euthanized pigs at d21 (8 pigs/diet) to assess gut histology and intestinal bacterial profiles, respectively. *Lactobacillus*-affiliated sequences were found to be the most highly represented across treatments. Accordingly, the 3 most abundant Operational Taxonomic Units (OTUs) were affiliated with *Lactobacillus*, with each showing a distinct abundance pattern with regards to dietary treatment. *L. amylovorus*, was found to be more abundant in NEG and POS samples, compared to MSBM samples, *L. johnsonii*, was more highly represented in POS and MSBM samples compared to NEG, *L. delbrueckii*, was found in highest abundance in ileal samples from MSBM-fed pigs. In trial 2, pens of weaned pigs (21d of age, 6.56 ±

0.87 kg; n=5 pens/diet; 7 pigs/pen) in 2 equal blocks of pigs were fed one of 4 complex experimental diets: 1) positive control, containing corn, soybean meal, spray dried plasma (SDP), and FM identified as CON, 2) SDP and MSBM ($MSBM_{+SDP}$), 3) FM and MSBM ($MSBM_{+FM}$), and 4) MSBM in both Phase I (d1-7 post-wean; 0, 12.75, 20.40, 34% MSBM inclusion, respectively) and II (d8-21; 0, 5, 8, 15% MSBM inclusion, respectively). Ileal digesta was collected from 5 pigs/diet at d21 (1 pig/pen). Ileal mucosa tissue was collected from 10 pigs/diet at d21 (1pig/pen). *Lactobacillaceae* appeared to be the dominant family in both luminal and mucosal block 1 populations, whereas *Clostridiaceae* was most prevalent in block 2 mucosal samples. A dominant *Lactobacillus* presence was demonstrated in 8 and 2 mucosal samples within block 1 and block 2, respectively. The dominant presence of *Clostridia* was comprised of only 2 OTU within the mucosal population; these were closely related to *C. ventriculi* and *C. saudiense*. Luminal populations were primarily comprised of 2 dominant OTU, which were closely related to *L. amylovorus* and *L. delbrueckii*. The mucosal population contained twice the level of species biodiversity and was dominated by only one OTU, which was closely related to *L. amylovorus*. Luminal populations remained more homogenous in their population with higher proportions of select species, primarily *Lactobacillus*. Neither trial demonstrated an effect of treatment ($P > 0.05$) on relative abundance of phyla, family, or genera within luminal or mucosal samples, with the exception of one OTU identified as *L. reuter* in $MSBM_{+FM}$ (0.05±0.03%). Bacterial profiles, in both simple and complex luminal populations, demonstrated a dominance of select *Lactobacillus* OTU in both trials. Furthermore, *Lactobacillus* remained a prominent presence in mucosal samples, but to a lesser degree as *Clostridia* increased its

population in block 2 samples. The identification of bacterial populations may provide evidence that unique location-based profiles exist and that certain non-dietary factors have the potential to alter mucosal populations, while having minimal influence on luminal populations.

CHAPTER 1

1.0 LITERATURE REVIEW

1.1 INTRODUCTION

Weaning is arguably the most influential period of a conventional production pig's life. Young pigs undergo a plethora of stressors that can lead to increased animal health risks and economic losses for producers. Certain changes are particularly problematic, because young pigs have underdeveloped immune systems (Blecha *et al.*, 1983) and digestive tracts (Shields *et al.*, 1980). The lack of key anatomical and physiological advancements, coupled with increased weaning stress, often results in reduced performance and post-weaning diarrhea (PWD). Weaning stressors can include, but are not limited to, early and abrupt separation from the sow, handling and transport, physical environmental factors, re-establishment of social hierarchy, introduction to dry feed, and exposure to novel pathogens. Stress and increased stimuli cause the animals to experience a reduction in voluntary feed intake. If pigs are going to consume less feed, it is imperative that their feed be more nutrient dense and palatable to maintain growth response. Conventional post-wean diets have included high-quality feedstuffs, such as fishmeal (FM), soybean meal (SBM), blood plasma products, and/or milk products. These products help to maintain maintenance energy and growth requirements. Furthermore, they act to inoculate the gastrointestinal tract with commensal, probiotic bacteria to competitively exclude pathogenic bacteria. If the stress of weaned pigs is not tempered appropriately, it could lead to pathogens colonizing the gut of young animals, which will result in disease, poor nutrient absorption, reduced growth, and/or death of these animals.

1.2 WEANING

Weaning is a time when piglets cease consumption of maternal milk in lieu of a solid feed source. In the wild, it is a slow and gradual process that typically begins after the first week of lactation when the sow decreases milk letdown and continues until weeks 11-14 when a majority of piglets are reliant on dry feed (Newberry and Wood-Gush, 1985, Jensen and Recén, 1989). The North American swine industry conventionally induces weaning of nursery pigs between 3-4 weeks of age (Robert *et al.*, 1999). Research has reported that piglets weaned any younger have drastically increased digestive upset and weaning growth check (Leibbrandt *et al.*, 1975, Blecha *et al.*, 1983, Metz and Gonyou, 1990, Ko *et al.*, 2015), while weaning older results in economic losses from a reduced number of sow breeding cycles and overall sow health (Elsley *et al.*, 1968). Most of the detrimental effects, contributed to weaning at 3-4 weeks of age, will occur within the first two week prior to weaning. Many of the health concerns and increased risk factors stem from a decreased appetite, which lasts no more than 24 hours, but can lead to intestinal cell damage and increased inflammation (Metz and Gonyou, 1990). Conventional weaning systems often observe a combined effect of young weaning age, intestinal degradation, inflammation, and several other factors that collectively create a "weaning growth check." One of the most widely recognized indicators of newly weaned pigs' growth and health inhibition is PWD syndrome.

Escherichia coli is a commensal resident of mammalian GIT (Tenaillon *et al.*, 2010); however, some strains have been identified in PWD, namely *β*-haemolytic enterotoxigenic *E. coli* (ETEC) or colibacillosis (Fairbrother *et al.*, 2005). The presence of this pathogen often manifests as diarrhea during the first 2 weeks post-wean (Amezcua *et al.*, 2002). The symptoms that characterize the infection are diarrhea, dehydration,

severe growth retardation, and sudden death (Amezcua *et al.*, 2002, Fairbrother *et al.*, 2005). ETEC produces two different kinds of plasmid-encoded heat stable (ST) and heat labile (LT) enterotoxins (Steinsland *et al.*, 2002, Crossman *et al.*, 2010, Fleckenstein *et al.*, 2010). The ETEC may also produce colonization factors, which can allow fimbrial adhesion to the mucosal lining within the small intestine (SI) (Nataro and Kaper, 1998). The toxins and fimbrial adherins (binding proteins) result in extreme fluid secretion within the SI causing watery diarrhea and dehydration. It was reported that ETEC fimbrial adherins, F4 and F18, comprise 36.1 and 18.2% of the PWD outbreaks in swine (Luppi *et al.*, 2016). Morphological decline, reported due to reduced voluntary feed intake, may present an ideal environment for ETEC to cross the intestinal gut barrier and induce an inflammatory response (Campbell *et al.*, 2013). The colonization and infection of PWD agents is a serious, world-wide issue that becomes exacerbated by multifactorial post-weaning stresses.

1.3 POST-WEANING STRESS

Stress can be defined as "the reaction of the body to stimuli that disturbs its normal physiological equilibrium or homeostasis, often with detrimental effects" (Khansari *et al.*, 1990, Hicks *et al.*, 1998). Nursery pigs experience stress factors through reduced voluntary feed intake (Bark *et al.*, 1986, Lindemann *et al.*, 1986). A decline in feed consumption will limit individuals' growth rate for up to 2 weeks (Madec *et al.*, 1998), at which point the pigs will return to a pre-wean growth rate (Robertson *et al.*, 1985, Pluske *et al.*, 1995). If the pigs are unable to adapt quickly to environmental, social, morphological, and physiological stressors, they will experience reduced performance and increase mortality.

1.3.1 Environment

Nursery pigs experience many environmental stressors as they undergo the weaning process. These can include changes in ambient temperature (drafts, ventilation, and humidity) (McCracken and Caldwell, 1980, Le Dividich, 1981, Becker *et al.*, 1985, Jordan *et al.*, 2010), novel equipment and surroundings (i.e. rattle paddles, sorting boards, flooring), transport procedures (Houpt *et al.*, 1983, Parrott and Misson, 1989, Hicks *et al.*, 1998), air contaminants, and pathogen diversity. Extensive barn management is required to reduce the occurrence and exposure time of young pigs to suboptimal environmental conditions. Some delay in growth potential is unavoidable as the animals are exposed to novel stimuli, but deliberate management practices can reduce incidence of stress-induced gastrointestinal (GI) disturbance (Kelley, 1980, Kelley *et al.*, 1985). Stressful novelties compromise growth and health in young pigs, leading to drastic economic reductions for producers.

1.3.2 Social

Conventional weaning systems remove piglets from the sow and transport them to alternate grower facilities. The practice is designed to limit pathogen transfer between piglets and dams (Alexander *et al.*, 1980). Transferring piglets from a farrowing to weaning facility may have advantages in limiting disease transfer, but it may progress social stress incurred by early piglet removal from the dam, random mixing of piglets, and social hierarchy re-establishment between new groups of animals (Morrow-Tesch *et al.*, 1994). Social stress has been documented in pigs separated from the sow earlier than 4 weeks of age, measured by increased vocalizations at high pitches and belly-nosing of

litter mates (Weary *et al.*, 1999). Social stress can influence body weight (Raab et al., 1986; House et al., 1988), decrease antibody synthesis, cellular immunity, and resistance to bacterial infections (reviewed by Kelley, 1980, 1985 and McGlone, 1990). Factors that influence social stress coupled with reductions in voluntary feed intake commonly result in an underdeveloped GIT structure.

1.3.3 Dietary Impact

Pre-weaned pigs consume maternal milk designed for optimal growth and health of nursery pigs for up to 3 weeks of age. After these initial weeks, the sow starts to produce a more variable product that may be unable to maintain the growth of her piglets. It is at this point that the pigs are weaned in North America. The weaning process shifts pigs from milk to a grain-based diet that typically incurs negative consequences due to the inclusion of allergenic proteins found in SBM. Pigs decrease and/or cease eating and drinking until they become acclimated to their surroundings and diet. This reduction in dietary intake results in a failure to maintain gut structure and function, resulting in a decreased absorption and paracellular barrier function (Wijtten *et al.*, 2011). However, it was reported that animals weaned at 3 weeks of age were decidedly worse in these factors compared to animals weaned at 4 weeks or later (Wijtten *et al.*, 2011). It has been suggested that these findings are a result of a more developed and enzymatically mature GIT. However, increasing the pre-wean period is not the only solution to mitigating the weaning growth check.

The transition experienced by weaned pigs can be greatly influenced by dietary factors. Due to weaning challenges, diet may have an increased opportunity to beneficially alter gut health. Select feed additives and high-quality feedstuffs can boost

pigs' metabolism and growth, which minimizes the effects observed during their first week (Pluske, 2013). Feed additives, such as prebiotics and/or non-starch polysaccharides, have been reported to stimulate the underdeveloped gut microbiome and reinforce gut barrier function (Knudsen *et al.*, 2012, Pluske, 2013). Furthermore, high-quality ingredients increase appetite and gut flow, which provides the necessary energy to maintain gut health and growth. However, any changes or limitations to the supply of required macronutrients, micronutrients, and/or energy will result in compromised health, development, and subsequent recovery time (Pluske, 2013). Thus, these underlying dietary concerns originate from morphological degradation and physiological repression within the intestinal tract.

1.3.3.1 Morphological

The reduced growth performance associated with weaning is linked to structural changes within the anatomy of the small intestine. The small intestine is composed of three sections: duodenum, jejunum, and ileum. In the lumen of the small intestine, small finger-like projections called villi line the interior of the tract. Microvilli are a structural component of the villi and act to increase surface area within the intestine. The villi cells are replenished by the Crypts of Lieberkuhn (crypts). The primary role of villi and microvilli are to aid in digestion and absorption; however, this is not their only function. The small intestinal epithelium is responsible for the selective absorption of nutrients and maintenance of the gut barrier against harmful luminal pathogens (Gibson *et al.*, 1996). Any damage to these cells will cause a reduction in nutrient digestion and absorption capacity, which will negatively affect growth and health.

Villus depression is caused by either an increased rate of crypt cell production (i.e. induced by microbial challenges or antigenic compounds from novel feedstuffs) and/or a failure to regenerate new cells at appropriate capacities due to fasting (Pluske *et al.*, 1997). It was suggested that reduced feed consumption at weaning can lead to sub-optimal protein and energy availability, which can limit crypt cell production rate and, consequently, prolong villus atrophy. The crypts work to replenish the villi's absorptive cells; however, this can result in a lag in the system while the cells mature into their function. Even a fasting period of 24 hours (48 hours is commonly observed at weaning) can have drastic effects on the gut morphology.

Pigs weaned in the United States experience a decline in feed and water intake, which commonly results a reduced growth performance, because the morphology of the gut has been degraded. The structure of villi will be altered the most at 5 days post-weaning, most-notably in the upper jejunum and lower ileum (Kelly *et al.*, 1991). Villus height was reported at 75% of its pre-wean values within the first 24 hours (Kelly *et al.*, 1991). The reduction continued at a slower rate for up to 5 days post-weaning with final villus heights only equating to 50% of their pre-wean values of 850 µm at d22 (Hampson, 1986, Pluske *et al.*, 1997). Hampson et al. (1986) suggested that villus atrophy was a resultant factor from reduced enterocytes lining the villus and not from villus contraction. As the animals get older they begin to acclimate to their new environment and diet; this results in the animals returning to their pre-weaning growth rates by d5-8 (Cera *et al.*, 1988b). Natural weaned pigs experienced minimal reductions in villus height (Pluske *et al.*, 1997).

1.3.3.2 Physiological

Changes within the gut morphology also have physiolological implications. Digestion occurs predominantly in the small intestine with digestive preparation occuring in cephalic section of the stomact. The reduction in villus height and crypt depth is directly correlated with reductions in brush border enzymes, namely lactase and sucrase (Pluske *et al.*, 1997). Reductions in brush border enzymes at weaning can lead to increased malabsorption and ultimately further reduced growth performance (Hampson and Kidder, 1986). Intestinal lactase activity drastically declined from 171 IU/g of mucosal protein at 21 d of age to 46 IU/g mucosal protein at d11 post-weaning (Bailey *et al.*, 1956, Hampson and Kidder, 1986). Sucrase activity is generally thought to reside in low levels within the intestinal tissues at birth and progressively increase with age (Bailey *et al.*, 1956). At weaning, it was reported to initially decline to as low as 20 IU/g of mucosal protein by d4-5 post-wean (Hampson and Kidder, 1986). It then surpassed pre-wean levels at 31 IU/d of mucosal protein by d11 post-wean (Hampson and Kidder, 1986). The production trends of lactase and sucrase provide insight into carbohydrate digestion and absorption that may provide some explanation of post-weaning growth check.

1.4 IMMUNE RESPONSE

1.4.1 Passive Immunity

Pigs are born with an underdeveloped immune system that requires them to seek alternate protection until they develop fully functional adaptive and innate immune systems. The sow provides passive immunity in the form of antibodies that are transferred to piglets via colostrum and milk (Pastoret *et al.*, 1998). Colostrum stimulates intestinal growth and function (Xu *et al.*, 2000), while providing temporary immunological antibody defense

(Rooke and Bland, 2002). The piglets are able to absorb maternal antibodies from colostrum for 24-36 hours post-parturition (Weström *et al.*, 1984). The direct absorption of antibodies is restricted when epithelial surfaces become impermeable to antigens in a process called gut closure (Weström *et al.*, 1984). Colostral compounds absorbed into the body are characterized by the presence of IgG antibodies that provide protection by increasing phagocytosis, via macrophage activity of foreign antigens. Colostral IgG antibodies have a half-life ranging from 12-14 days (Curtis and Bourne, 1971), which provides the necessary time for young pigs to start developing a functional immune system.

Post-colostrum, maternal milk is still able to provide a measure of protection to the developing immune system. Milk contains high concentrations of IgA antibodies that bind to the mucosal lining within the GIT (Porter *et al.*, 1970). IgA acts to neutralize toxins and pathogens, but unlike IgG, IgA does not stimulate gut inflammation. Furthermore, IgA physically inhibits bacteria from utilizing the epithelial cells as an entrance into the body (Galdeano and Perdigon, 2006, Mantis *et al.*, 2011). The ligand binding between IgA and gut microbes allows M cells to transcytose sampled microbial particles across the epithelial lining leading to the activation of the innate and adaptive immune responses (Isolauri *et al.*, 2001, Mantis *et al.*, 2011).

At weaning, it is not uncommon for pigs to experience an immunity gap. This is marked by a significant delay in maternal passive immunity (Miller *et al.*, 1962) and a failure of the adaptive immune system to provide full protection against novel antigens (Brown *et al.*, 1961). A young pig's immune system does not reach maturity until 6 weeks of age (Stein, 1996). Many foreign antigens belonging to alternative feedstuffs can

illicit an immune response. Recognition of foreign dietary antigens has been reported to increase the expression of inflammatory cytokines in 28 d old pigs; inflammation peaked from d0-2 post-weaning and then return to pre-wean levels by d8 (Pié *et al.*, 2004). To combat these effects, production managers typically include antibiotics in the diet to manage growth and limit opportunistic pathogens that may compound the inflammatory effects of dietary antigens. However, the usage of antibiotics has gained increased notoriety and regulation, which may create managerial challenges as production facilities are required to keep more stringent records and a standing relationship with a veterinarian.

1.4.2 Antibiotic Effect

The use of antibiotics in nursery pig diets has been a staple for more than 50 years of swine production. They are used in the diet to reduce the potential for pathogenicity when animals may be immunocompromised. Until recently, antibiotics were also added in nursery diets at sub-therapeutic levels as growth promotants (Close, 2000). Antibiotics approved for use in swine diets have been reported to improve growth performance and feed efficiency by 16.4% and 6.9%, respectively (Cromwell, 2002). Additionally, dietary antibiotic usage has been reported to reduce mortality from 4.3 to 2.0% (Cromwell, 2002). The mechanism of action of feed-grade antibiotics has not been well documented. However, it has been suggested that antibiotics help control intestinal pathogens that cause clinical and/or subclinical infections, limit bacterial metabolism, and allow for increased nutrient uptake (Jacela *et al.*, 2010). The mode of action for antibiotics has not previously been a primary concern for producers; however, increased regulation on

antibiotic use in livestock has created a demand to understand the mechanism of action to allow for the creation of antibiotic alternatives in swine diets.

Antibiotic usage in post-weaned pig diets was considered the norm in the United States until January 2017 when the Food and Drug Administration (FDA) initiated Veterinary Feed Directive (VFD). Under Guidance # 209 and 213, the FDA has outlined the restrictions pertaining to the use of compounds considered as medically important for humans in animal agriculture. VFDs restrict the use of these human medically important antibiotics for growth promotion purposes, while also requiring veterinary oversight and authorization for clinical use of in-feed antibiotics. The FDA's increased regulation of agricultural antibiotics has the potential to drastically reduce antimicrobial resistance; however, these changes have forced producers to depend more heavily on high quality feeds while imposing stricter herd management practices with the objective of identifying comparable alternatives for combatting the post-wean growth check.

1.5 POST-WEAN DIET COMPOSITION

Weaned pigs can be fed diets that meet their nutritional requirements in the form of either simple corn and SBM diets or complex multi-phase diets (Mahan *et al.*, 2004). However, simple diets are discouraged, because SBM contains anti-nutritional factors (i.e. trypsin inhibitor and lectins) and intrinsic allergenic compounds (i.e. glycinin and beta-conglycinin) that negatively affect weaned pigs' growth performance and health, respectively (Li *et al.*, 1990, Li *et al.*, 1991). Therefore, it is common to feed complex diets until weaned pigs (> 6.8 kg) become more tolerant of soy protein allergens without growth repercussions. Complex diets contain expensive, specialty ingredients, such as milk products (Graham *et al.*, 1981), plasma proteins (Hansen *et al.*, 1993), fishmeal

(Stoner *et al.*, 1990), and other ingredients that help maximize feed efficiency and growth performance. Corn and SBM are still included in complex starter diets but at low initial inclusion levels. As the pigs age, dietary ingredients can be phased out for the more conventional and less expensive swine diet staples.

1.5.1 Dried Whey

The utilization of dried whey in complex nursery diets provides a highly digestible energy source due to the high availability of lactase in the 3-week-old weaned pig (Kim and Allee, 2001). Dried whey is composed of 72% lactose, making it an almost ideal ingredient for weaned pig diets due to their available enzymes (Tokach *et al.*, 1989). Gradually, weaned pigs become accustomed to a less complex diet and develop the necessary enzymes, such as protease, amylase, maltase, and sucrase, to digest macronutrients; however, these enzymes will not reach adequate quantities until 6-8 weeks of age (Hartma *et al.*, 1961, Lindemann *et al.*, 1986). The inclusion of dried whey in starter diets has been reported to increase feed intake, feed efficiency, and growth when administered immediately post-wean (Danielson *et al.*, 1960, Kornegay *et al.*, 1974, Cera *et al.*, 1988a, Lepine *et al.*, 1991). Therefore, the inclusion of dried-whey provides a highly digestible and palatable energy source capable of assisting in the transition from sow milk to grain-based diets.

1.5.2 Spray-Dried Plasma

Spray-dried plasma (SDP) is a specialty protein source that has been utilized within the initial phases of weaned pig diets. It is a highly digestible amino acid source that is derived from bovine and/or porcine origins. The optimal inclusion rate equates to

6% of the diet and has been reported to result in increased feed intake and average daily gain, while reducing PWD (van Dijk, 2002, De Lange *et al.*, 2010). Dietary inclusion of SDP is commonly recognized as benefiting post-weaned pigs; however, its specific mode of action remains unclear. It was suggested that SDP has an increased palatability that results in the observed increase in feed intake. This was later reported not to be the case as the palatability of SDP was only moderately palatable when compared to a variety of other protein sources (Sola-Oriol *et al.*, 2009). SDP was also found to be less palatable than fishmeal (FM) (Sola-Oriol *et al.*, 2009). Then it was suggested (Coffey and Cromwell, 1995) and confirmed (Pierce *et al.*, 2005) that feeding SDP was advantageous due to an influx of IgG antibodies that limited the proliferation of pathogens on the mucosal lining of the GIT. The exact mode of action is still unclear, but diet inclusion of SDP is still a successful feeding strategy against post-wean growth check.

There has been some relatively recent hesitation towards using SDP for fear that it may play a role in disease transmission in pigs with immature immune systems. These concerns are not unfounded, but the stringent disease transmission prevention practices utilized by the ingredient processing companies dramatically reduce their likelihood of fault. However, many attributed the North American porcine epidemic diarrhea virus (PEDV) outbreak from May 2013 to have originated from contaminated SDP (Huang *et al.*, 2013). Gerber et al. (2014) reported that the experimental spray-drying process, used to replicate the ingredient processing companies' process, was sufficient to nullify any infectious PEDV that could have been present in the plasma. In contrast, Pasick *et al.* (2014) is still pursuing PEDV-contaminated feed as a possible mode of transmission.

Regardless, swine producers have limited the inclusion of SDP in weaned pig starter diets, thus contributing to the demand for alternatives.

1.5.3 Fishmeal

Fishmeal is a dried ingredient derived from wild-caught fish for the purpose of extracting fish oil; however, FM is a valuable by-product that can be obtained and utilized in other agriculture feeding industries, too. Fishmeal is a highly digestible protein source with an ideal essential amino acid composition. The NRC (2012) has identified it as a comparable replacement for SBM when analyzed for lysine, methionine, tryptophan, threonine, and histidine. The inclusion of FM in nursery diets has been reported to stimulate feed intake and growth performance during the first few weeks post-wean (DeRouchey *et al.*, 2010). Pigs fed menhaden FM at 8% inclusion levels observed an 11.5% increase in average daily gain when compared to pigs not fed FM (Stoner *et al.*, 1990). In addition, FM contains omega-3 fatty acids (eicosatetraenoic and docosahexaenoic acid) (Hasan, 2012). The omega-3 fatty acids harvested from FM have been reported to modulate the immune system by reducing secretion of inflammatory cytokines, like TNF-α, when fed to pigs (Carroll *et al.*, 2003). Furthermore, Gaines *et al.* (2003) reported that fish oil, and not corn oil, modulated the immune system, while preventing growth suppression associated with lipopolysaccharide (LPS)-challenged animals. However, FM is expensive and in limited supply due to overfished wild fish stocks (FAO, 2014) and competitive usage within aquaculture industries (Olsen and Hasan, 2012). Other concerns have been expressed regarding altered carcass tastes and large concentrations of heavy metals in wild caught fish (Dorea, 2006, Jaturasitha *et al.*, 2009). However, many of these concerns would be more relevant to finisher pig diets,

rather than starter diets, but the high cost excludes its inclusion in finisher diets. While the benefits of FM are significant, an economic evaluation of the cost (animal-based proteins are typically more expensive than plant-based proteins), supply (FAO, 2014), and product variability (Kim and Easter, 2001) will be necessary upon consideration of dietary inclusion (Jones *et al.*, 2010). These concerns have encouraged interest in pretreated SBM products.

1.5.4 Soybean Meal: Conventional vs. Pretreated

Conventional SBM is a co-product derived from the cleaning, flaking, and oil extraction of soybeans (NRC, 2012). In North America, SBM is the most widely used protein source within swine diets due to its availability and relatively low cost. SBM is high in lysine, tryptophan, histidine, and threonine but contains low levels of methionine (NRC, 2012). Furthermore, SBM compliments the amino acid profile of corn because corn contains high levels of methionine and low levels of lysine and threonine (NCR, 2012). The result is a relatively inexpensive and available diet that provides a complete amino acid profile for pigs. However, the use of SBM in the early nursery phase feeding program is restricted (<15%) due to its inherent anti-nutritional factors (DeRouchey *et al.*, 2010). Anti-nutritional factors contain heat stable compounds that are able to withstand standard heat-treatment practices used to degrade indigestible carbohydrates found within the soybeans (Knudsen, 1997). These anti-nutritional factors often damage the intestinal tract of weaned pigs, while preventing them from maintaining pre-wean growth potential (Li *et al.*, 1990). It has been suggested that the anti-nutritional qualities associated with non-starch polysaccharides can result in a decreased rate of gastric emptying that creates an optimal environment for pathogens, especially enterotoxigenic

E. coli (ETEC) (Choct *et al.*, 2010). Furthermore, soy protein antigens, conglycinin and beta-conglycinin, both pose concerns due to immune-mediated gut hypersensitivities in young pigs (Lalles, 1993). Thus, the potential modification of SBM as a means of obtaining an economic advantage while limiting harmful compounds has become an appealing objective.

1.5.5 Alternative Feed Ingredients

Since the 1990's, pretreated SBM has been under increasing investigations. SBM has been processed via heat treatment (Hancock *et al.*, 1990), extrusion (Burnham *et al.*, 2000, Qiao *et al.*, 2003), purification (Hancock *et al.*, 1989), enzyme treated (Jones *et al.*, 2018), and microbial fermentation (Hong *et al.*, 2004). The microbial fermentation process utilizes an increased quantity of proteases to partially degrade large proteins and increase nutrient absorption in the duodenum and jejunum (Feng *et al.*, 2007b). Furthermore, the fermentation of SBM has been reported to increase the bioavailability of nutrients (Hotz and Gibson, 2007) and decrease soybean anti-nutritional factors (Egounlety and Aworh, 2003), thus creating the potential for increased digestibility and growth performance in nursery pigs (Walker *et al.*, 1986, Zhang *et al.*, 2013). Cervantes-Pahm and Stein (2010) reported that both fermented and enzymatically treated SBM contained increased quantities of small peptides and digestible AA than conventional SBM. However, fermented and enzymatically treated SBM did not contain a higher SID or AID of AA (excluding Lys) when compared to conventional SBM and/or FM (Cervantes-Pahm and Stein, 2010). Hong *et al.* (2004) reported similar findings, but also reported that the SID of some essential AA were higher in fermented SBM than conventional SBM. Sinn *et al.* (2016) reported that the ideal digestible crude protein and

AA from a fungal-processed SBM were comparable to those of FM in both 10 kg and 30 kg pigs, supporting its continued usage beyond the nursery phase and into grow-finisher diets. In accordance with AA digestibility, treated SBM is a viable alternative to conventional SBM and/or FM.

Favorable results have been obtained from fermented SBM treated with bacteria and/or fungus. A variety of microorganisms have been used in the processing of SBM; a complete list has been provided in a review by Mukherjee *et al.* (2016). The diversity found in processing species can vary the resultant products (Frias *et al.*, 2008). Both fungal and bacterial species have been used to obtain processed SBM with varied levels of improved nutritional advantages. However, fungal *Aspergillus* has become the most widely used microorganism for feed fermentation processes.

Aspergillus species produce participating enzymes that include hemicellulases, hydrolases, proteases, amylases, lipases, and tannases (Pinto *et al.*, 2001, Mathivanan *et al.*, 2006). Some of the *Aspergillus* species that have been used in the fermentation process include *A. oryzae* (Feng *et al.*, 2007a, Feng *et al.*, 2007b, Liu *et al.*, 2007), *A. usamii* (Hirabayashi *et al.*, 1998), *A. awamori* (Kishida *et al.*, 2000), *A. niger* (Mathivanan *et al.*, 2006). *A. oryzae* has been reported to completely eliminate trypsin inhibitors from SBM (Feng *et al.*, 2007b, Liu *et al.*, 2007). Furthermore, it has been reported to reduce stachyose and raffinose, while eliminating sucrose for the production of α-galactosidases (Cervantes-Pahm and Stein, 2010). The fungal breakdown of carbohydrates often results in the increase in nutritive value reported in fermented SBM products, namely increases in crude fat, crude ash, dry matter, and CP (Hong *et al.*, 2004, Feng *et al.*, 2007a, Feng *et al.*, 2007b). Liu *et al.* (2007) reported an increase in average

daily gain, average daily feed intake, serum phosphorus, serum IgM and serum IgA after feeding *A. oryzae*-treated feed to young pigs. However, fungal species are not the only candidate capable of creating these advantageous results from fermented SBM.

Many bacterial species have been used in SBM fermentation. These species are able to decrease SBM's anti-nutritional factors and increase the nutritive value of the end-products. Bacterial species, such as *Bacillus subtilis* and *Lactobacillus plantarum*, can decrease overall protein size, which increasing absorption within non-ruminant digestive tracts. *B. subtilis* increases the availability of several amino acids, but a decrease in proline was reported as a limitation to its usage (Teng *et al.*, 2012). Bacterial fermentation increases antioxidant properties of SBM (Amadou *et al.*, 2011). Song *et al.* (2008) reported that *L. plantarum* and *Bifidobacterium lactis* significantly reduced the allergenic effects of soy proteins. Similar to fungus, bacteria-based fermentation can yield reductions in anti-nutritional factors and increases in nutrient availability in young pigs. However, the different processing agents have varying production requirements in temperature and metabolic substrate that ultimately change the final product values and responses in animal models. Therefore, the final decision involved in choosing which processing microorganisms to use depends on the desired nutrient profile desired for the final product.

An experimental microbially-enhanced SBM (MSBM) has been under investigation at South Dakota State University (SDSU). MSBM was created from a unique incubation process involving a yeast-like strain of *Aureobasidium pullulans*. *A. pullulans* is a common yeast-like fungus that occurs within a diverse array of environmental settings (Samson *et al.*, 2004). *A. pullulans* has been reported to have the

potential to increase various enzymes (i.e. amylase, protease, lipase, cellulase, xylanase) that become biologically relevant within feed processing and other niche markets as reviewed by Gaur *et al.* (2010). However, its use in the fermentation of SBM was originally investigated as a replacement for FM in aquaculture diets. MSBM was reported to contain lower levels of anti-nutritional factors and a higher AA digestibility than conventional SBM in fish. Sinn *et al.* (2016) investigated and reported MSBM as a viable alternative to FM in weaned pig diets. The inclusion of MSBM in the diet can pose as a replacement for FM without compromising growth performance and/or health (Sinn *et al.*, 2016, Koepke *et al.*, 2017). Furthermore, pigs fed a diet containing MSBM had reduced incidence and severity of PWD (Sinn *et al.*, 2016). Therefore, it has been suggested that the microbiome may act as a primary contributor to the improved growth performance and health parameters observed in prior studies (Sinn *et al.*, 2016, Koepke *et al.*, 2017).

1.6 SWINE MICROBIOME

The pig GIT is populated with complex communities of microorganisms, referred to as the microbiome. This includes bacteria, fungi, archaea, and viruses (Conlon and Bird, 2015). These organisms, specifically bacteria, play an essential role in health, digestion, and nutrition within the host (Gustafsson, 1959). Within the microbiome, bacteria represent the most diverse and abundant cell type, followed by archaea (primarily methanogens) and eukaryotes (e.g. protozoa, fungi). It has been estimated that the microbiome is composed of 10^{14} bacteria, which are then divided into 500-1000 different species (Xu and Gordon, 2003, Sonnenburg *et al.*, 2004). Bacterial species are

reported to have the highest level of species diversity (Savage, 1977) and the widest range of metabolic activities (Savage, 1977), thus making them the focus of most microbiome research.

The highest abundance of bacterial species is found in the colon of the GIT; however, the small intestine provides a stronger link between host, diet, and intestinal bacterial populations. Furthermore, the ileum of the small intestine contains the densest bacterial population (10^8 cfu × mL^{-1}) within the small intestine, while providing an environment for bacteria to interact with a site of high nutrient absorption (~90%). Its biologically relevant location and population density has increased investigations into which, and how, these bacterial species are influenced by dietary changes followed by their subsequent effects on digestive, absorptive, and immune processes (Blaut and Clavel, 2007, Leser and Molbak, 2009). However, a comprehensive investigation of gut bacterial population has only recently been made possible by culture-dependent, culture-independent, and sequencing technological advancements.

1.6.1 Studying the Microbiome

The available technology used to investigate animal microbial ecology has previously been limited. The microbiome is a vast network of colonization that has evaded direct study due its vast, dynamic, and microscopic. These challenges have been exacerbated by its location within the GIT. Conventional methods for studying the microbiome (Figure 1.1) have employed culture-dependent approaches that primarily isolate bacteria and grow them within a petri dish. This method is restrictive to organisms that can be cultured, isolated, and systematically studied in a laboratory setting (Zoetendal *et al.*, 2004). Thus, a fundamental need for culture-independent analyses have

developed. Culture-independent approaches have their own challenges and restrictions, but currently their utilization has drastically increased the available information on the GIT's microbiome. The utilization of both techniques may ultimately be the key to deciphering the complexities of the microbiome, but current research has yet to thoroughly investigate this combined approach.

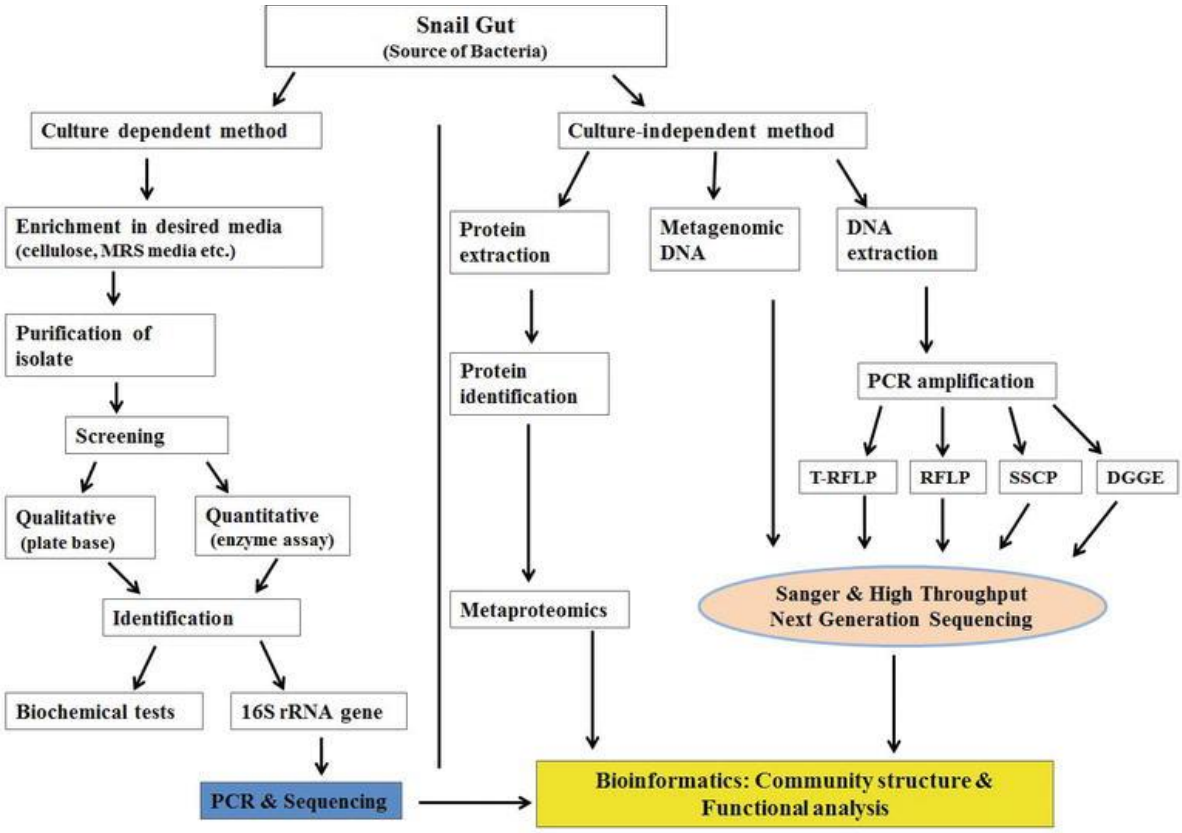

FIGURE 1.1 DEPENDENT AND INDEPENDENT CULTURING TECHNIQUES USED TO IDENTIFY BACTERIA. COPIED FROM DAR *ET AL.* (2017).

1.6.1.1 Culture-Dependent

Culture-dependent approaches investigate known bacteria that can be isolated, cultured, and studied in aerobic conditions (Zoetendal *et al.*, 2008). Bacteria are cultured in either a broth or on a solid media; the media will encourage the proliferation of bacteria that utilize the provided nutrients. These bacteria are typically pathogenic species

that have stimulated symptoms within their host and caused the public to demand a thorough understanding and cessation of this organism's growth. The majority of culturable bacteria are gram-positive, strict anaerobes (i.e. *Streptococci, Lactobacilli, Eubacteria, Clostridia,* and *Peptostreptococci*) (Allison *et al.*, 1979, Robinson *et al.*, 1981). Culturable gram-negative bacteria, within the microbiome, most commonly belonged to Bacteroidetes (Allison *et al.*, 1979, Robinson *et al.*, 1981). However, it has been estimated that only about 80% of the GIT's microbial ecology is able to be cultured (Rajilic-Stojanovic and Vos, 2014). Thus, culture-dependent analyses often underestimate microbial diversity. While these techniques were not all-encompassing, they were an excellent approach to initial microbial studies that have led researchers to culture-independent approaches.

1.6.1.2 Culture-Independent

Culture-independent approaches, as the name suggests, utilizes DNA sequencing and molecular markers to characterize the microbiome instead of culturing techniques. Some of the more common techniques used are traditional PCR, quantitative PCR, fluorescent *in situ* hybridization, DNA sequencing, and DNA microarray (Gong *et al.*, 2008). Other PCR-based DNA profiling techniques, such as temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) have been developed to monitor shifts in the microbial ecology after an environmental disturbance (Muyzer *et al.*, 1993). Standard gel electrophoresis is used to isolate target regions of DNA based on fragment size and flow rate. Several molecular markers exist for these techniques; however, the most common are 16S ribosomal RNA (16S rRNA) gene (Sekirov *et al.*, 2010) and 60 kDa chaperonin protein subunit gene (cpn60) (Choresh *et*

al., 2004). Targeted DNA regions, that have been amplified, can then be sequenced via high-throughput sequencing techniques, including Illumina, Roche 454 GS FLX+, SOLiD 5500 series, and Ion Torrent/Ion Proton Platforms (Zhou *et al.*, 2015). This review will focus on the techniques pertinent to the following studies: traditional PCR, gel electrophoresis, and high-throughput sequencing on Illumina.

Traditional polymerase chain reaction (PCR) is a commonly used method to amplify known regions of sample DNA. Currently, PCR is the ideal method for identifying composition and diversity of the gut microbiota. Once the target DNA has been amplified, it can then be run through gel electrophoresis. PCR amplicons are placed into the gel and an electric charge is used to separate DNA segments from largest to smallest. The target DNA region can then be verified, purified, and sent for high-throughput sequencing. The sequencing results can be analyzed and cross-referenced for known bacterial taxonomies that will help identify bacterial communities.

Some taxonomic resolution has been forgone, but with minimal consequences, due to the hypervariable regions of the targeted amplified region. While it is ideal to sequence an entire bacterial genome to identify its taxonomic classification (Kim *et al.*, 2011), it is not practical and/or economical. However, within these constraints, hypervariable regions have demonstrated what appears to be adequate taxonomic differentiation between OTU. But some controversy exists between which of the nine variable regions of the 16S rRNA gene demonstrate the highest level of identification and differentiation within sequence data.

The concern stems from a lack of even distribution associated with sequence divergence, which could result a non-uniform analysis of diversity and species richness

(Kim *et al.*, 2011). Variable regions pertaining to the V1–V3 region or the V3–V5 region are the most common, Kim *et al.* (2011) suggested the V1-V4 region be targeted if the newest sequencing, 454 FLX, system was being used due to its low distance level 0.01 or 99% similarity between sequences. It was also recommended within the same study that a V1-V3 region be targets if the current FLX Titanium system was being used to sequence the data due to its distance level of 0.03 or 97% species identification level. The sequencing technology limits the total number of base pairs that can be sequences because it is using paired-end sequencing techniques.

The complex and largely unknown bacterial composition presents some problems assigning bacteria to a designated genus, species, and strain. Sequencing data returned for taxonomic assignment can often be clustered into operational taxonomic units (OTU). An OTU is a group of similar sequence variants from the gene marker, typically 16S rRNA gene, that codes for a ribosomal sub-unit found within all prokaryotes (Konstantinidis and Tiedje, 2005). The formation of an OTU is initiated by the selection of a single representative sequence that is assigned a unique code. All sequences with a level of 97% sequence similarity are typically identified as being highly related at that genus level, whereas a 99% similarity can suggest species and strain-level distinctions (Konstantinidis and Tiedje, 2005). The primary advantage associated with OTU clustering is computational analyses of data due to the high volume of sequences reads from a single sample (Nguyen *et al.*, 2016). OTU clustering can provide some confidence in grouping sequences together, which can ultimately assist in identifying compositional trends and potential functionality.

1.6.2 Early Development and Colonization

The swine microbiome is highly dynamic and under-established immediately after birth and proceeds through a series of population shifts as a stable ecosystem is established. Development of the microbiome begins with the colonization of the gut by a few pioneering microbial species from the vaginal canal and surrounding environment shortly after birth (Stark and Lee, 1982). The initial colonizing bacteria are lactic acid bacteria, *Enterobacteria*, and *Streptococci* (Ducluzeau, 1983, Lalles *et al.*, 2007). These bacteria then progress through successive metabolic cycles in response to environmental factors, such as diet, resulting in an increased population density and bacterial diversity (Ducluzeau, 1983, Deplancke *et al.*, 2002). Though the microbiome is highly dynamic and animal-specific, trends and shifts can be identified as animals age and develop a more stable bacterial population (Richards *et al.*, 2005).

The first of these trends have been identified by the colonization of aerobic and facultative anaerobic bacteria between d0-5, at which point, a decline in aerobic bacteria will be observed (Swords *et al.*, 1993). In their place, obligate anaerobes begin to proliferate and diversify (Konstantinov *et al.*, 2006). Inoue *et al.* (2005) reported the next major change between d7 and d22 after parturition. This change is marked by an overall colonic increase in anaerobic bacterial diversity and volatile fatty acid (VFA) production (Murray *et al.*, 1987); the abundant genera are *Eubacterium*, *Fusobacterium*, and *Propionibacterium* (Swords et al., 1993). An increase in intestinal diversity occurs by d16 as noted by fecal populations (Inoue *et al.*, 2005). Between d22-25 days old, just prior to weaning, a third shift occurs as aerobic bacteria, such as *E. coli* and other coliforms, are greatly reduced in number (Inoue *et al.*, 2005). The shift was suggested to mark the complete transition from aerobic and facultative anaerobic bacteria to anaerobic

bacteria (Inoue *et al.*, 2005). Swords *et al.* (1993) reported that a large percentage of the anaerobic bacteria at this time point belonged to *Bacteroides* species. The change may be due to the initial immunological maturation stages of the piglets' GIT (Butler and Brown, 1994, Bailey *et al.*, 2001, Bollinger *et al.*, 2003). Bacterial shifts also occurred in pig's fecal samples at d35 and d49 suggesting that weaning diets altered the available substrates (Inoue *et al.*, 2005). The influence of diet on pig GIT and the microbiome has been demonstrated in many studies (Mackie *et al.*, 1999, Favier *et al.*, 2002, Inoue and Ushida, 2003). However, the influence of diet on small intestine, luminal and mucosal, bacterial populations may suggest different time points associated with bacterial profile shifts and development.

1.6.3 Luminal Phylogeny

The lumen of the GIT is characterized by various types of bacteria, including *Streptococcus, Lactobacillus, Eubacterium, Fusobacterium, Bacteroides, Peptostreptococcus, Bifidobacterium, Selenomonas, Clostridium, Butyrivibrio, Escherichia, Prevotella,* and *Ruminococcus* (Moore *et al.*, 1987, Stewart, 1997, Jensen, 2000, Gaskins *et al.*, 2002, Leser *et al.*, 2002). The presence and proliferation of bacteria is dependent on environmental factors that regulate surrounding populations. These micro-environments are controlled by an extensive array of factors from the luminal bacteria and/or host, including pH, peristalsis, redox potential, bacterial adhesion, bacterial interactions, mucin secretion, nutrient and substrate availability, diet, and bacterial antagonism (Hao and Lee, 2004). Specifically, commensal bacteria utilize and maintain their micro-environments within the lumen to effectively prohibit the colonization of pathogenic bacteria. Bacterial profiles are subject to change; however,

due to these micro-environments, the bacteria typically form a stable, location-based ecosystem that resists further colonization by alternate bacteria (Richards *et al.*, 2005). As the digestive tract progresses towards the colon there is a prominent decrease in several regulatory factors, including peristalsis, acidity, and redox reactions. The decrease in regulation allows for a more abundant and diverse colonization of both the luminal and mucosal locations of the GIT (Hao and Lee, 2004). The bacterial populations colonizing the distal portion of the small intestine provide opportunistic insight for researchers investigating the bacteria influencing digestion and absorption within the body.

The ileum is comprised of more than 200 species of primarily anaerobic bacteria, but a small contingency is comprised of aerobic species (Donaldson Jr, 1964). These bacteria can be classified as either autochthonous (indigenous) or allochthonous (transient) depending on their commensal nature. Due to the rapid gastric flow of the SI, it is imperative that true autochthonous species find adherence sites either on the epithelium or within the mucus above the epithelium (Donaldson Jr, 1964). Failure to adhere to the intestinal lining has been suggested to limit a specific bacteria's ability to influence the host before the luminal digesta is removed. The colonization of autochthonous species is typically dependent on initial gut microbiome establishment, thus making it rare, but not impossible, for pathogenic bacteria to become autochthonous. Stable colonizing populations have been established all along the GIT; however, each location is characterized by a unique bacterial profile creating highly regulated micro-environments.

The luminal bacteria located within the ileum are distinct from other regions of the gastrointestinal tract (Hill *et al.*, 2002). Swine's ileal digesta is predominated by

gram-positive bacteria classified within the Firmicutes and Bacteroidetes phyla (Hill *et al.*, 2005, Schmidt *et al.*, 2011, Buzoianu *et al.*, 2012, Schokker *et al.*, 2014, Mach *et al.*, 2015). Similar results were found in humans via The Human Microbiome Project (2012). The Firmicutes phylum, in both mammalian groups, maintains the largest portion of the bacterial population (Ley *et al.*, 2006). Mach *et al.* (2015) reported a much lower presence of *Proteobacteria* (5.14%), *Spirochaetes* (1.49), and *Fusobacteria* (0.76%) phyla. The prominence of distinguished phyla, combined with the absence of other phyla, suggests that under normal circumstances the host-bacteria relationship is both highly selective and stable. Furthermore, Hill *et al.* (2005) reported that *Lactobacillales* (Firmicutes phyla) was the most highly represented order within the ileal digesta-*Clostridiales* and *Bacillales* being the next relative abundant groups. Similar findings have been documented (Leser *et al.*, 2002, Li *et al.*, 2003, Konstantinov *et al.*, 2004). Low bacterial diversity was reported when the ileal digesta samples were compared to the colonic region samples (Hill *et al.*, 2005). Hill *et al.* (2005) reported a small number of sequences (14% of unique sequences) with a high frequency (81% of the sequences). This trend within the ileal sequence data was similarly reported in a study performed by Li *et al.* (2017); they reported 42 bacterial classes, and of those, seven of them comprised 97% of the sequence data. The identification of a few bacterial taxa comprising most of the sequences may indicate which bacteria are functionally active and able to modulate the host. However, the classification trends in the higher taxa may provide an overly simplistic and misleading view. While it has been proposed that more highly abundant bacteria are likely to play integral roles in modulation of health and growth performance,

these larger taxonomic classification groups fail to identify functions that are linked specifically to species and strain.

Identifying bacteria based on species and strain has become easier since the development of culture-independent approaches, but limitations persist in reference-based databases and mass data analyses. Furthermore, once operational taxonomic units (OTU), or potential species, have been identified, it becomes exponentially more difficult to link them to potential functions due to the complex array of extenuating circumstances and variables. Regardless of these difficulties, some OTU have been identified in unique situations that may suggest a bacteria's involvement. However, it is still unclear as to how useful the study of the luminal bacteria can be in relation to predicting or explaining host response compared to the mucosal bacteria, which has been strongly indicated as the likely candidates for host immune modulation.

1.6.4 Mucosal Phylogeny

The intestinal lining of the GIT is composed of several layers with the innermost portion belonging to the mucosa. The three layers are a mucus epithelium, lamina propria, and muscularis mucosae. The mucus coating of the epithelium is used to protect the underlying layer of epithelial cells from the abrasive nature of food particles and to act as a selective barrier for nutrients, water, and ions. The epithelial layer contains the nutrient absorptive villi and microvilli that characterize the lumen of the small intestine. Within the epithelial cells, the lamina propria provides both gut-associated lymphoid tissue (GALT) and an intra-villi blood supply that participates in the removal and distribution of absorbed nutrients. Finally, the muscularis mucosae aids in the maintenance of peristaltic gut movements that encourages the continued downstream

progression of digesta. However, in healthy animals, bacteria only colonize the mucus and the epithelial layers of the mucosa.

Many bacteria consider either the epithelium and/or the mucus their niche; however, in relation to luminal population density, it is a select population (Morowitz *et al.*, 2011). Levesque *et al.* (2014) suggested that mucosal-associated bacteria colonize the GIT tract early and are highly stable when exposed to dietary shifts. Mucosal bacteria are regionally unique (Pryde et al. 1999; Gong et al. 2002a) and are often characterized by a bacterial species ability to adhere to the GIT lining without being removed upon gastric emptying (Fuller, 1989). Pedersen and Tannock (1989) reported that *in vitro* studies were not capable of predicting adhesion capability in an *in vivo* model due to extenuating environmental factors. Thus, researchers are not able to use adhesion capabilities as a means of distinguishing mucosal and luminal populations. However, via culture-independent approaches, a strong distinction has been identified between the luminal and mucosal population regardless of adhesion.

Recently, through the advancements in culture-independent technologies, researchers have tried to identify distinct differences between mucosal and luminal populations (Levesque *et al.*, 2014, Zhang *et al.*, 2018). Zhang *et al.* (2018) reported data on luminal and mucosal bacteria populations of pigs (11.05 ± 0.11 kg BW) fed corn-SBM diets. Firmicutes and Proteobacteria were the most highly represented phyla within the ileum; these phyla were reported to be composed of 95% and 80% of the resident populations in the lumen and mucosa, respectively. These results were supported by others (Schmidt *et al.*, 2011, Isaacson and Kim, 2012, Levesque *et al.*, 2014). Ileal mucosal populations contained lower abundances of Firmicutes bacteria and higher levels

of Proteobacteria and Bacteroidetes than ileal luminal populations (Zhang *et al.*, 2018). Ileal mucosal bacterial variation within family and genus was reduced when compared to other mucosal populations in the cecum and colon; however, ileal mucosal populations had an increased phylogenic diversity compared to ileal luminal samples (Zhang *et al.*, 2018). Families that colonized the mucosa were identified as *Pseudomonadaceae, Enterococcaceae, Caulobacteraceae, Xanthomonadaceae,* and *Enterobacteriaceae* within Proteobacteria and Firmicutes. Levesque *et al.* (2014) identified three prominent genera within the mucosal population at 8 weeks post-wean that comprised 81% of the total population sequences; these included *Clostridium, Lactobacillus* and *Sarcina*. Similar results were reported for mucosal populations at 2 weeks post-wean (*Lactobacillus*, *Turicibacter*, and *Clostridiales*). Mucosal populations appear to be more stable than luminal populations, but that does not limit their ability to fluctuate under certain environmental conditions.

Schmidt *et al.* (2011) reported findings on mucosal populations of pigs raised in isolation and excessively hygienic environmental condition from d2-d56. Two piglets (one used within each treatment group) from five sows (same paternal genetics) were utilized in this study. It was reported that ileal mucosal populations varied depending on if the piglets were born inside or outside prior to being isolated. The abundance of Firmicutes in mucosal populations was lower in animals raised inside as compared to outside-reared animals; these populations were replaced by Bacteroidetes and Proteobacteria. Schmidt *et al.* (2011) also suggested that dominant *Lactobacillus* species change as animals progress from neonates to weanlings (Pieper *et al.*, 2008). Schmidt *et al.* (2011) reported the presence of various *Lactobacillus* phylotypes in both indoor and

outdoor-reared pigs; these included *L. reuteri*, *L. amylovorous*, *L. johnsonii*, *L. brevis*, *L. pentosus* and *L. plantarum*. However, in animals colonized outdoors, *Lactobacillus* comprised only 7.8% of the total number of OTU, whereas they comprised 27.4% of the identified bacteria in indoor-reared animals. These phylogenic results are similar to those reported by Mulder *et al.* (2009). Thus, the *Lactobacillus* species originally colonizing the gut may not be the most adapt at using post-wean substrate for continued colonization. Furthermore, indoor environments with increased hygiene parameters may limit the acquisition of bacterial biodiversity required for the strong gut barrier defense that mucosa-associated bacteria are primarily known to provide to their host.

1.6.5 Modulation by Diet

The young pig's bacterial composition and diversity are most susceptible to dietary factors that can include feed ingredients, additives, and antimicrobials (Brulc *et al.*, 2009). As pigs age, their microbiome becomes more stable and less susceptible to dietary influence (Holman and Chenier, 2014). Levesque *et al.* (2014) suggested that luminal bacterial populations have an increased susceptibility to alteration by the inclusion of either novel or increased qualities of ingredients, whereas mucosal bacterial populations are less likely to respond to dietary stimuli. The first major gut modulation due to diet occurs from pre- to post-weaned pigs. Pre-weaned pigs consume highly digestible sow milk while post-wean pigs experience changes in their bacterial composition due to grain-based diets that are largely different in the source of nutrients (Holman and Chenier, 2014, Mach *et al.*, 2015). Zhao *et al.* (2015) reported that dietary shifts from pre- to post-weaning resulted in decreased Proteobacteria and increased Firmicutes populations, thus demonstrating that initial colonizing bacteria are less

representative of post-wean population exposed to grain-based diets. However, early post-weaning is not the only opportunity to induce bacterial changes based on dietary shifts.

The use of in-feed antimicrobials and antibiotics in swine diets has been suggested to alter the microbiome. It was reported that lincomycin (110 mg/kg feed) decreased bacterial diversity in the ileal population over the course of 3 weeks when compared to individuals on a basal diet (Namkung *et al.*, 2004, Gong *et al.*, 2008). These results were similar to those reported by Thymann *et al.* (2007) who administered amoxicillin (20 mg/kg feed) and zinc oxide (2500 mg ZnO/kg feed). However, it was reported that amoxicillin (600 mg/kg feed), doxycycline (300 mg/kg feed), or tilmicosin (400 mg/kg feed) did not alter jejunal bacterial composition in 3 week old pigs; however, average daily gain was improved by antibiotic supplementation between d0-7 and d0-21 post-wean (Bosi *et al.*, 2011). Janczyk *et al.* (2007) analyzed the effects of a single dose of intramuscular amoxicillin (15 mg/kg feed) on d1 post-parturition; they identified an increase in several pathogenic bacteria, while *Lactobacillus spp.* thrived only in control groups. The authors suggest that the prophylactic benefits associated with antibiotics may not outweigh the consequences of a disrupted bacterial population. The use of antibiotics may induce beneficial growth responses, but the mode of action does not appear to perpetuate a healthy, diverse microbiome. It could be suggested that the identification of colonizing bacteria in dietary feedstuffs, known to result in increased growth performance, would yield potential species correlating with the advantageous effects. High quality proteins often yield improved performance in post-weaned pigs making such diets optimal candidates for studying the resident and proliferating bacterial

populations. In the interest of this review, only SDP, FM, and fermented SBM products will be discussed.

The mode of action associated with spray-dried plasma has typically been associated with an increase in the protective immunoglobulins that control specific pathogenic *E. coli* strains. However, a thorough understanding of how SDP influences other surrounding bacteria has only recently been investigated. In a study by Hedegaard *et al.* (2016), a purified pig plasma IgG product was analyzed for its effect on the bacterial populations. It was reported that the plasma product contained specific binding activities that aided in the prevention of *E. coli* and *S. enterica* from adhering to mucosal intestinal lining, while maintaining the superior growth performance commonly observed in weaned pigs supplemented with plasma products. *E. coli* challenged pigs were reported to have a significantly lower population of *Enterobacteriaceae* within the plasma treated group. Conversely, ileal bacterial profiles were reported to contain significantly higher proportions of pathogenic bacteria, even than the control group containing plasma. The author speculated that these unexpected results are only likely to produce a pathogenic response when combined with the presence of ETEC strains (Hermann-Bank *et al.*, 2015). In a fecal microbiome analysis, Tran *et al.* (2018) studied the effects of SDP on nursery pig intestinal microbiome and reported that feeding SDP resulted in a greater population of lactic acid producing bacteria and cellulytic bacteria. Diets containing plasma products were also associated with decreased populations of *Clostridiaceae*, *Veillonellaceae*, and *Lachnospiraceae* (Tran *et al.*, 2018). The performance (ADG and BW) results associated with SDP may be the result of a synergistic combination of increase immunoglobulins and optimization of the microbiome populations. However, its

inclusion in complex diets often makes isolating its effect on bacterial composition difficult.

There is a scarcity of literature investigating the effects of isolated protein ingredients as the sole protein source on post-weaned pig microbiome. Simple diets used to isolate the effect of protein source on bacterial population is rare, because complex diets (including a combination of whey, FM, and SDP) are associated with improved growth performance and health. Only recently has it been acknowledged that diet has the potential to practically benefit production industry. Cao *et al.* (2016) reported the dominant phylum comprising the FM-based luminal bacterial profiles was Proteobacteria (65%). It was also reported that 6% FM-based diets in the same weaned pigs was associated with a high relative abundance for *Escherichia*/*Shigella* and *Serratia* species within each of the three sections of the small intestine (Cao *et al.*, 2016). Heo *et al.* (2013) suggested that pathogenic bacteria prefer AA as a viable energy source once fermentable carbohydrates have been metabolized.

Aquaculture conventionally utilizes FM as their primary protein source, thus making ingredient based microbiome studies more abundant in this species. Gajardo *et al.* (2017) performed a bacterial composition analysis on the ileal luminal and mucosal populations of salmon. It was reported that the luminal bacterial profile (phyla) in salmon given a FM-based diet was composed of Firmicutes (38%), Proteobacteria (32%), and Fusobacteria (21%). The luminal genera composition was composed of *Photobacterium* (27%), *Peptostreptococcus* (14%), *Clostridiales* (13%), and *Cetobacterium* (10%). FM-based diets were associated with the lowest mean luminal bacterial diversity score when compared to population associated with SBM-based diets. The mucosal population of

animals fed FM was comprised of Proteobacteria, Bacteroidetes, Parcubacteria, and Firmicutes. Proteobacteria dominated the FM mucosa population, and *Firmicutes* was least represented when compared to SBM-based treatment groups.

FM is utilized within chicken diets to improve growth performance and gut health, thus encouraging an understanding of the bacterial composition. Stanley *et al.* (2014) reported that the ceca luminal bacterial population in chickens was comprised of *Clostridia*, *Lactobacillus, Eubacteria,* and *Ruminococcus*. It was reported that FM increased overall gut pH and was a likely candidate in the pathogenicity of necrotic enteritis in chickens (Stanley *et al.*, 2014). Though limited research is available for the specific analysis of FM's influence on bacterial composition of swine, Hanning and Diaz-Sanchez (2015) suggested that the diet, rearing environment, and GIT anatomy may be more responsible for bacterial populations than the host organism due to uniformity in agricultural practices. Therefore, host and bacterial species may be comprised of various bacterial profiles with similar functional metagenomes (Qu *et al.*, 2008).

Research exists on the bacterial profile of pigs fed SBM (discussed in sections 1.6.2-3); however, limited results have been published on the bacterial ecology of pigs fed further processed SBM. Xie *et al.* (2017) identified bacterial profiles within the cecum and colon of weaned pigs fed fresh fermented SBM; four dominant phyla, (Firmicutes, Bacteroidetes, Proteobacteria and Tenericutes) were reported to comprise a majority of the population. Furthermore, Firmicutes increased as Bacteroidetes and Proteobacteria decreased in animal fed fermented soybean meal. Relative abundance levels of *Lactobacillus* and *Prevotella,* butyrate-producing genera, were higher in both the cecum and colon of treatment pigs when compared to the control animals on a

standard corn-SBM diet. Furthermore, van Winsen *et al.* (2001) reported a reduction in *Enterobacteriaceae* within luminal contents of animals fed fermented SBM within the stomach, ileum, cecum, colon, and rectum.

Therefore, the objective of this research is to investigate the bacterial composition of luminal and mucosal ileal populations of weaned pigs fed a microbially enhanced SBM within both simple and complex nursery diets. It is hypothesized that microbially enhanced SBM diets, both simple and complex, will encourage the proliferation of potentially probiotic species while competitively excluding pathogenic species.

CHAPTER 2

2.0 Comparative analysis of the ileal bacterial composition of post-weaned pigs fed different high-quality protein sources.

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2.1 ABSTRACT

To further understand the contribution of feedstuff ingredients to gut health in swine, the objectives of this research were to assess gut histology and intestinal bacterial profiles associated with the use of two high quality protein sources, microbially-enhanced soybean meal (MSBM) and Menhaden fishmeal (FM). Weaned pigs were fed 1 of 3 experimental diets: 1) basal diet containing corn and soybean meal (Negative Control - NEG), 2) basal diet + fishmeal (FM; Positive Control - POS), and 3) basal diet + MSBM (MSBM). Phase I POS and MSBM diets (d0 – d7 post-wean) included FM or MSBM at 7.5%, while Phase II POS and MSBM diets (d8 – d21) included FM or MSBM at 5.0%.

Host tissue and ileal digesta were collected from euthanized pigs at d21 (8 pigs/diet) to assess gut histology and intestinal bacterial profiles, respectively. Data was analyzed using Proc Mixed in SAS, with pig as the experimental unit and pig (treatment) as the random effect. Histological and immunohistochemical analyses of stomach and small intestinal tissue using H&E, PAS, and inflammatory cell staining did not reveal differences in host response to dietary treatment. Ileal bacterial composition profiles were obtained from next-generation sequencing of PCR generated amplicons targeting the V1-V3 region of the 16S rRNA gene. *Lactobacillus*-affiliated sequences were found to be the most highly represented across treatments, with an average relative abundance of 64.0%, 59.9% and 41.80% in samples from pigs fed the NEG, POS and MSBM diets, respectively. Accordingly, the 3 most abundant Operational Taxonomic Units (OTUs) were affiliated to *Lactobacillus*, with each showing a distinct abundance pattern with regards to dietary treatment. SD_Ssd_00001, most closely related to *L. amylovorus*, was found to be more abundant in NEG and POS samples, compared to MSBM samples (23.5 and 35.0 vs 9.2%). SD_Ssd_00002, whose closest relative was *L. johnsonii*, was more highly represented in POS and MSBM samples compared to NEG (14.0 and 15.8 vs 0.1%). Finally, Sd_Ssd-00011, which showed highest sequence identity to *L. delbrueckii*, was found at 1.9, 3.3, and 11.3% relative abundance in POS, NEG and MSBM ileal samples, respectively. There was no effect of treatment ($P > 0.05$) on bacterial composition; however, the 5-fold difference in relative abundance may demonstrate practical relevance of dietary protein source on bacterial composition in ileal digesta.

2.2 IMPLICATIONS

Minor dietary inclusion of high quality protein can alter ileal bacterial profiles of weaned pigs. Predominance of *Lactobacillus* affiliated bacteria, previously described as having probiotic-like properties (*L. amylovorus, L. johnsonii, and L. debrueckii*), suggests bacterial populations capable of immune modulation in contributing to gut health. A balanced distribution of these three prominent *Lactobacillus* species, across MSBM-fed animals, suggests communities that may be more resilient to perturbations and dysbiosis.

2.3 INTRODUCTION

The period initially following weaning is a critical phase of development for the nursery pig associated with gastrointestinal instability that often results in low feed intake and decreased body weight (Hötzel *et al.*, 2011). Pigs undergo a substantial series of stressful events that include dietary and environmental changes, hierarchical re-establishment, as well as increased pathogen exposure (Hötzel *et al.*, 2011). Stress, coupled with both an underdeveloped immune system and transitioning microbial communities, can result in structural alterations of intestinal morphology and physiology, resulting in sub-par growth, health, and production (Pluske *et al.*, 1997).

Conventionally, a combination of antibiotics and high-quality protein ingredients have been included in the diet of weaned pigs to manage these performance and health concerns in the early weaning period (Berrocoso *et al.*, 2012, Yuan *et al.*, 2016). However, recent legislation in the U.S. and Canada (FDA, 2013, Canada, 2018) has limited the use of medically important antimicrobials. Restrictions on the prophylactic use of antibiotics have thus increased the need to develop effective alternative strategies, such as improved dietary protein sources that would benefit both the health and nutrition of young animals.

Traditionally, fish meal (FM) has been utilized as a high-quality protein source in nursery pig diets (Yuan *et al.*, 2016). Due to concerns with sustainability of wild fish populations (FAO, 2014) and feed costs, further processed soybean meal (SBM) products, such as generated by pretreatment with enzymes, fermentation, or microbial conversion, have become increasingly attractive as substitutes for FM (Sinn *et al.*, 2016, Yuan *et al.*, 2016). While the value of FM and further processed SBM as high-quality protein sources has been well established (NRC, 2012), their effects on gut physiology remain largely unknown. For instance, these ingredients may modulate the histology and physiology of the gastrointestinal tract (GIT). Alternatively, or in combination with this host response, the type of protein source may affect the GIT microbiome, i.e. the communities of symbiotic microorganisms, such as bacteria, archaea, fungi, and viruses, which populate the gut of animals (Conlon and Bird, 2015). Among these, bacteria are considered to have the largest impact on gut health and immune modulation, because they have the highest level of species diversity and display the widest range of metabolic activities. In non-ruminants, an important focus area has been within the ileum, as it is a highly active site for nutrient absorption by the host and houses the highest density of bacteria in the small intestine (10^8 cfu × mL−1). Furthermore, a number of investigations have reported that symbiotic bacteria can affect intestinal absorptive and immune processes, while in turn their presence in the GIT can be influenced by changes in host diet (Blaut and Clavel, 2007, Leser and Molbak, 2009).

In this context, we took advantage of a companion study by (Sinn *et al.*, 2016) to compare the respective effects of FM, conventional SBM and MSBM on the histology and bacterial communities of the ileum. Considering the levels of anti-nutritional factors

present in SBM, it was hypothesized that the MSBM-fed animals would harbor similar, but more beneficial bacteria, when compared to conventional SBM-fed animals. Also, in light of the similar growth performance and diarrhea incidence between FM and MSBM (Sinn *et al.*, 2016), it was hypothesized that nursery pigs fed these ingredients would display similar ileal histological features and bacterial profiles.

2.4 MATERIALS and METHODS

2.4.1 Animals, Diets, and Experimental Design

The analyses presented in this report were performed on samples collected as part of a previously reported companion study (Sinn *et al.*, 2016). Briefly, all experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at South Dakota State University (SDSU; IACUC #13-044A, 13-052A). Pens of pigs (7 pigs/pen; initial BW: 6.1 ± 0.8 kg; weaning age: 21 ± 1 d), with 8 pens/dietary treatment, were randomly assigned to one of 6 dietary treatments for 35 days in a 3 x 2 factorial design: 3 feedstuff protein ingredients (conventional SBM, FM or MSBM), with or without a dietary acidifier (0.2%, KEMIN, Des Moines, Iowa, USA). The 3 dietary treatments included: 1) basal diet containing corn, SBM and whey (negative control - NEG), 2) basal diet supplemented with Menhaden FM (positive control - POS), and 3) basal diet supplemented with MSBM (experimental diet - MSBM). All diets were formulated to meet or exceed nutrient requirements for nursery pigs from wean to 25 kg in a 3-phase feeding program. The FM and MSBM were provided by Prairie AquaTech, (Brookings, South Dakota, USA) and included at 7.5 and 5% in Phases I and II, respectively. Details related to diet formulation and production of MSBM have been reported elsewhere (Sinn *et al.*, 2016).

2.4.2 Tissue Sample Collection, Preparation, and Histological Analyses

On d7 (end of Phase I) and d21 (end of Phase II), one representative pig per pen, selected based on average pen BW and performance (balancing for gender between treatments), was euthanized for sample collection. On each collection day, a total of twelve pigs were processed individually at 20 min intervals, beginning at 0900 h. Following euthanasia via captive bolt stunning and exsanguination, the entire gastrointestinal tract was carefully excised from the abdominal cavity. The stomach was segregated at both the cardiac and pyloric regions prior to its removal. Tissue samples from the pyloric and corpus regions were dissected, rinsed with saline, blotted dry, then placed in buffered formalin for a minimum of 24 h. These were then dehydrated and infiltrated with paraffin wax (South Dakota Animal Disease Research & Diagnostic Laboratory, Brookings, SD) for histological staining. The duodenum, jejunum, and ileum were separated by hemostats, and the small intestine was detached from the large intestine marked by the cecum. The upper duodenum was divided into two segments: one section was gently rinsed with saline, blotted dry, then snap frozen in liquid nitrogen for further analysis, while the other was paraffin-embedded as described above for the stomach tissue. Digesta pH was measured at six locations along the gastrointestinal tract (stomach, proximal duodenum, mid jejunum, distal ileum, cecum, and colon) using a Thermo Orion digital pH meter (Model #360; Hogentogler&Co, Inc, Columbia, MD).

Histological and immunohistochemical analyses were conducted on the stomach and duodenal tissues. Periodic acid Schiff (PAS) staining of stomach tissue was used to assess mucin type according to a 5-point scale, ranging from 1 (pink - neutral) to 5 (blue - acidic). A mean slide score was calculated from 3 sections/tissue per slide. Duodenal

sections were stained with Haematoxylin and Eosin (H&E) to measure villus height and crypt depth. A mean slide value for each parameter was calculated from 10 sections/tissue/slide. For goblet cells, mucin staining was performed on duodenal sections using the Alcian blue/PAS stain kit (Newcomer Supply, Middleton, WI) following the manufacturer's instructions. The area (μm^2) occupied by goblet cells was calculated by dividing the mucin stained area (blue or dark purple color) by the total tissue area. Images were captured at 200X magnification using a BX53F Olympus microscope, then analyzed using the Olympus Cellsense software (Olympus America Inc., Center Valley, Pennsylvania). The goblet cell percentage was calculated based on the total slide coverage of the tissue that was populated by goblet cells per field, and the average was determined from all fields for a group comparison.

For inflammatory analysis, 4 μm tissue sections were mounted on charged slides, deparaffinized, then rehydrated by sequential immersion in xylene, 100% ethanol, 80% ethanol then phosphate buffer saline. Antigen retrieval was performed by boiling in 10 mM sodium citrate buffer (pH 6.0). The DAB Substrate Kit (Abcam, Cambridge, MA) was used for immunohistochemistry staining following the manufacturer instructions, with an additional 1 h blocking step with goat serum (1:200) followed by incubation with an anti-CD45 primary antibody (1:500 Abcam, Cambridge, MA) for 2 h at ambient temperature. Inflammatory cells clusters were counted in all fields occupied by tissue at 200X magnification. Scores were assigned to each field as: 1 (clusters only at the base of the gland), 2 (clusters displacing tissue up to the neck of the gland) or 3 (inflammatory infiltrate fully displacing the gland). Each field was evaluated by multiplying the number of clusters by the score per field. For intestinal samples, the same procedure was followed

with another possible score of 4 given to fields where the inflammatory infiltrate was invading the submucosa. Field values were averaged per animal for final group comparisons.

2.4.3 Microbial Analysis

Microbiome analysis was performed on ileal samples collected at the end of Phase II (d21) from pigs selected from each pen fed non-acidified diets. Digesta samples were obtained from a 30 cm section proximal to the ileo-cecal junction, snap frozen in liquid nitrogen, then stored frozen (-80°C) until processed. Microbial genomic DNA was extracted and purified using a commercial kit (PowerSoil DNA Isolation Kit, MoBio Laboratory Inc) according to the manufacturer's recommended protocol, which included cell lysis by bead beating.

The V1–V3 region of bacterial 16S rRNA gene sequences was PCR-amplified using the 27F forward (Edwards *et al.,* 1989) and 519R reverse (Lane *et al.*, 1985) primer pair. PCR reactions were performed with the Phusion *Taq* DNA polymerase (Thermo Scientific, Waltham, MA, USA) under the following conditions: hot start (4 min, 98 °C), followed by 35 cycles of denaturation (10 s, 98 °C), annealing (30 s, 50 °C) and extension (30 s, 72 °C), then ending with a final extension period (10 min, 72 °C). PCR products were separated by agarose gel electrophoresis, and amplicons of the expected size (~500 bp) were excised for gel purification using the QiaexII Gel extraction kit (QIAGEN, Hilden, Germany). For each sample, approximately 400 ng of amplified DNA was submitted to Molecular Research DNA (MRDNA, Shallowater, TX, USA) for sequencing with the MiSeq 2x300 platform (Illumina, San Diego, USA) to generate overlapping paired-end reads.

Unless specified otherwise, computational analysis of PCR-generated 16S rRNA gene amplicon sequences were performed using custom written Perl scripts (available upon request). Raw bacterial 16S rRNA gene V1–V3 amplicon sequences were provided by Molecular Research DNA as assembled contigs from overlapping MiSeq 2x300 paired-end reads from the same flow cell clusters. Reads were selected to meet the following criteria: presence of both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nt, and a minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15. Following quality screens, sequence reads were aligned, then clustered into Operational Taxonomic Units (OTU) at a genetic distance cutoff of 5% sequence dissimilarity. It was previously assessed (St-Pierre *et al.*, 2015) that a 5% dissimilarity cutoff for 16S rRNA gene was more representative of the genetic variation within the V1–V3 hypervariable regions, as determined by the meta-analysis of Kim *et al.* (2011).

OTU were screened for DNA sequence artifacts using the following methods. Chimeric sequences were first identified with the chimera.uchime and chimera.slayer commands from the MOTHUR source software package (v.1.36.1, University of Michigan, Ann Arbor, USA) open (Schloss *et al.*, 2009). Secondly, the integrity of the 5′ and 3′ ends of OTU was evaluated using a database alignment search-based approach. When compared to their closest match of equal or longer sequence length from the NCBI nt database, as determined by BLASTN (2.5.0) (Altschul *et al.*, 1997), OTU with more than five nucleotides missing from the 5′ or 3′ end of their respective alignments were discarded as artifacts. Single read OTU were subjected to an additional screening, where only sequences that had a perfect, or near perfect, match to a sequence in the NCBI nt

database were kept for analysis. Thus, the alignment had to span the entire sequence of the OTU and a maximum of 1% nucleotide dissimilarity was tolerated.

After removal of sequence chimeras and artifacts, the bacterial composition of each sample was determined by calculating the relative abundance of valid OTU. This was defined as the number of sequence reads assigned to an OTU in a given sample, divided by the number of total reads in that sample. Taxonomic assignment of valid OTU was determined using a combination of RDP Classifier (Wang *et al.*, 2007) and BLAST (Altschul *et al.*, 1997). The List of Prokaryotic Names with Standing in Nomenclature (LPSN) was also consulted for information on valid species belonging to taxa of interest (Euzéby, 2013, Parte, 2014).

2.4.4 Accession Numbers for Next Generation Sequencing Data

Raw unprocessed sequence data are available from the NCBI Sequence Read Archive (SRA) under BioProject PRJNA474940. Read numbers and SRA accession numbers for each sample are provided in Table 2.1.

2.4.5 Statistical Analyses

pH, histology, and immunohistochemistry data were analyzed using the PROC MIXED procedure of SAS (v9.3, SAS Inst. Inc.) with pen as the experimental unit. Fixed effects were diet and their interaction. For the random variable, pen was nested within block. Statistical significance was established at $P < 0.05$. For all ANOVA analyses, the Tukey-Kramer adjustment was used to test mean separation where main effects were significant. All data were tested *a priori* for normality and homogeneity of variances using SAS (v9.3, SAS Inst. Inc.), and data are presented as least squares means ± SE.

Fisher's exact test compared OTU profiles across treatments. Principle component analysis (PCA), performed using MOTHUR open source software package (v.1.36.1, University of Michigan, Ann Arbor, USA) (Schloss *et al.*, 2009), was utilized to assess bacterial profile relationships between samples and treatments.

2.5 RESULTS

Comparative Histological Analyses of Stomach and Intestinal Segments in

2.5.1 Response to Diet

Histological data collected and reported by Sinn (2018) has been replicated here to provide a comprehensive understanding of results. At the end of Phase I, the mean pH along the GIT (pyloric region of the stomach, duodenum, jejunum, ileum, cecum, and colon, respectively) across all diets was 3.98, 5.38, 6.47, 7.08, 6.17, and 6.41 compared to 4.18, 5.55, 6.25, 6.74, 5.60, and 5.93 at the end of the Phase II. There was an effect of location on pH ($P < 0.0001$), with the lowest pH observed in the pyloric region of the stomach, while each measured location in the small intestine increased progressively until the distal end of the ileum ($P < 0.05$) in Phase I and II (Table 2.2; reproduced from Sinn (2018)). As a function of dietary treatments in Phase I, there was no difference in pH between the stomach and duodenum in pigs fed the NEG (-Acid) and POS (+Acid) diets, while all other groups had lower pH in the stomach compared to the duodenum ($P < 0.05$). Furthermore, no difference in duodenum and jejunum pH was observed in pigs fed MSBM (-Acid) and POS (-Acid), while pigs fed NEG (+Acid) or POS (+Acid) had lower pH in the duodenum compared to the jejunum ($P < 0.05$). Within dietary treatments from Phase II, all treatments had lower pH in the stomach compared to the duodenum ($P < 0.05$). Pigs fed NEG (+Acid), POS (+Acid), and MSBM (+Acid) had similar pH in the

duodenum compared to the jejunum, while pigs fed MSBM had lower pH in the duodenum compared to the jejunum ($P < 0.05$). Neither protein source nor dietary acidifier were found to be associated with differences in VH, CD, VH:CD, goblet cell area, Ki-67, inflammation, or mucin accumulation (Table 2.2).

2.5.2 Taxonomic Composition of Ileal Bacterial Communities

To assess the possible effect of protein source on intestinal microbial communities, bacterial composition of ileal digesta was determined at d21. A total of 462,534 high quality 16S rRNA gene sequence reads were generated across 21 digesta samples (NEG, n=8; POS, n=7; MSBM, n=6). As PCR-amplification of the 16S rRNA gene V1-V3 region did not yield sufficient amounts of amplicons for 3 ileal digesta samples (P8, M7 and M8), these were not included in this analysis. At the phylum level, Firmicutes were the highest represented group (Table 2.3; Figure 2.1), with *Lactobacillaceae* found overall to be the most abundant family-level taxon identified. While there was a wide range in representation of *Lactobacillaceae* across all samples (0.3% - 99.1%), only two samples showed relative abundances for this family that were less than 1%, in contrast to 14 samples that ranged between 42.4% and 99.1%. While not as prominent as *Lactobacillaceae*, members of other Firmicutes families, such as *Clostridiaceae, Erysipelotrichaceae, Ruminococcaceae,* and *Peptostreptococcaceae*, were also identified. Proteobacteria was the second most prominent phylum, with the majority of sequences found to be affiliated to either *Pasteurellaceae, Campylobacteraceae or Enterobacteriaeae*. Within Bacteroidetes, *Prevotellaceae* were the most highly represented family. Actinobacteria were only identified in high

abundance in one ileal sample (29.8 %, sample M6), compared to 0 – 0.9% in any of the other samples.

Based on phylogenetic affiliations, different bacterial composition profiles could be distinguished amongst samples (Figure 2.1). The most common profile was predominance of *Lactobacillaceae*, which was defined as a relative abundance of 75% or greater. This profile was observed in 5 samples from each of the POS- and NEG-fed pigs, in contrast to only 2 samples from the MSBM-fed pigs. While the frequencies of these profiles were numerically lower for MSBM-fed pigs compared to the other diets, these differences were not found to be significant based on Fisher's exact test. Predominance of Proteobacteria (75% or greater) was observed in one sample from each diet (P3, N2, and M4). High abundance of *Prevotellaceae* (50% or greater) was observed in two samples, one from the POS diet and one from the NEG diet; while below this threshold, *Prevotellaceae* were well represented in two MSBM samples (28.9 – 36.0%). The distinctive feature of the remaining samples (N4, M6) was a high proportion of *Peptostreptococcaceae* (31.8 - 52.5%).

OTU Composition Analysis of Ileal Bacterial Communities

Across all samples, a total of 3006 OTU were identified. Among *Lactobacillaceae*, OTU SD_Ssd-02 displayed the highest abundance in a single sample (94.5%, P4), followed by SD_Ssd-01 (84.1%, N8) and SD_Ssd-11 (60.2%, M5). For Proteobacteria, the most highly represented OTU were SD_Ssd-07 in sample M4 (53.0%, *Enterobacteriaceae*), SD_Ssd-04 in sample P3 (45.2%, *Campylobacteraceae*), and SD_Ssd-05 in sample N2 (18.1%, *Pasteurellaceae*). Because taxonomic profiling

revealed shared bacterial families amongst digesta samples from animals on different diets (Figure 2.1), a comparative OTU composition analysis was performed to determine whether this overlap also applied at the OTU level as well. Consistent with taxonomic profiling, results from Principal Component Analysis (Figure 2.2) were indicative of shared OTU across samples. While there was no obvious distribution of samples based on treatment, PCA analysis suggested the existence of two main profile groups across all samples based on OTU composition.

Further analyses were conducted on the 16 most abundant OTU, which were selected based on a mean relative abundance of 10% or greater in at least 1 treatment. Consistent with the taxonomic profiles, 8 of the most abundant OTU were assigned to *Lactobacillaceae*, with the others distributed amongst *Clostridiaceae, Peptostreptococcaceae,* Proteobacteria, and Bacteroidetes (Table 2.4). Based on BLAST analysis, their sequence identity to their closest respective valid taxon was greater than the 95% cutoff used for OTU clustering (95.6 to 99.8%; Table 2.4). While numerical differences in relative abundance were observed for the most highly represented OTU among treatment groups, they were not found to be statistically significant.

2.6 DISCUSSION

Conventional pig diets typically include high levels of corn and SBM. However, in nursery pig diets, an allergenic response associated with high SBM inclusion can result in a plethora of reduced gut health indices, including villous atrophy, crypt elongation, maldigestion and malabsorption, increased gastric emptying, and diarrhea (Dunsford *et al.*, 1989, Lalles, 1993). Thus, high quality protein sources, such as FM and further processed SBM, are used in a transient manner until tolerance to soy proteins has been

developed in an attempt to minimize the detrimental effects of high SBM inclusion. As pressure to reduce reliance on antimicrobials continues, there is an increasing need to understand the mechanistic effects of feedstuffs on gut physiology in addition to providing nutrients. Recent evidence suggests that further processed SBM can increase feed enzyme activity and lactic acid bacteria counts (Yuan *et al.*, 2016); as well as reduce diarrhea incidence (Sinn *et al.*, 2016) and enhanced antibody counts during the post-weaning (Koepke *et al.*, 2017) in pigs. Thus, to gain further insight into the phenotypic responses to these feedstuffs, the current study aimed to determine the effects of two high-quality protein sources (FM and MSBM) on histomorphological parameters and ileal bacteria composition.

Multiple histological variables from the duodenum were assessed as a means of measuring of gut health. Duodenal VH in pigs across all dietary treatments were found to be in accordance with Shen *et al.* (2009) who fed diets containing similar levels of corn, SBM, and whey in their basal diet; however, CD values reported here were much larger than those reported Shen *et al.* (2009). Duodenal VH and CD reported in this study were lower when compared to values reported by Gu *et al.* (2002). The histological values reported here were within an acceptable range reported by Wiese *et al.* (2003); however a lack of congruency may demonstrate the requirement for additional histological parameters to adequately elucidate gut health.

Goblet cells reside in the intestinal epithelium and produce a viscoelastic mucin coat to protect mucosal epithelium and house secretory IgA antibodies (Kindon *et al.*, 1995). The lack of difference in the goblet cell area is consistent with the lack of difference reported between treatments in the mucin scores of the stomach and upper

duodenum. Ki-67 is a marker of intestinal cell proliferation where the positive (brown) cells can be counted and calculated with total cell count as an indicator of tissue growth (Wiyaporn *et al.*, 2013). The lack of difference between treatments for cell proliferation is consistent with the lack of difference in the morphological assessment of the duodenum (i.e. VH, CD, and VH:CD) for all treatments.

Inflammation in the GIT may be influenced by multiple factors, including pathogenic microorganisms, toxins, and antigenic activity (i.e. feedstuffs) (Campbell *et al.*, 2013). Inflammation occurs when the intestinal barrier is disrupted, which increases gut permeability and allows harmful toxins, bacteria, or antigens to cross the gut barrier; thus, resulting in malabsorption, diarrhea, and reduced growth performance (Campbell *et al.*, 2013). The lack of differences in inflammation in the stomach and upper duodenum, observed in the current study, suggest minimal influence of experimental diets on these select locations. Combined histological data may indicate that impact of protein source reported previously (Sinn et al., 2016) is minimally associated with enhancements in intestinal morphology.

Firmicutes, Proteobacteria, and Bacteroidetes were found to be the dominant phyla across the three diets used in this study. These results are in accordance with previous investigations, such as the ileal and fecal bacterial profiles in pigs reported by Quan *et al.* (2018) and Molist *et al.* (2012), respectively. The results from Quan *et al.* (2018) also support our findings that *Lactobacillaceae* (Firmicutes) can represent more than half of the bacterial profile in most swine ileal digesta samples. The predominance of this family, mostly consisting of cultured and uncultured members of the genus *Lactobacillus*, was likely the result of high inclusion of corn and soybean meal in

experimental diets fed to animals sampled in this study (54.8% - 64.87% throughout Phases I and II) (Sinn *et al.*, 2016). Indeed, since *Lactobacilli* as a group tend to metabolize complex carbohydrates as preferred substrates, ileal digesta would have provided optimal conditions for their growth in this study, as these nutrients would have been readily accessible from the hydrolysis of non-starch polysaccharides and oligosaccharides provided by the diets (Graham *et al.*, 1986, Schwab and Ganzle, 2011). *Lactobacilli* are of particular interest in post-weaned pigs, as they limit pathogenic bacteria, such as colibacillosis and enterotoxigenic *Escherichia coli,* from initiating GIT infections (Nemcova *et al.*, 1999, Gustavo Hermes *et al.*, 2013). It is interesting to note that, in the present study, samples with low abundance of *Lactobacilli* were mostly comprised of bacteria from the phylum Proteobacteria. As the main OTU affiliated with Proteobacteria (SD_Ssd-03, SD_Ssd-13, SD_Ssd-14, and SD_Ssd-23) were assigned to families within this phylum that include a number of known pathogens (e.g. *Enterobacteriaceae, Campylobacterceae,* and *Pasteurellaceae*), their predominance in certain samples may have been indicative of dysbiosis-like conditions in corresponding animals. While further investigations will be required to determine whether these ileal Proteobacteria OTU represent pathogens or undesirable commensals, the apparent mutual exclusion of *Lactobacilli* and Proteobacteria observed in this study are consistent with the current models that beneficial bacterial species can competitively exclude and/or provide metabolic environments that are non-conducive to the success of pathogenic species (Nollet *et al.*, 1999).

The *Lactobacillus* genus is composed of hundreds of cultured and uncultured species and strains, which as a group have the potential to provide a diverse array of

functions (Collins *et al.*, 1991, Kant *et al.*, 2011a). While metabolic capabilities can be difficult to assign to individual species based on genus-level characteristics, further insights can be provided by comparisons with well characterized species that are very closely related to specific OTU. In this study, the main *Lactobacillus*-affiliated OTU were respectively found to be more closely related to either *L. amylovorus*, *L. johnsonii*, or *L. delbrueckii*. Each of these species has been linked to antimicrobial effects within the ileum of the post-weaned pig (Sillanpää *et al.*, 2000, Pridmore *et al.*, 2004, Omar *et al.*, 2013). Interestingly, each was observed in different relative proportions within individuals fed different protein sources in this study.

As *L. amylovorus* was isolated from corn silage, and it is currently classified within the *L. acidophilus* group, it may represent a beneficial resident of the mammalian gut (Kant *et al.*, 2011b). Its cellular structure includes Surface (S)-layered proteins that maintain structural integrity of the cell and provide protection against environmental hazards (Avall-Jaaskelainen and Palva, 2005). The S-layer proteins contribute to a number of probiotic benefits often assigned to *Lactobacilli*, such as strong adherence to enterocytes and the host's extracellular matrix (Toba *et al.*, 1995, Avall-Jaaskelainen and Palva, 2005). These proteins may aid in activating the innate immune system of the host to optimize gut health (Taverniti *et al.*, 2013), notably by acting as antigen delivery vehicles for their host (Hynönen and Palva, 2013). The very high prevalence of *L. amylovorus* in the NEG group may explain the low incidence of intestinal damage and inflammation in this group, despite dietary levels of conventional SBM previously reported to initiate intestinal damage (Friesen *et al.*, 1993).

L. johnsonii abundance was found at 14% or greater in both POS and MSBM treatment groups. Identified as a normal resident of both the human and swine GIT (Leser *et al.*, 2002), *L. johnsonii* is classified within the *L. acidophilus* Group B, because it has both probiotic qualities and aggregation promoting factor proteins (Apf) (Ventura *et al.*, 2002). While the exact role of these structural adhesion proteins remains unknown, it has been suggested based on structure and location that they provide similar functions to S-layer proteins found in other members of the *L. acidophilus* Group A (Ventura *et al.*, 2002).

L. delbueckii is a nonpathogenic lactic acid producing bacteria that is commonly utilized in dairy food production. In accordance with Gong *et al.* (2008) who reported *L. delbrueckii* as highly susceptible to dietary influence, its abundance was found at greater than 10% in the ileal digesta of MSBM-fed animals, compared to 2% or less in NEG and POS dietary groups. As a member of the genus *Lactobacillus* that is closely related to *L. amylovorus* and *L. acidophilus* (Schleifer and Ludwig, 1995), *L. delbueckii* would be expected to perform similar functions and provide analogous probiotic benefits. Interestingly, *L. delbueckii* was additionally found to induce B cell activation via immunostimulatory oligonucleotides in mice (Kitazawa *et al.* (2003), suggesting that increased abundance of this species could improve immune response.

The congruencies between *Lactobacilli* function, in combination with altered proportions of these prominent species, may lend credence to Functional Redundancy Theory. Functional Redundancy Theory can be defined as the potential for different species within a taxonomic group to perform the same function, but via alternate pathways, thus ensuring ecologically relevant functions are maintained within an

organism (Sonnenburg *et al.*, 2005). The presence of these three prominent *Lactobacillus* species suggests an ecological insurance of a keystone function within the population. Furthermore, the balanced coexistence of each of these three *Lactobacillus* species in MSBM-fed animals suggests a fortification of their function within the ecosystem against disruption. Thus, it may explains the improved performance observed by Sinn *et al.* (2016) in MSBM-fed animals as they demonstrated a proliferation of multiple ileal *Lactobacillus* species, rather than the dominance of a single strain.

While histological data has commonly been used as an indicator of gut health, especially in newly weaned pigs experiencing a plethora of various stressors that create an immunological challenge (Heinritz *et al.*, 2016), their effectiveness as indicators of an individual's gut health may be too limited. Thus, the combination of histological data and bacterial profiles may provide a more thorough assessment of gut health. Most commonly luminal, or fecal, bacterial populations are evaluated, because these bacterial populations are relatively simpler to obtain for composition identification, but their relevance to the bacteria's impact on gut health may be limited as their direct interaction with the host is minimal. Relative to luminal populations, mucosal bacteria are a select population often characterized by their adhesion capabilities (Fuller, 1989, Morowitz *et al.*, 2011). Levesque *et al.* (2014) suggested that mucosal-associated bacteria colonize the GIT tract early and are highly stable when exposed to dietary shifts. Evidence from this study suggests that luminal bacteria can provide information on commensal organisms and bacterial groups being altered by diet, but evidence of the influence of these population changes on pig phenotypic response to the feedstuffs was unclear. Further investigation

into mucosal OTU data will be required to fully understand the impact of protein source on bacterial species' gut morphology and gut health.

2.7 CONCLUSION

In conclusion, dietary protein source, even at relatively low inclusion levels, can result in variable bacteria profiles. In light of these results, the improved growth performance reported by (Sinn *et al.*, 2016) could have been the result of promoting the proliferation of combinations of ileal *Lactobacillus* species, rather than dominance of a single strain. However, the presence of similar species and strains, observed in the *Lactobacillus* population at varying degrees across all samples, suggests a necessity to maintain certain ecological functions. The limited morphological damage observed in all dietary groups suggests predominant ileal species have an influence on competitive exclusion against the growth of pathogens. The proliferation these beneficial bacterial species may thus promote the growth and colonization of mucosa-associated microorganisms, which have a direct interaction with the host. Additional investigations are required to further characterize the composition and functional relevance involved in identifying the effects of dietary ingredients on gut health.

TABLE 2.1 NUMBER OF HIGH-QUALITY (Q15) AND NON-CHIMERIC 16S RRNA READS USED TO DETERMINE THE BACTERIAL COMPOSITION OF ILEAL SAMPLES COLLECTED FROM PIGS FED ONE OF THREE DIETS: NEG (N: CONVENTIONAL SOYBEAN MEAL), POS (P: FISH MEAL), OR MSBM (M: FURTHER PROCESSED SOYBEAN MEAL).

Diet	Sample	Q15[1] reads	Non chimeric[2] reads	SRA accession numbers[3]
NEG	N1	4886	4011	SRR7275043
NEG	N2	19894	17922	SRR7275044
NEG	N3	16602	11717	SRR7275041
NEG	N4	42477	38177	SRR7275042
NEG	N5	74432	66157	SRR7275039
NEG	N6	21114	20007	SRR7275040
NEG	N7	2300	2232	SRR7275037
NEG	N8	17426	16774	SRR7275038
POS	P1	56888	50813	SRR7275035
POS	P2	63186	48374	SRR7275036
POS	P3	15085	12298	SRR7275051
POS	P4	30927	30392	SRR7275052
POS	P5	3329	2099	SRR7275049
POS	P6	38390	33369	SRR7275050
POS	P7	23571	21226	SRR7275047
MSBM	M1	24292	19244	SRR7275048
MSBM	M2	25680	16353	SRR7275045
MSBM	M3	3383	2833	SRR7275046
MSBM	M4	15836	14807	SRR7275054
MSBM	M5	12945	12219	SRR7275055
MSBM	M6	7186	6814	SRR7275053

[1] Number of reads for each sample that had both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nt, and a minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15.

[2] Number of reads used for bacterial composition analysis (i.e. after chimera check, 5' and 3' end quality check, as well as single-read OTU check).

[3] Accession numbers for raw, unprocessed sequence reads with barcodes.

TABLE 2.2 ***DIGESTA PH AND DUODENAL HISTOLOGICAL PARAMETERS AT THE END OF PHASE I (7D) AND PHASE II (14D) IN WEANED PIGS FED DIETS CONTAINING CONVENTIONAL SOYBEAN MEAL (NEG) AND MENHADEN FISHMEAL (POS) OR MICROBIALLY-ENHANCED SOYBEAN MEAL (MSBM)[1] WITH OR WITHOUT DIETARY ACIDIFIER.***

	NEG		POS		MSBM		SEM
Item	- Acid	+ Acid	- Acid	+ Acid	- Acid	+ Acid	
Location, d 7 pH							
Stomach	4.20[a]	3.99[a]	3.74[a]	4.11[a]	3.92[a]	3.97[a]	0.24
Duodenum	5.09[a,c]	5.56[b]	5.65[b]	4.74[a]	5.60[b]	5.62[b]	0.24
Jejunum	6.36[b,d]	6.62[b,c]	6.57[b,c]	6.78[b]	6.23[b]	6.28[b,c]	0.26
Ileum	6.95[d]	6.88[c]	7.10[c]	7.15[b]	7.29[c]	7.10[c]	0.26
Cecum	5.95[b,c]	6.29[b]	6.27[b,c]	6.31[b]	6.21[b,c]	6.00[b]	0.24
Colon	6.24[b,d]	6.59[b,c]	6.51[b.c]	6.37[b]	6.49[b]	6.28[b,c]	0.24
Location, d 21 pH							
Stomach	4.56[a]	4.16[a]	4.17[a]	4.31[a]	3.86[a]	4.00[a]	0.19
Duodenum	5.61[b]	5.17[a,c]	5.53[b]	6.08[b]	5.37[b]	5.55[b]	0.25
Jejunum	6.11[b]	6.17[b,c]	6.17[b]	6.25[b]	6.36[c]	6.43[b,d]	0.19
Ileum	6.52[b]	6.54[b]	6.79[b,c]	6.98[b]	6.54[c,d]	7.07[c,d]	0.20
Cecum	5.65[b]	5.62[b,c]	5.76[b]	5.47[b,c]	5.63[b,d]	5.51[b]	0.19
Colon	5.96[b]	6.06[b,c]	5.94[b]	5.84[b,c]	6.01[b,d]	5.78[b]	0.19
Histology, d 21[3]							
Villus height, μm	300	281	286	271	305	263	37.30
Crypt depth, μm	282	259	263	258	311	276	33.10
VH:CD, μm:μm	1.09	1.10	1.19	1.07	1.10	1.09	0.13
Goblet cell area (%)	3.20	4.10	3.00	3.5	3.80	3.50	0.50
Ki-67 (%)	61.20	61.60	63.30	63.10	60.90	59.40	6.07
Inflammation scores							
Stomach	1.48	1.41	0.62	1.34	1.30	1.33	0.38
Duodenum	0.90	1.06	0.75	0.77	0.88	0.57	0.27
Mucin scores							
Stomach bottom	3.45	3.45	2.93	3.31	3.17	2.99	0.56

Stomach neck	3.49	3.78	3.24	3.76	3.67	3.85	0.61
Stomach pit	3.04	2.87	2.99	3.67	2.67	3.38	0.42
Brunner's gland	2.63	2.98	2.29	2.99	2.64	2.82	0.45

[1]Experimental diets contained fishmeal and MSBM at 7.5 and 5% in Phase 1 (7d) and II (14d), respectively.
[2]Reported SEM is largest among measurements.
[3]Diet x acidifier interaction was not significant (P>0.10).
[a,b,c] For pH data, means within a column and day without a common superscript letter differ ($P < 0.05$).
Note: Data reproduced from Table 3.2 within Sinn (2018) thesis manuscript

TABLE 2.3 ***MEAN RELATIVE ABUNDANCE (%) OF THE MAIN TAXONOMIC GROUPS FROM ILEAL DIGESTA OF 21D POST-WEANED PIGS FED DIETS CONTAINING SOYBEAN MEAL ALONG (NEG), MENHADEN FISHMEAL (POS), OR MICROBIALLY-ENHANCED SOYBEAN MEAL (MSBM)[1].***

Taxonomy	NEG	POS	MSBM	SEM[2]	*P*-value
Firmicutes (%)	74.10	63.50	60.30	14.64	0.77
Lactobacillaceae	64.00	59.90	41.80	15.02	0.58
Clostridiaceae	0.90	0.10	4.10	0.03	0.19
Erysipelotrichaceae	0.20	0.20	1.40	0.11	0.34
Ruminococcaceae	0.30	0.50	1.50	0.19	0.23
Peptostreptococcaceae	6.70	0.60	6.10	0.43	0.64
Lachnospiraceae	0.20	0.70	1.70	0.46	0.30
Other Firmicutes	1.90	1.30	3.60	0.51	0.15
Proteobacteria (%)	14.20	23.40	20.80	8.10	0.83
Pasteurellaceae	7.90	9.80	6.20	8.05	0.91
Campylobacteraceae	2.10	11.30	0.40	0.06	0.22
Enterobacteriaceae	3.40	0.50	11.00	11.24	0.44
Other Proteobacteria	0.70	1.90	3.20	4.42	0.19
Bacteroidetes (%)	11.40	12.60	13.10	7.51	0.98
Prevotellaceae	11.10	12.40	12.70	0.30	0.98
Other Bacteroidetes	0.30	0.20	0.40	0.76	0.75
Actinobacteria (%)	0.20	0.04	5.00	0.01	0.31
Other Bacteria (%)	0.30	0.60	0.80	0.28	0.34

[1]Experimental diets contained fishmeal and MSBM at 7.5 and 5% in Phase 1 (7d) and II (14d), respectively. Ileal digesta was collected at the end of Phase II. Sample size (NEG, n=8; POS, n=7; MSBM, n=6).

[2]Reported SEM is largest among measurements.

TABLE 2.4 ***MOST ABUNDANT OPERATIONAL TAXONOMIC UNIT (OTU)[1] FOUND IN ILEAL DIGESTA OF 21D POST-WEANED PIGS FED DIETS CONTAINING SOYBEAN MEAL ALONG (NEG), MENHADEN FISHMEAL (POS), OR MICROBIALLY-ENHANCED SOYBEAN MEAL (MSBM)[1].***

Taxonomy[2]	OTU[3]	NEG	POS	MSBM	SEM[4]	P-value	Closest Valid Taxon[5]
Firmicutes							
Lactobacillaceae	SD_Ssd-01	35.00	23.50	9.20	0.11	0.250	*L. amylovorous*
	SD_Ssd-02	0.10	15.80	14.40	0.12	0.482	*L. johnsonii*
	SD_Ssd-11	3.30	1.90	11.30	0.02	0.436	*L. delbrueckii*
	SD_Ssd-06	2.50	1.90	0.20	0.01	0.385	*L. crispatus*
	SD_Ssd-08	2.60	1.50	0.70	0.01	0.527	*L. mucosae*
	SD_Ssd-09	2.70	2.90	0.10	0.02	0.453	*L. delbrueckii*
	SD_Ssd-20	2.10	1.60	0.20	0.02	0.633	*L. delbrueckii*
	SD_Ssd-34	1.60	0.70	0.08	0.01	0.563	*L. delbrueckii*
Clostridiaceae	SD_Ssd-13	0.60	0.03	3.50	0.02	0.169	*S. ventriculi*
Peptostreptococcaceae	SD_Ssd-23	4.90	0.08	0.08	0.04	0.468	*R. ilealis*
	SD_Ssd-14	0.20	0.40	5.60	0.05	0.275	*T. mayombei*
Bacteroidetes							
Prevotellaceae	SD_Ssd-03	7.00	6.30	5.60	0.05	0.977	*P. copri*
Proteobacteria							
Pasteurellaceae	SD_Ssd-24	2.30	0.90	0.50	0.02	0.683	*A. porcinus*
	SD_Ssd-05	2.30	4.60	2.30	0.02	0.671	*A. minor*
Campylobacteraceae	SD_Ssd-04	1.60	9.70	0.30	0.06	0.221	*C. lanienae*
Enterobacteriaceae	SD_Ssd-07	2.30	0.20	8.90	0.02	0.412	*E. coli*

[1]Experimental diets contained fishmeal and MSBM at 7.5 and 5% in Phase 1 (7d) and II (14d), respectively. Ileal digesta was collected at the end of Phase II. Sample size (NEG, n=8; POS, n=7; MSBM, n=6).
[2]As determined by RDP classifier at an 80% bootstrap cutoff.
[3]OTU representing at least 10% of sequence reads in at least one sample with internally assigned # identifiers.
[4]Reported SEM is largest among measurements
[5]Nucleotide sequence identity as determined by BLAST (Altschul *et al.*, 1997). All taxa are listed at <6% sequence divergence or at a 94% similarity to known species

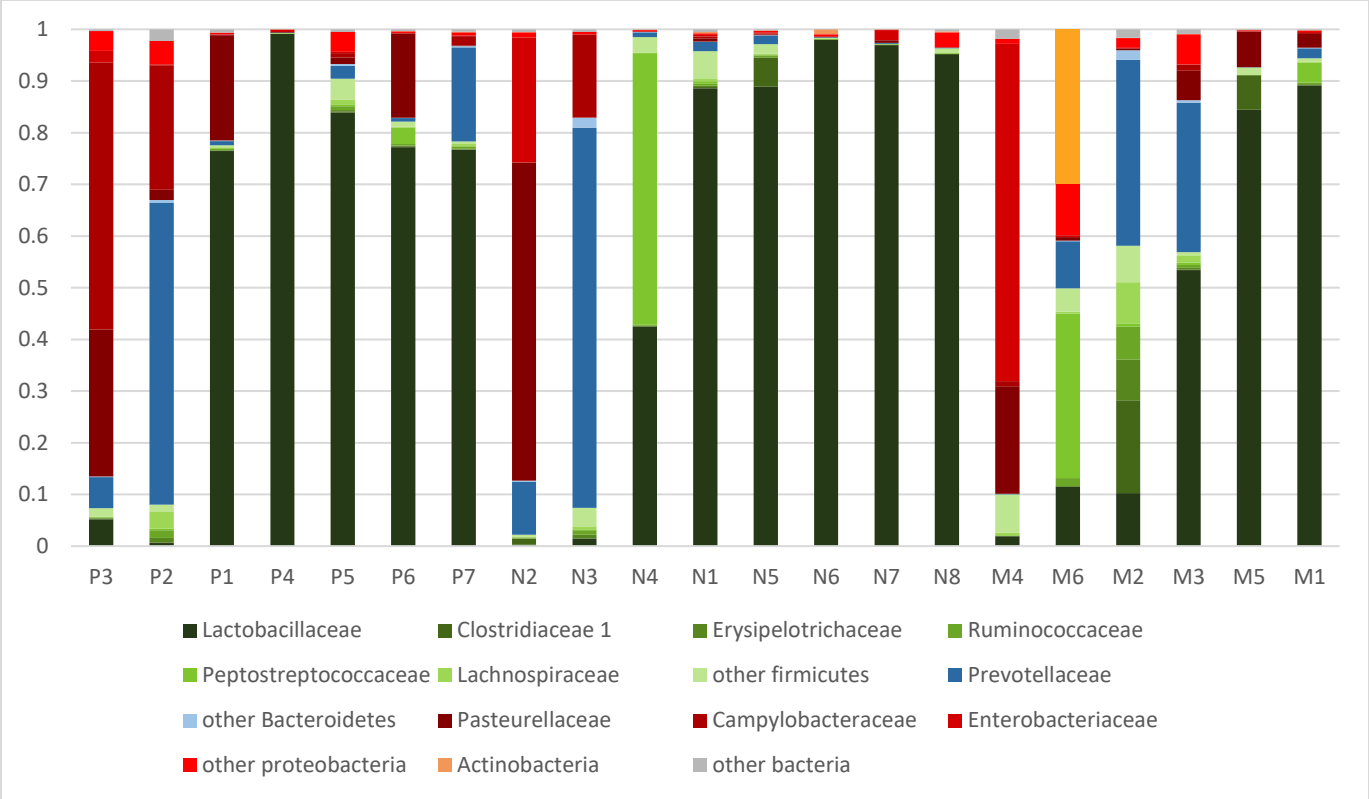

FIGURE 2.1 BACTERIAL COMPOSITION PROFILES OF ILEAL DIGESTA ISOLATED FROM 21D POST-WEANED PIGS FED DIETS CONTAINING CONVENTIONAL SOYBEAN MEAL (N), MENHADEN (P), OR MICROBIALLY-ENHANCED SOYBEAN MEAL (M). BACTERIAL COMPOSTION WAS DETERMINED BY 16S rRNA GENE PYROSEQUENCING. DIGESTA SAMPLES WERE COLLECTED AT 21D POST-WEAN.

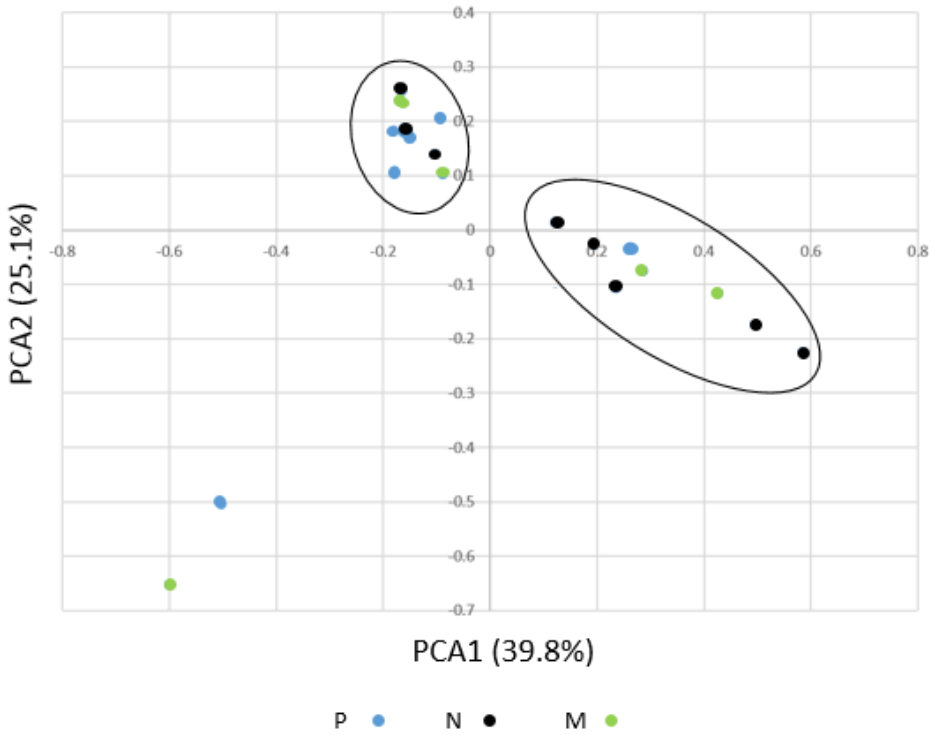

FIGURE 2.2 PRINCIPAL COMPONENT ANALYSIS OF UNI FRAC DISTANCE METRIC. PLOTTED POINTS REPRESENT PIG DIGESTA SAMPLES COLLECTED ON D21. SAMPLES ARE COLOR CODED ACCORDING TO TREATMENT: P, N, AND M REPRESENTING PIGS FED DIETS CONTAINING MENHADEN FISHMEAL, CONVENTIONAL SOYBEAN MEAL, OR MICROBIALLY-ENHANCED SOYBEAN MEAL, RESPECTIVELY.

Chapter 3

3.0 Comparative analysis of the luminal and mucosal bacteria populations in the ileum of weaned pigs fed different protein sources in complex nursery diets.

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3.1 ABSTRACT

The objective was to investigate differences between regionally-based bacterial populations and histomorphology to identify potential diet-induced modes of action to improve gut health in weaned pigs. Pens of weaned pigs (21d of age, 6.56 ± 0.87 kg; n=10 pens/diet; 7 pigs/pen X; 2 blocks of 20 pens) were fed one of 4 experimental diets: 1) positive control, containing corn, soybean meal, SDP, and FM (CON), 2) SDP and

MSBM ($MSBM_{+SDP}$), 3) FM and MSBM ($MSBM_{+FM}$), and 4) MSBM in both Phase I (d1-7 post-wean; 0, 12.75, 20.40, 34% MSBM inclusion, respectively) and II (d8-21; 0, 5, 8, 15% MSBM inclusion, respectively). Ileal digesta was collected from 5 pigs/diet at d21 (1 pig/pen). Ileal mucosal tissue was collected from 10 pigs/diet at d21 (1pig/pen). Luminal and mucosal ileal microbial gDNA was used for PCR amplification of the 16S rRNA gene (V1-V3 region) and sequenced on the Illumina Miseq 2x300 platform. Data produced approximately 302,000 and 670,000 high quality sequences in luminal and mucosal populations, respectively. Samples were analyzed using Proc GLM in SAS with pig as the experimental unit and pig as the random effect according to a completely random design or a complete randomized block design in luminal and mucosal samples, respectively. There was no effect of treatment ($P > 0.05$) on relative abundance of phyla, family, or genera within luminal or mucosal samples, with the exception of one operational taxonomic unit (OTU) identified as being closely related to *Lactobacillus reuteri* in $MSBM_{+FM}$ (0.05 ± 0.59; $P=0.0003$). Firmicutes was the highest represented group across all treatment groups representing 67% or greater of the total relative abundance. *Clostridiaceae* and *Lactobacillaceae* were found to be the most abundant family-level taxa identified, each representing more than 25% of the phyla abundance. When blocks were analyzed separately, *Lactobacillaceae* was the dominant profile in 44% of the samples (9 of 19) in block 1, whereas *Clostridiaceae* was the most highly represented family in 55% of the samples (10 of 18) in block 2. Furthermore, *Lactobacillaceae* appeared to be the dominant family in block 2 luminal and block 1

mucosal sample populations, whereas *Clostridiaceae* was the most prevalent in block 2 mucosal sample populations. The luminal population was primarily comprised of 2 OTU, which were closely related to *L. amylovorus* and L. *delbrueckii*, which contributed 31, 11, 23, and 18% of the CON, MSBM$_{+\mathbf{SDP}}$, MSBM$_{+\mathbf{FM}}$, and MSBM, respectively. Mucosal bacteria contained twice the species biodiversity compared to luminal populations. Mucosal *L. amylovorus* contributed 15, 24, 10, and 17% of the CON, MSBM$_{+\mathbf{SDP}}$, MSBM$_{+\mathbf{FM}}$, and MSBM, respectively. Luminal populations remained more homogenous in their comparison with higher proportions of select species, primarily *Lactobacillus*, while mucosal populations flourished with more than twice the number of predominant species. Thus, the retention and further development of a heterogeneous mucosal population may act as an ecological protective mechanism in what was a suspected disease challenge.

3.2 IMPLICATIONS

Luminal and mucosal bacterial populations in the ileum of post-weaned pigs develop bacterial polycultures unique to their anatomic locations. These polycultures suggest a location-specific response to opportunistic pathogens. Dietary and/or management strategies that aid in maintaining polyculture mucosal bacterial populations in the early post-weaned pig may provide a useful strategy ensure gut, and thus pig, health.

3.3 INTRODUCTION

Weaning is a critical phase of development associated with gastrointestinal instability that often results in low feed intake and decreased body weight in nursery pigs (Hötzel *et al.*, 2011). Pigs undergoing a substantial series of stressful events commonly demonstrate gastrointestinal disturbances and reduced gut health (Hötzel *et al.*, 2011). The microbiota can be highly indicative of gut health and immune modulation as bacteria are reported to have the highest level of species diversity while having the widest range of metabolic activities. Thus, the primary focus area in swine has been within the ileum, because it is a highly active site for nutrient absorption by the host and demonstrates the highest density of bacteria in the small intestine. Furthermore, a number of investigations have reported that symbiotic bacteria can affect intestinal absorptive and immune processes, while in turn be influenced by changes in host diet (Blaut and Clavel, 2007, Leser and Molbak, 2009). However, some controversy has persisted as to if the influence of bacteria on the host is dependent on their respective niches.

The microbiota establishes itself all along the gastrointestinal tract of its host according to the ecological niches present in different segments of the gut (Li *et al.*, 2008). However, host interaction does have an effect beyond provision of habitat; if bacterial communities become disrupted, opportunistic pathogens may proliferate, posing a potential risk to the health of the host, thus necessitating activation of an immune response (Suzuki *et al.*, 2004, Li *et al.*, 2012). Bacterial communities on the mucosa likely play an important role during competitive exclusion of pathogenic organisms due to closer proximity to host cells (Li *et al.*, 2008, Collado *et al.*, 2010). While more distant

from host cells than mucosal bacteria, luminal bacteria also form a beneficial relationship with the host, as they breakdown undigested nutrients and produce secondary metabolites that can be utilized by the host (Thibault *et al.*, 2010, LeBlanc *et al.*, 2013, Bedford and Gong, 2017). They can also be involved in competitive exclusion by the production of bacteriocins and modulation of pH (Šušković *et al.*, 2010). Bacteria colonizing either lumen or mucosa are functionally different, thus necessitating the need to characterize their respective composition profiles to better understand their influence on gut health.

Conventionally, evidence of gut health has been associated with certain clinical symptoms, such as low incidence of diarrhea. As researchers work to improve performance, this simple definition of gut health needs to be expanded to incorporate not only clinical but also subclinical gastrointestinal disturbances. Thus, researchers have included a wider range of factors that could potentially be modulated to improve gut function, such as immune modulation via antibody testing (Pierce *et al.*, 2005, Koepke *et al.*, 2017), identification and improvement of gut histomorphology (Coffey and Cromwell, 1995), and reduction of diarrhea incidence (Sinn *et al.*, 2016). However, understanding how bacteria, the agents that are in direct contact with the host, are responsible for gastrointestinal disturbances has not been well defined as a mode of action.

As a follow-up study to Koepke *et al.* (2017), a unique opportunity has been presented to investigate the effects of complex, high-quality protein source diets on intestinal bacteria without variation in performance as a confounding factor, which was

demonstrated previously. The objective was to investigate differences between regionally-based bacterial populations and histomorphology to identify potential diet-induced modes of action for improved gut health in weaned pigs. It was hypothesized that complex nursery diets would demonstrate a bacterial profile representative of gut health and growth performance capable of explaining performance data reported by Koepke *et al.* (2017).

3.4 MATERIALS AND METHODS

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at South Dakota State University (SDSU; IACUC #15-114A) and experiments were completed at the large animal research unit at South Dakota State University in Brookings, South Dakota. Animal trial specifics have been published by Koepke *et al.* (2017), but some animal feeding details have been included here for ease of understanding. The research presented here acts as an extension of the previous publication.

Animals, diets, and experimental design

Pigs were obtained from a commercial herd (Claremont Hutterite Colony, Castlewood, SD, USA) whose genetics stemmed from Landrace × Large White sows mated with Hampshire × German Large White boars. A total of 239 pigs were weaned at 21 ± 1 d of age (initial BW 6.56 ± 0.87 kg) into 48 pens (3 barrows and 3 gilts/pen; n = 10 pens/treatment) for a 35-d study. The study was conducted in 2 blocks, with 120 and 119 pigs in blocks 1 and 2, respectively. Upon arrival of block 2 pigs, the total number of

pigs failed to meet experimental expectations; thus, one treatment pen in the second block contained only 3 barrows and 2 gilts. At weaning, pigs were randomly allotted to one of 4 dietary treatments: 1) Control (**CON**; corn, SBM and whey-based diet containing FM and SDP), 2) control with MSBM replacing FM (**MSBM$_{+SDP}$**), 3) control with MSBM replacing SDP (**MSBM$_{+FM}$**), and 4) control with MSBM replacing both FM and SDP (**MSBM**). Experimental diets were fed in a 3-phase feeding program, where Phase I was fed from d 0 to 7 and Phase II was fed from d 8 to 21 post-wean (Table 2.1). Phase III consisted of a common corn/SBM diet for d 22 to 35 post-wean. All diets were fed in a meal form and were formulated to meet or exceed NRC (2012) requirements for weaned pigs.

3.4.1 Tissue Sample Collection, Preparation, and Analyses

On d21 and d22 (end of Phase II) of each block, one representative pig per pen, selected based on average pen BW and performance (balancing for gender between treatments), was euthanized for sample collection. On each collection day, a total of 10 pigs were processed individually at 20 min intervals beginning at 0900 h. Following euthanasia via captive bolt stunning and exsanguination, the entire small intestine was carefully excised from the abdominal cavity. Prior to removal, tissue was clamped via hemostats at the pyloric sphincter (demarcating the duodenum) and ileal-cecal junction (demarcating the ileum). It was then equally divided into three sections to identify the midpoint of the small intestine (demarcating the jejunum). Digesta and tissue were

collected for bacterial analysis, histology and gene-expression. Ileal digesta was collected from pigs in block 2 only from a 30 cm region proximal to the ileal-cecal junction. Mucosal tissue was then collected in a 10 cm increment at 70 cm distal to the pylorus. Histology samples were collected in a 5 cm increment distal to the mucosal tissue samples. Mucosal tissue was stored in saline until the day's collections were complete.

Histological samples were gently rinsed with saline, then immediately placed in buffered formalin for a minimum of 24 h prior to their processing. They were then dehydrated and infiltrated with paraffin wax (South Dakota Animal Disease Research & Diagnostic Laboratory, Brookings, SD) prior to slide mounting and staining with Haematoxylin and Eosin (H&E) to measure villus height (VH) and crypt depth (CD). A mean slide value for each parameter was calculated from 10 sections/tissue per slide. Mucosal tissues were immediately taken to the lab and processed according to Levesque *et al.* (2013) with some modifications (use of 10 sterile beads with 15 mL of buffer to separate mucosal bacteria from the tissue).

3.4.2 Microbial Analysis

Microbiome analysis was performed on ileal samples collected at the end of Phase II (d21-22) from one representative pig/pen on treatment diets (n=10 pigs/treatment). Microbial genomic DNA was extracted and purified using a commercial kit (PowerSoil DNA Isolation Kit, MoBio Laboratory Inc) according to the manufacturer's recommended protocol, which included cell lysis by bead beating.

The V1–V3 region of bacterial 16S rRNA gene sequences was PCR-amplified using the 27F forward (Edwards *et al*., 1989) and 519R reverse (Lane *et al.*, 1985) primer pair. PCR reactions were performed with the Phusion *Taq* DNA polymerase (Thermo Scientific) under the following conditions: hot start (4 min, 98 °C), followed by 35 cycles of denaturation (10 s, 98 °C), annealing (30 s, 50 °C) and extension (30 s, 72 °C), then ending with a final extension period (10 min, 72 °C). PCR products were separated by agarose gel electrophoresis, and amplicons of the expected size (~500 bp) were excised for gel purification using the QiaexII Gel extraction kit (QIAGEN). For each sample, approximately 400 ng of amplified DNA was submitted to Molecular Research DNA (MRDNA, Shallowater, TX, USA) for sequencing with the MiSeq 2x300 platform (Illumina, San Diego, USA) to generate overlapping paired-end reads.

Unless specified otherwise, computational analysis of PCR-Generated 16S rRNA amplicon sequences were performed using custom written Perl scripts (available upon request). Raw bacterial 16S rRNA gene V1–V3 amplicon sequences were provided by Molecular Research DNA as assembled contigs from overlapping MiSeq 2x300 paired-end reads from the same flow cell clusters. Reads were selected to meet the following criteria: presence of both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nt, and a minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15. Following quality screens, sequence reads were aligned, then clustered into Operational Taxonomic Units (OTU) at a genetic distance cutoff of 5% sequence dissimilarity. It was previously

assessed (St-Pierre and Wright, 2015) in consideration of the meta-analysis by Kim *et al*. (2011) that a 5% dissimilarity cutoff for 16S rRNA is more representative of the genetic variation in 16S rRNA gene sequences for the V1–V3 hypervariable regions.

OTU were screened for DNA sequence artifacts using the following methods. Chimeric sequences were first identified with the chimera.uchime and chimera.slayer commands from the MOTHUR (v.1.36.1, University of Michigan, Ann Arbor, USA) open source software package (Schloss *et al*., 2009). Secondly, the integrity of the 5′ and 3′ ends of OTU was evaluated using a database alignment search-based approach; when compared to their closest match of equal or longer sequence length from the NCBI nt database, as determined by BLASTN (2.5.0) (Altschul *et al*., 1997), OTU with more than five nucleotides missing from the 5′ or 3′ end of their respective alignments were discarded as artifacts. Single read OTU were subjected to an additional screen, where only sequences that had a perfect or near perfect match to a sequence in the NCBI nt database were kept for analysis; the alignment had to span the entire sequence of the OTU, and a maximum of 1% of dissimilar nucleotides was tolerated.

After removal of sequence chimeras and artifacts, the bacterial composition of each sample was determined by calculating the relative abundance of valid OTU. This was defined as the number of sequence reads assigned to an OTU in a given sample, divided by the number of total reads in that sample. Taxonomic assignment of valid OTU was determined using a combination of RDP Classifier (Wang *et al*.,2007) and BLAST (Altschul *et al*., 1997). The List of Prokaryotic Names with Standing in Nomenclature

(LPSN) was also consulted for information on valid species belonging to taxa of interest (Parte, 2014; Euzeby 2017).

3.4.3 Statistical Analyses

The PROC GLM procedure of SAS (v9.3, SAS Inst. Inc.) was used, with pig as the experimental unit, and fixed effects as diet, block, and their interaction. The random variable was pig. Statistical significance was established at $P < 0.05$ for ANOVA. An adjusted Tukey comparison was employed to identify significance $(P<0.05)$ between each treatment. Correlation between histology and OTU, weight gain and OTU data within intestinal location, as well as, between luminal and mucosal OTU from block 2 pigs was tested using PROC CORR. Luminal OTU, mucosal OTU, and histological parameters were reported with adjusted *P-values*. Luminal correlations were reported as statistically significant where $P_{adj} \leq 0.0006$ and a tendency was $P_{adj} \leq 0.0013$. Mucosal correlations were reported as statistically significant if $P_{adj} \leq 0.0001$ and as a tendency if $P_{adj} \leq 0.00026$. All data is presented as least squares means ± SE.

3.5 RESULTS

3.5.1 Histology and correlation among histology, bacterial profiles, and performance

There was no significant effect between treatment and villus height ($P = 0.134$) and/or crypt depth ($P = 0.808$; Table 3.2). No interaction between treatment and block was detected in either CD and/or VH of the histological variables. Significant correlations between OTU and histological variables in luminal microbial populations were not

observed. A significant correlation (P = 0.0001; r = 0.587) between the VH and CD and a tendency for a correlation between *Prevotella ruminicola* (Prevotella81) and VH (P < 0.0001; r=0.580) was observed (Table 3.3). Correlations were identified between location-based OTU (Table 3.3) where correlations among OTUs within the same genera comprised 50% of the significant correlations (i.e. Actinobacillus3 and Actonibacillus4). In addition, significant correlations (P < 0.0001) were observed between luminal *Prevotella23* and *Campylobacter7* OTU in the luminal population (r = 1), and between *Clostridium* and 4 species of *Actinobacillus* (r = 0.786, 0.794,0.784, 0.796), *Prevotella* and *Lactobacillus* (r = 0.978), and *Terrisporobacteria* and *Romboutsia* (r = 0.737; Table 3.3) in the mucosal population. All correlation coefficients between location-based OTU were supportive of strong and positive associations. Correlations between the first three weeks (d0, d7, d14, d21) of gain post-wean and location-specific OTU were analyzed, but no significant correlations were identified within either luminal or mucosal OTU.

3.5.2 Luminal Taxonomic Composition Analysis

Bacterial profiles were determined at d21 from a total of 301,961 high quality 16S rRNA gene sequence reads that were obtained from 17 luminal digesta samples. Samples contained a range of 5,844 to 70,555 high quality sequences/animal. Three samples failed to yield sufficient amounts of V1-V3 PCR amplicons for sequencing. At the phylum level, Firmicutes were the most highly represented taxonomic group within all treatments (CON, $MSBM_{+SDP}$, $MSBM_{+FM}$, MSBM; 97.41, 95.57, 73.74, 96.26, respectively), with

Lactobacillaceae comprising the most abundant family-level taxon. Luminal sequence data demonstrated a wide range of *Lactobacillus*–affiliated sequences across all samples (2.8 - 96.3%). While not as prominent as the family *Lactobacillaceae*, other families within the Firmicutes phyla were represented, such as *Aerococcaceae, Clostridiaceae*, and *Ruminococcaceae* (Table 3.4). Furthermore, Bacteroidetes composed the second most prominent phyla (2.32, 3.64, 25.34, 2.01% in CON, $MSBM_{+SDP}$, $MSBM_{+FM}$, MSBM-fed pigs, respectively), which was predominantly comprised of *Prevotellaceae*.

Based on phylogenetic affiliations, a number of distinct bacterial population profiles were identified amongst samples (Figure 3.1). Within a given sample, a bacterial taxonomic group was designated as predominant if its relative abundance was 75% or greater. The most common predominant group was *Lactobacillaceae*, which was observed in 14 of the 17 samples, indicating an overall high level of similarity amongst luminal bacterial profiles across treatments. Of the other 3 samples, two were found in animals fed $MSBM_{+FM}$ ($MSBM_{+FM7}$ and $MSBM_{+FM9}$), and primarily consisted of *Prevotellaceae* and *Aerococcaceae*, respectively. The remaining sample, $MSBM_{+SDP5}$, was primarily comprised of *Lactobacillaceae* and *Ruminococcaceae*, at 48.5% and 36.4% relative abundances, respectively. While numerically different amongst treatments, the relative abundances of these bacteria were not found to be significant based on Fisher's exact test (Table 3.5).

3.5.3 Luminal OTU Composition Analysis

Across all samples, a total of 1010 species-level OTU were identified. Among *Lactobacillaceae* OTU, *Lactobacillus15* displayed the highest abundance in a single sample (93.11%, SDP8), followed by *Lactobacillus13* (89.9%, FM8; 89.0%, CON10). Furthermore, *Clostridiaceae* maintained a presence within all samples (0.1 - 96.0%); the two predominant OTU were *Clostridium sensu stricto8* (96.0%, FM7) and *Clostridium sensu stricto9* (77.1%, CON8). For Bacteroidetes, the most highly represented OTU was *Prevotella23* in sample FM9 (47.3%, *Prevotellaceae*).

Additional analyses were conducted on the 11 most abundant OTU, which were defined as having a mean relative abundance of at least 1% for at least one treatment group. Consistent with the taxonomic profiles, 5 of the most abundant OTU were assigned to *Lactobacillaceae*, with the other OTU distributed amongst *Aerococcaceae, Clostridiaceae, Ruminococcaceae, Prevotellaceae* (Table 3.5). BLAST analyses revealed that the respective levels of sequence identities of these abundant OTU to their respective valid taxon were greater than the 95% cutoff used for OTU clustering in this study (Table 3.5). While numerical differences were observed for the most abundant OTU among treatment groups, none were found to be statistically significant.

3.5.4 Mucosal Bacterial Composition Analysis

A total of 673,315 high quality 16S rRNA gene sequence reads were obtained from 37 tissue samples (n = 10, n = 9, n = 9, n = 9 for CON, $MSBM_{+SDP}$, $MSBM_{+FM}$, and MSBM, respectively). Three samples failed to yield sufficient amounts of V1-V3 PCR amplicons

for sequencing. At the phylum level, Firmicutes were the highest represented group across all treatment groups representing 67% or greater of the total relative abundance (Table 3.6). Within the Firmicutes phylum, *Clostridiaceae* and *Lactobacillaceae* were found to be the most abundant family-level taxon identified, each representing more than 25% of the phylum abundance. Other Firmicutes families identified were *Ruminococcaceae*, *Streptococcaceae*, *Veillonellaceae*, and *Peptostreptococcaceae*. There was a significant effect of block for *Clostridiaceae* and *Lactobacillaceae* where relative abundance of *Clostridiaceae* was higher in block 2 (μ_{B1}= 9.85± 7.29, μ_{B2}=47.68±8.70, *P*=0.0004) while abundance of *Lactobacillaceae* was lower in block 2 (μ_{B1}= 40.93±7.69, μ_{B2}=16.23±7.36, *P*=0.02). The phylum Proteobacteria and Bacteroidetes represented the remaining 20 – 30% abundance. The majority of sequences within the Proteobacteria phylum were found to be affiliated to either *Pasteurellaceae*, *Rhodobacteraceae*, *Helicobacteraceae*, *Ampylobacteraceae*, and *Enterobacteriaceae*. Within Bacteroidetes, *Prevotellaceae* was the most highly represented family. *Cyanobacteria* were only identified in high abundance in one ileal mucosal sample (39.78%, sample MSBM1), compared to 0 – 0.20% in any of the other samples.

Based on phylogenetic affiliations, a more diverse composition of bacteria, on an individual sample basis, was observed for mucosal populations than that detected in luminal samples where one family (*Lactobacillaceae*) was predominant in 14 out of 17 samples (Figure 3.1 and 3.2). Within a given sample, a bacteria phylogenetic group was designated as dominant if it represented 50% or more of an individual's bacterial profile.

Of the 39 total samples, *Clostridiaceae* dominance was observed in 12 samples, and *Lactobacillaceae* was dominant in 11 samples. Dominance of other Firmicutes families occurred in 3 samples at relative abundance levels well above the 50% threshold (77.25%, 99.70%, and 91.10%, respectively). Dominance of *Pasteurellacaceae*, *Heliobacteriaceae*, and *Campylobacteraceae* occurred in 1 sample each, dominance of *Prevotellaceae* was observed in 2 samples and no dominant family was observed in 6 samples. Consistent with the effect of block, there was a difference in dominance profiles between blocks, as *Lactobacillaceae* was the most common dominance profile observed within block 1, whereas Clostridiaceae was more prevalent within block 2 samples. Based on Fisher's exact test, no statistical difference was found at the phyla or family levels with regard to treatment and no effect of interaction between block and treatment was identified.

3.5.5 Mucosal OTU Composition Analysis

Across all samples, a total of 1692 OTU were identified. Taxonomic profiling revealed shared bacterial families amongst mucosal samples with varying proportions of these bacteria, which were dependent on levels and ingredient inclusion (Figure 3.2). A comparative OTU composition analysis was performed on the 26 most abundant OTU within these samples; OTU selection criteria were maintained from luminal data analyses. Consistent with mucosal taxonomic profiles, 2 of the most abundant OTU were assigned to *Clostridiaceae* and 8 were assigned to *Lactobacillaceae*. Other OTU were distributed

amongst *Pasturellaceae*, *Campylobacteraceae*, *Enterobacteraceae*, *Helicobacteraceae*, *Prevotellaceae*, and *Streptococcaceae* (Table 3.7). BLAST analyses revealed that the respective levels of sequence identities of abundant OTU to their respective valid taxon were greater than the 95% cutoff used for OTU clustering in this study (Table 3.7). While numerical differences were observed for the most abundant OTU among treatment groups, only *Lactobacillus48* (P = 0.0003) was found to be statistically significant. This OTU not only demonstrated an effect of treatment, but also demonstrated an effect of block where it was observed in higher abundances in block 1 (μ_{B1}=1.0 ± 0.04%, μ_{B2}=0.01 ± 3.75%, P=0.0008). Furthermore, two other OTU, *Clostridia20* and *Lactobacillus39*, were found to be influenced by an effect of block; *Clostridia20* (μ_{B1}=7.82 ± 4.27%, μ_{B2}=40.43 ± 7.40%, P=0.001) and *Lactobacillus39* (μ_{B1}=24.43 ± 5.27%, μ_{B2}=51.56 ± 3.85%, P=0.03) were both observed in higher abundance within block 2.

3.6 DISCUSSION

Minimizing the effects of weaning has long been the shared goal of producers and researchers alike. Young pigs typically experience environmental, social, and physiological stress that generally results in reduced growth performance and gut health (Hötzel *et al.*, 2011). Diet has been demonstrated to successfully modulate some of these detrimental occurrences (Danielson *et al.*, 1960, Lepine *et al.*, 1991), but the mode of action that is responsible remains unclear and ingredient specific. Thus, in this follow-up study to Koepke *et al.* (2017), the objective was to identify a potential diet-induced mode

of action via bacterial populations as a means of improving gut health through their influence on histological and performance parameters.

Weaning has a consistent impact on the histomorphological parameters, villus height and crypt depth (Hampson and Smith, 1986, Kelly *et al.*, 1991), which has previously justified their use as indicators of gut health. While the ileal VH values obtained in this study were consistent with previous research, the CD values were atypical of 6 week old pigs (Gu *et al.*, 2002). Since the latter were observed across all treatments, they were likely not effected by diet. No significant correlations were observed between histomorphological parameters and body weight (d0, d7, d14, d21; reported by Koepke *et al.* (2017)) as they pertained to bacteria.

This study has shown different bacterial profiles between luminal and mucosal communities. While luminal samples were mainly comprised of Firmicutes and Bacteroidetes, mucosal bacteria were distributed across 3 different phyla, including Firmicutes, Bacteroidetes, and Proteobacteria. At the family level, the luminal bacteria were distributed amongst 5 dominant groups (*Lactobacillaceae, Aerococcaceae, Clostridiaceae, Ruminococcaceae,* and *Prevotellaceae*), whereas mucosal bacteria were distributed across 13 families with varying combinations of individual relative abundances. Luminal populations were represented with fewer bacterial families and higher proportions of these families. This suggests that luminal populations adapt faster to nutrient shifts, and thus function more efficiently without extensive competitive exclusion pressure commonly observed in mucosal populations. Luminal and mucosal

families identified in this study were consistent to those reported by Zhang *et al.* (2018), with the exception of *Aerococcaceae*. *Aerococcaceae* is a family of bacteria that is commonly associated with sediment and marine environments. It was highly represented in only one sample (FM9; 11.87%) suggesting a link to the FM in the diet.

The significant effect of block on relative abundance of *Clostridiaceae* and *Lactobacillaceae* of the mucosal population may have been the result of a moderate disease challenge observed in block 2, as reported by Koepke *et al.* (2017). Thus, mucosal populations within block 1 may be more representative of their dietary treatment than those in block 2. When blocks were analyzed separately, *Lactobacillaceae* was the dominant profile in 44% of the samples (9 of 19) in block 1, whereas *Clostridiaceae* was the most highly represented family in 55% of the samples (10 of 18) in block 2. The inverse relationship observed in this study was at odds with the results described by Levesque *et al.* (2014), whom observed a direct relationship between genera in these two groups. The incongruencies may pertain to intrinsic differences in taxonomic families and genera that may be linked to ecological functions that are species and strain specific. The species included in the *Lactobacillaceae* and *Clostridiaceae* family may occupy the same mucosal niche (Finn *et al.*, 1984, Ward *et al.*, 2006), and potentially share common metabolic activities, such as catabolic substrates (Lam-Himlin *et al.*, 2011), whereas those species included in the genera reported by Levesque *et al.* (2014) may demonstrate various other functions.

Dietary treatments containing FM appeared to favor *Clostridiaceae* over *Lactobacillaceae* in mucosal samples. Finn *et al.* (1984) suggests that *Clostridiaceae* demonstrated a greater relative abundance in the presence of high protein diets, specifically FM. Therefore, animal-based proteins may be the preferred metabolic substrate for *Clostridiaceae*, which would have been a disadvantage in animals fed diets containing higher inclusion levels of plant-based diets. The current study demonstrated preference towards *Lactobacillaceae* over *Clostridiaceae* in MSBM-fed animals. These distinctions between luminal and mucosal population profiles may lead to further investigations on how diet can influence bacteria and how bacteria can conversely influence their host.

Lactobacillus spp. have been reported to promote an environment that competitively excludes pathogenic bacteria. At the mucosal location, a high prevalence of *Lactobacillus spp*. in block 1 samples suggests the formation of a polyculture capable of utilizing available substrate, producing select metabolites counter to pathogenic species proliferation, and occupying available adhesion sites. The *Lactobacillus spp*. identified as prominent OTU included *L. amylovorus* (5 strains; 2 luminal, 3 mucosal), *L. delbrueckii* (4 strains; 2 luminal, 2 mucosal), *L. gasseri* (2 strains; 1 luminal, 1 mucosal), *L. mucosae* (1 strain; mucosal), and *L. reuterii* (1 strain; mucosal). These findings were similar to those reported by Mann *et al.* (2014). Within luminal populations, 2 OTU within the *Lactobacillus* genera, *L. amylovorus* (99%, ID%) and *L. delbrueckii* (99% ID%), contained a relatively high abundance across all treatments (*L. amylovorus*: 32%, 11%,

23%,18% in CON, MSBM$_{+SDP}$, MSBM$_{+FM}$, and MSBM, respectively; *L. delbrueckii*: 31%, 46%, 23%, 37% in CON, MSBM$_{+SDP}$, MSBM$_{+FM}$, and MSBM, respectively). A high abundance of these two carbohydrate-digesting OTU in the lumen can be explained by their monogastric host's limited ability to digest and absorb insoluble carbohydrates and thus providing a metabolic substrate which such bacteria are capable of utilizing.

Within mucosal populations, only 1 OTU from the *Lactobacillus* genera, *L. amylovorus*, contained a relatively high abundance across treatments (15%, 26%, 9%, 17% in CON, MSBM$_{+SDP}$, MSBM$_{+FM}$, and MSBM, respectively) in block 1 data. However, another OTU, *L. reuteri* (99%, ID%) demonstrated a significant ($P = 0.0003$) increase in this OTU relative abundance in MSBM$_{+FM}$ over other treatment groups, but relative abundance within all treatment groups were below 1% relative abundance (0.16%, 0.39%, 0.05%, and 1.64% in CON, MSBM$_{+SDP}$, MSBM$_{+FM}$, and MSBM, respectively), which suggests this difference had minimal effect on the overall population. However, in block 2, a *Clostridia* OTU, closely related to *C. ventriculi*, appeared to have a higher relative abundance across all treatments (24%, 22%, 35%, 15%, 15% in CON, MSBM$_{+SDP}$, MSBM$_{+FM}$, and MSBM, respectively) as opposed to the dominant *Lactobacillus* OTU previously identified in block 1.

The positive correlations demonstrated by *Lactobacillus spp*. suggest the colonization of one probiotic *Lactobacillus* species, specifically *L. amylovorus*, may provide an opportunity for the development of a larger *Lactobacillus* polyculture. The host benefits from a diverse array of species adhering to its mucosal lining and thus lower

relative abundance OTU competing against *Lactobacillus* for space and metabolic substrate could improve antigen sampling diversity and, consequently, host performance (Willing *et al.*, 2010). These polycultures of *Lactobacillus spp.* have the potential to limit the presence of pathogenic species, such as *Clostridia* OTU.

C. ventriculi (formally *Sarcina ventriculi*; 99%, ID%), is a rare anaerobic bacterial species that utilizes carbohydrates as their sole metabolic substrate (Claus and Wilmanns, 1974). This species has also been implicated in the pathogenicity of several animal-based incidences of gastric disturbance, including gastric dilation (Edwards *et al.*, 2008), stomach ulcers (Lam-Himlin *et al.*, 2011), and emphysematous gastritis (Laass *et al.*, 2010). According to Lam-Himlin *et al.* (2011), *C. ventriculi* does not influence direct mucosal damage, but rather capitalizes on the presence of additional gastrointestinal problems that increase an individual's risk of developing complications. Observations of greater than 10% relative abundance of *C. ventriculi*, in both luminal and mucosal populations, suggests that the moderate health challenge demonstrated in block 2 may have been due to an exacerbation of conventional weaning stress by the presence of this organism. Furthermore, this species may explain the lack of histological damage between treatment groups as a result of its opportunistic pathogenicity. The results reported by Lam-Himlin *et al.* (2011) also provide evidence to identify *C. ventriculi* as an opportunistic pathogen within the gut, while explaining the inverse relationship between it and *Lactobacillaceae* in intestinal mucosal profiles.

Other prominent mucosal OTU were *S. alactolticus* (99%, ID%) and *P. copri* (98%, ID%). These OTU demonstrated notable biological differences within relative abundance of one or more treatment groups. *S. alactolticus* increased presence within both CON and $MSBM_{+SDP}$-fed animals (7.68% and 8.89%, respectively) and lack of presence in both $MSBM_{+FM}$ and MSBM-fed animals (0.95% and 2.3%, respectively) suggests a utilization of SDP over FM or MSBM. Finally, *P. copri* (98%, ID%) comprised 4 of 5 OTU belonging to the most abundant Bacteriodetes phyla and *Prevotella* family. This OTU was observed at 7.25% in $MSBM_{+FM}$, whereas all other OTU representing the *Prevotellaceae* family did not surpass a 3% relative abundance. Therefore, the presence of this OTU demonstrates that, even at the species level, bacteria may be preferential to location and dietary ingredient.

3.7 CONCLUSION

In conclusion, complex nursery diets containing increasing levels of MSBM can alter the microbiota; however, variation in bacteria profiles were more apparent based on location than dietary treatment. Luminal and mucosal bacterial populations share many of the same phyla, family, genera, and OTU, but mucosal populations display more species heterogeneity than the luminal populations. Luminal populations may be more responsive to host dietary changes than mucosal populations. Thus, the development of bacterial polycultures suggests a location-specific response to opportunistic pathogens. However, stress and disease may act to disrupt the mucosal polyculture, resulting in pathogenic

species establishing populations on the mucosal border. The maintenance of a polyculture mucosal barrier in early post-weaned pigs is paramount to gut health.

TABLES

TABLE 3.1 INGREDIENT COMPOSITION AND NUTRIENT CONTENT OF CONTROL AND EXPERIMENTAL DIETS FED TO WEANED PIGS, AS FED BASIS[1].

Item	Phase I				Phase II			
	CON	**MSBM$_{+SDP}$**	**MSBM$_{+FM}$**	**MSBM**	**CON**	**MSBM$_{+SDP}$**	**MSBM$_{+FM}$**	**MSBM**
Ingredient (%)								
Corn	42.65	41.07	37.57	34.40	56.40	54.93	51.31	48.02
Soybean meal, dehulled (46.5%)	15.00	15.00	15.00	15.00	22.00	22.00	22.00	22.00
Whey, dried	25.00	25.00	25.00	25.00	10.00	10.00	10.00	10.00
Blood plasma[2]	6.50	6.50	-	-	3.00	3.00	-	-
Select Menhaden fishmeal	7.50	-	7.50	-	5.00	-	5.00	-
MSBM[3]	-	7.50	12.00	20.00	-	5.00	8.00	15.00
Soya oil	1.25	1.45	1.00	3.40	1.00	1.50	1.40	1.80
$_{L}$-Lys-HCl	0.05	0.33	0.04	0.33	0.18	0.37	0.09	0.21
$_{DL}$-Met	0.19	0.19	0.14	0.17	0.17	0.15	0.10	0.10
$_{L}$-Thr	-	0.03	-	0.08	-	0.07	-	-
$_{L}$-Trp	-	0.03	-	0.07	-	0.03	-	0.02
Limestone	0.70	1.20	0.60	1.10	1.00	1.20	0.85	1.20
Salt	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Monocalcium phosphate	0.30	0.85	0.30	1.00	0.60	1.10	0.60	1.00

Vitamin premix[4]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Mineral premix[5]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Zinc oxide	0.40	0.40	0.40	0.40	0.20	0.20	0.20	0.20
Formulated content								
Total Lys %	1.78	1.72	1.76	1.72	1.55	1.51	1.56	1.53
SID Lys %	1.58	1.56	1.55	1.55	1.37	1.36	1.37	1.36
Met + Cys:Lys ratio, %	0.97	0.86	0.91	0.83	0.85	0.75	0.80	0.75
Thr:Lys ratio, %	1.01	0.93	0.98	0.95	0.81	0.80	0.84	0.80
Ile:Lys ratio, %	1.10	1.12	0.97	1.01	0.87	0.89	0.88	0.94
Val:Lys ratio, %	1.15	1.04	1.15	1.03	0.95	0.87	1.00	0.97
Trp:Lys ratio, %	0.33	0.31	0.31	0.32	0.27	0.26	0.27	0.26
Digestible energy (kcal/kg)	3614	3573	3559	3559	3541	3533	3549	3537
Lysine: digestible energy (g/Mcal)	4.91	4.17	3.93	3.15	4.38	3.86	3.72	3.07
Analyzed composition (%)								
Dry matter	91.89	91.70	91.85	92.98	91.13	91.00	90.78	92.03
CP	22.16	23.33	25.80	25.98	23.93	21.28	22.16	25.88
Ash	6.88	6.67	6.24	6.47	6.20	5.67	6.11	6.12
Crude fat	3.73	3.72	4.31	3.64	4.13	3.65	4.06	3.59
Crude fiber	1.42	2.05	2.01	2.64	1.96	2.07	2.51	3.52
Lys	1.88	1.58	1.53	1.45	1.42	1.46	1.80	1.95

[1]Experimental diets were fed in Phase I (d7) and Phase II (d21) post-weaning. All pigs received a common Phase III diet (d35).

[2] Appetein (APC, Ankeny, IA, USA).

[3] MSBM, microbially-enhanced soybean meal (Prairie AquaTech, Brookings, SD, USA).

[4]Provided per kg of complete diet: 11,002 IU vitamin A supplement, 1651 IU vitamin D_3 supplement, 55.1 IU vitamin E supplement, 0.044 mg vitamin B_{12} supplement, 4.4 mg menadione as menadione dimethylpyrimidinol bisulfite, 9.91 mg riboflavin supplement, 60.6 mg $_D$-pantothenic acid as $_D$-calcium, 55.1 mg niacin supplement, 1.1 mg folic acid, 3.3 mg pyridoxine as pyridoxine hydrochloride, 3.3 mg thiamine as thiamine mononitrate and 0.171 mg biotin.
[5]Provided per kg of the complete diet: 165 mg Zn as zinc sulfate, 165 mg Fe as ferrous sulfate, 43.5 mg Mn as manganese sulfate, 16.5 mg Cu as basic copper chloride, 0.36 mg I as ethylenediamine dihydriodide and 0.3 mg of Se as sodium selenite.

Table 3.2 VILLUS HEIGHT (VH) AND CRYPT DEPTH (CD) FROM THE ILEUM OF 21d POST-WEANED PIGS FED MSBM DIETS CONTAINING DIFFERENT PROTEIN SOURCES[1].

Item	CON	MSBM$_{+SDP}$	MSBM$_{+FM}$	MSBM	SEM[2]	*P-value*	Blk 1	Blk 2	SEM[2]	*P-value*
VH	324	262	331	314	31	0.13	329	287	18	0.054
CD	153	166	151	147	26	0.81	170	138	14	0.044

[1]Experimental diets contained corn, SBM, whey (25%), FM (7.5%), and SDP (6.5%) in the CON diet with decreasing inclusion levels of whey (10%), FM (5%), and SDP (3%) within Phase I (d7) and Phase II (d21), respectively. MSBM replaced FM in the MSBM+SDP diet (7.5 and 5.0%), SDP in MSBM+FM diet (12 and 8%), and both FM and SDP in MSBM diet (20 and 15%) in Phase 1 and Phase II, respectively. Block 2, n=19; treatment sample size: CON, n=3; MSBM$_{+SDP}$, n=5; MSBM$_{+FM}$, n=4; MSBM, n=5. Blk 1 (n=120) and Blk 2 (n=119) contained animals divided into 5 pens/treatment.
[2] Reported SEM was the largest among measurements.

TABLE 3.3 PEARSON CORRELATIONS[1] OF THE MAIN OPERATIONAL TAXONOMIC UNITS IN THE ILEAL LUMENAL AND MUCOSAL BACTERIA POPULATIONS AND VILLUS HEIGHT (VH) AND CRYPT DEPTH (CD) OBTAINED FROM 21D POST-WEAN PIGS FED MSBM DIETS CONTAINING DIFFERENT PROTEIN SOURCES[2].

Correlations		**r[1]**	***P-Value***
Luminal[3]			
Prevotella23	Campylobacter7	1.000	<.0001
Mucosal[4]			
Actinobacillus3	Actinobacillus2	0.992	<.0001
Actinobacillus4	Actinobacillus3	0.995	<.0001
Actinobacillus4	Actinobacillus2	0.986	<.0001
Actinobacillus5	Actinobacillus2	0.991	<.0001
Actinobacillus5	Actinobacillus3	0.999	<.0001
Actinobacillus5	Actinobacillus4	0.994	<.0001
Clostridiumsso26	Actinobacillus2	0.786	<.0001
Clostridiumsso26	Actinobacillus3	0.794	<.0001
Clostridiumsso26	Actinobacillus4	0.784	<.0001
Clostridiumsso26	Actinobacillus5	0.796	<.0001
*Lactobacillus*40	*Lactobacillus*39	0.696	<.0001
*Lactobacillus*41	*Lactobacillus*39	0.587	0.0001
Prevotella93	*Lactobacillus*51	0.978	<.0001
Prevotella96	*Lactobacillus*51	0.912	<.0001
Prevotella74	Prevotella72	0.962	<.0001
Prevotella96	Prevotella93	0.937	<.0001
Terrisporobacter132	Romboutsia105	0.737	0.0002
Prevotella81	VH	0.580	<.0001
CD	VH	0.587	0.0001

[1] Only correlations with significant *P-values* were reported.

[2] Experimental diets contained corn, SBM, whey (25%), FM (7.5%), and SDP (6.5%) in the CON diet with decreasing inclusion levels of whey (10%), FM (5%), and SDP (3%) within Phase I (d7) and Phase II (d21), respectively. MSBM replaced FM in the $MSBM_{+SDP}$ diet (7.5 and 5.0%), SDP in $MSBM_{+FM}$ diet (12 and 8%), and both FM and SDP in MSBM diet (20 and 15%) in Phase 1 and Phase II, respectively. Block 2, n=19; treatment sample size: CON, n=3; $MSBM_{+SDP}$, n=5; $MSBM_{+FM}$, n=4; MSBM, n=5

[3] Luminal correlations reported as statistically significant where $P \leq 0.0006$ and tendency where $P \leq 0.0013$.

[4] Mucosal correlations reported as statistically significant where $P \leq 0.000$ and tendency where $P \leq 0.00026$.

TABLE 3.4 MEAN RELATIVE ABUNDANCE (%) OF THE MAIN PHYLUM AND FAMILY FROM THE ILEAL LUMEN OF 21D POST-WEANED PIGS FED MSBM DIETS CONTAINING DIFFERENT PROTEIN SOURCES[1].

Luminal Taxonomy[4]	CON	MSBM$_{+SDP}$	MSBM$_{+FM}$	MSBM	SEM[2]	*P-value*[3]
Firmicutes	97	96	74	96	23	0.42
Aerococcaceae	0.05	0.11	12	0.06	12	0.39
Clostridiaceae	3.0	8.9	12	9.2	6.5	0.50
Lactobacillaceae	93	77	49	85	24	0.13
Ruminococcaceae	0.83	8.2	0.67	0.65	7.1	0.49
Bacteroidetes	2.0	4.0	25	2.0	24	0.42
Prevotellaceae	2.0	4.0	25	2.0	24	0.42

[1] Experimental diets contained corn, SBM, whey (25%), FM (7.5%), and SDP (6.5%) in the CON diet with decreasing inclusion levels of whey (10%), FM (5%), and SDP (3%) within Phase I (d7) and Phase II (d21), respectively. MSBM replaced FM in the MSBM+SDP diet (7.5 and 5.0%), SDP in MSBM+FM diet (12 and 8%), and both FM and SDP in MSBM diet (20 and 15%) in Phase 1 and Phase II, respectively. Block 2, n=19; treatment sample size: CON, n=3; MSBM$_{+SDP}$, n=5; MSBM$_{+FM}$, n=4; MSBM, n=5.

[2] Reported SEM was the largest among measurements.

[3] No effect of blk*trt was observed.

TABLE 3.5 MEAN RELATIVE ABUNDANCE (%) OF THE MAIN PHYLA AND FAMILY FROM THE ILEAL LUMEN OF 21D POST-WEANED PIGS FED MSBM DIETS CONTAINING DIFFERENT PROTEIN SOURCES[1].

Luminal Taxonomy[4]	OTU[5]	CON	MSBM$_{+SDP}$	MSBM$_{+FM}$	MSBM	SEM[2]	*P-value*[3]	Closest Valid Taxon (ID%[6])
Firmicutes		97	96	74	96	23	0.42	
Lactobacillaceae	13	32	11	23	18	29	0.87	*L. amylovorus (99%)*
	15	31	46	23	37	28	0.85	*L. delbrueckii (99%)*
	16	0.59	0.69	0.88	4.4	2.9	0.36	*L. gasseri (99%)*
	17	0.79	8.1	0.58	0.20	7.1	0.48	*L. amylovorus (96%)*
	18	0.35	1.5	1.8	0.62	1.4	0.59	*L. delbrueckii (94%)*
Pasteurellaceae	2	1.81	5.1	0.06	7.2	4.7	0.55	*A. minor (97%)*
Campylobacteraceae	7	0.03	0.04	4.3	0.05	4.3	0.38	*C. lanienae (98%)*
Clostridiaceae	8	1.87	2.2	24	1.6	24	0.43	*C. beijerinckii (96%)*
	9	26	13	0.70	13	26	0.67	*S. ventriculi (98%)*
Peptostreptococcaceae	32	2.28	0.84	0.09	8.9	8.7	0.59	*T. mayombei (98%)*
Bacteroidetes		2.32	3.7	25	2.0	23	0.42	
Prevotellaceae	23	0.05	0.10	12	0.06	12	0.38	*P. copri (99%)*

[1] Experimental diets contained corn, SBM, whey (25%), FM (7.5%), and SDP (6.5%) in the CON diet with decreasing inclusion levels of whey (10%), FM (5%), and SDP (3%) within Phase I (d7) and Phase II (d21), respectively. MSBM replaced FM in the MSBM+SDP diet (7.5 and 5.0%), SDP in MSBM+FM diet (12 and 8%), and both FM and SDP in MSBM diet (20 and 15%) in Phase 1 and Phase II, respectively. Block 1 n=18, block 2 n=19; treatment sample size: CON, n=10; MSBM$_{+SDP}$, n=9; MSBM$_{+FM}$, n=9; MSBM, n=9.

[2] Reported SEM was the largest among measurements.

[3] No effect of blk*trt was observed.

[4] As determined by RDP classifier at an 80% bootstrap cutoff.

[5] OTU representing a sample mean relative abundance >1% in at least one treatment group and/or taxonomic groups.

[6] Nucleotide sequence identity as determined by BLAST (Altschul *et al.*, 1997).

TABLE 3.6 MEAN RELATIVE ABUNDANCE (%) OF THE MAIN PHYLA AND FAMILY FROM THE ILEAL MUCOSA OF 21D POST-WEANED PIGS FED MSBM DIETS CONTAINING DIFFERENT PROTEIN SOUCES[1].

Mucosal Taxonomy[4]	CON	MSBM$_{+SDP}$	MSBM$_{+FM}$	MSBM	SEM[2]	*P-value*[3]
Firmicutes	67	85	81	70	11	0.55
Clostridiaceae	29	27	40	19	15	0.52¥
Lactobacillaceae	18	37	27	33	13	0.57¥
Streptococcaceae	9.7	12	1.1	1.8	7.5	0.40
Ruminococcaceae	0.43	0.59	11	0.71	11	0.42
Peptostreptococcaceae	7.5	5.0	1.2	7.7	4.5	0.43
Veillonellaceae	0.27	0.17	0.47	5.9	4.9	0.27
Proteobacteria	23	8.0	0.51	20	9.7	0.11
Pasteurellaceae	7.0	0.51	0.45	0.31	6.8	0.49
Rhodobacteraceae	0.14	0.4	0.00	5.42	5.6	0.42
Helicobacteraceae	11	2.8	0.26	0.63	2.9	0.30
Campylobacteraceae	0.27	1.5	0.00	6.9	7.1	0.47
Enterobacteriaceae	0.23	0.80	0.00	5.8	3.9	0.12
Bacteroidetes	8.0	5.0	19	4.1	9.7	0.31
Prevotellaceae	7.5	4.7	18	3.7	10	0.30
Cyanobacteria	0.27	0	0	4.3	4.4	0.43
Chloroplast	0.20	0.11	0	4.3	4.4	0.43

[1] Experimental diets contained corn, SBM, whey (25%), FM (7.5%), and SDP (6.5%) in the CON diet with decreasing inclusion levels of whey (10%), FM (5%), and SDP (3%) within Phase I (d7) and Phase II (d21), respectively. MSBM replaced FM in the MSBM+SDP diet (7.5 and 5.0%), SDP in MSBM+FM diet (12 and 8%), and both FM and SDP in MSBM diet (20 and 15%) in Phase 1 and Phase II, respectively. Block 1 n=18, block 2 n=19; treatment sample size: CON, n=10; $MSBM_{+SDP}$, n=9; $MSBM_{+FM}$, n=9; MSBM, n=9.
[2] Reported SEM was the largest among measurements.
[3] No statistical differences between treatment and luminal taxonomic family-level taxa. No effect of blk*trt was observed.
[4] As determined by RDP classifier at an 80% bootstrap cutoff.
[¥] Interaction by block occurred in 2 family-level taxa: *Clostridiaceae* (μ_{B1}= 9.85± 7.29, μ_{B2}=47.68±8.70, P=0.0004), *Lactobacillaceae* (μ_{B1}= 40.93±7.69, μ_{B2}=16.23±7.36, P=0.02).

TABLE 3.7 MEAN RELATIVE ABUNDANCE (%) OF THE MAIN TAXONOMIC GROUPS FROM THE ILEAL MUCOSA OF 21D POST-WEANED PIGS FED MSBM DIETS CONTAINING DIFFERENT PROTEIN SOURCES[1].

Mucosal Taxonomy[2]	OTU[3]	CON	MSBM$_{+SDP}$	MSBM$_{+FM}$	MSBM	SEM[4]	*P-value*[5]	Closest Valid Taxon (ID%)[6]
Firmicutes		67	85	81	70	11	0.55	
Clostridiaceae	20	24	22	35	15	13	0.43¥	*C. ventriculi (99%)*
	26	1.3	0.65	0.60	1.3	1.1	0.87	*C. saudiense (97%)*
Ruminococcaceae	21	0.01	0.04	9.8	0.08	11	0.49	*C. merdae (92%)*
Lactobacillaceae	39	15	26	9.1	17	10	0.41¥	*L. amylovorus (99%)*
	40	0.61	2.89	0.56	2.4	1.6	0.37	*L. amylovorus (99%)*
	41	0.33	1.49	0.18	1.4	0.89	0.26	*L. amylovorus (99%)*
	45	0.27	0.24	2.1	0.03	2.4	0.57	*L. delbrueckii (99%)*
	51	0.07	0.49	1.6	0.01	1.6	0.44	*L. delbrueckii (95%)*
	43	0.27	1.84	3.7	7.7	5.4	0.40	*L. gasseri (99%)*
	44	0.39	0.55	4.9	0.7	4.9	0.49	*L. mucosae (100%)*
	48	0.16[a]	0.39[a,b]	0.05[a]	1.6[b]	0.59	0.0003¥	*L. reuteri (99%)*[5]
Streptococcaceae	117	7.7	8.9	0.95	2.3	6.3	0.48	*S. alactolyticus (99%)*
Peptostreptococcaceae	132	1.7	1.4	0.57	2.5	1.8	0.71	*T. mayombei (98%)*
Proteobacteria		23	8.0	0.51	20	9.7	0.11	
Pasteurellaceae	2	2.3	0.38	0.04	0.09	2.3	0.51	*A. porcinus (98%)*
	3	2.0	0.09	0.01	0	2.0	0.46	*A. porcinus (97%)*
	4	1.4	0.20	0.07	0	1.4	0.51	*A. minor (97%)*
	5	1.0	0.01	0	0	1.0	0.45	*A. minor (97%)*
Campylobacteraceae	16	0.26	1.4	0.04	5.7	6.3	0.57	*C. lanienae (98%)*
Enterobacteriaceae	33	0.16	0.73	0.04	5.4	3.8	0.15	*S. sonnei (100%)*
Helicobacteraceae	36	11	2.7	0.07	0.49	7.5	0.33	*H. pullorum (98%)*
Rhodobacteraceae	105	4.9	2.2	0.62	4.6	2.7	0.38	*R. timonensis (99%)*

Bacteroidetes		8.0	5.0	19	4.1	10	0.31	
Prevotellaceae	81	1.1	0	0	0.7	1.1	0.62	*P. ruminicola (95%)*
	74	0.07	0.13	2.7	0.4	1.7	0.13	*P. copri (97%)*
	72	1.5	1.7	7.3	0.9	4.7	0.25	*P. copri (98%)*
	93	0	0	1.5	0	1.6	0.46	*P. copri (98%)*
	96	0.01	0.66	1.4	0.04	1.6	0.60	*P. copri (96%)*

[1] Experimental diets contained corn, SBM, whey (25%), FM (7.5%), and SDP (6.5%) in the CON diet with decreasing inclusion levels of whey (10%), FM (5%), and SDP (3%) within Phase I (d7) and Phase II (d21), respectively. MSBM replaced FM in the $MSBM_{+SDP}$ diet (7.5 and 5.0%), SDP in $MSBM_{+FM}$ diet (12 and 8%), and both FM and SDP in MSBM diet (20 and 15%) in Phase 1 and Phase II, respectively. Block 1 n=18, block 2 n=19; treatment sample size: CON, n=10; $MSBM_{+SDP}$, n=9; $MSBM_{+FM}$, n=9; MSBM, n=9.

[2] As determined by RDP classifier at an 80% bootstrap cutoff.

[3] OTU representing a sample mean relative abundance >1% in at least one treatment group and/or taxonomic

[4] Reported SEM was the largest among measurements.

[5] No effect of blk*trt was observed, except in *Lactobacillus48* ($\mu_{Trt*Blk}$= 0.56% ± 0.21%).

[6] Nucleotide sequence identity as determined by BLAST (Altschul *et al.*, 1997).

[a,b] Different superscripts within a row represent differences between lsmeans $P < 0.05$.[¥] Effect of block occurred in 3 OTU: *clostridia20* (μ_{B1}= 7.82± 4.27, μ_{B2}=40.43±7.40, *P*=0.001), *lactobacillus39* (μ_{B1}= 24.43±5.28, μ_{B2}=8.56±3.85, *P*=0.03), *lactobacillus48* (μ_{B1}= 0.10±0.04, μ_{B2}=1.02±0.38, *P*=0.0008).

FIGURES

FIGURE 3.1 BACTERIAL COMPOSITION PROFILES OF BACTERIA FOUND IN ILEAL LUMINAL SAMPLES

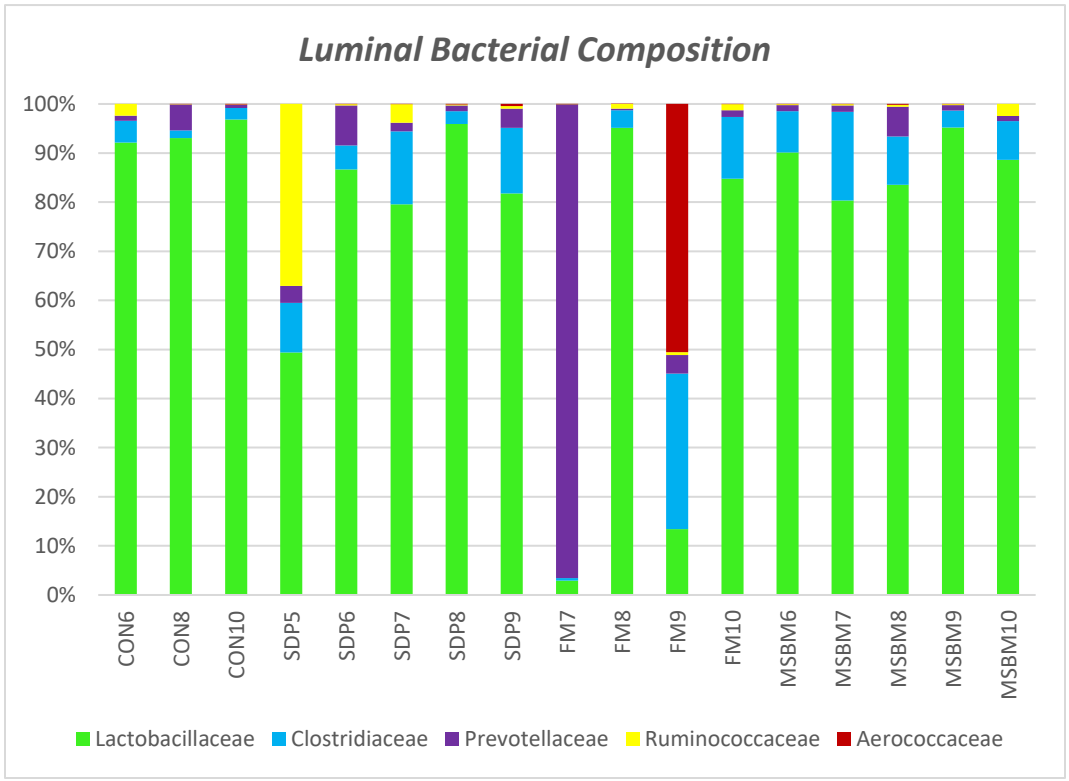
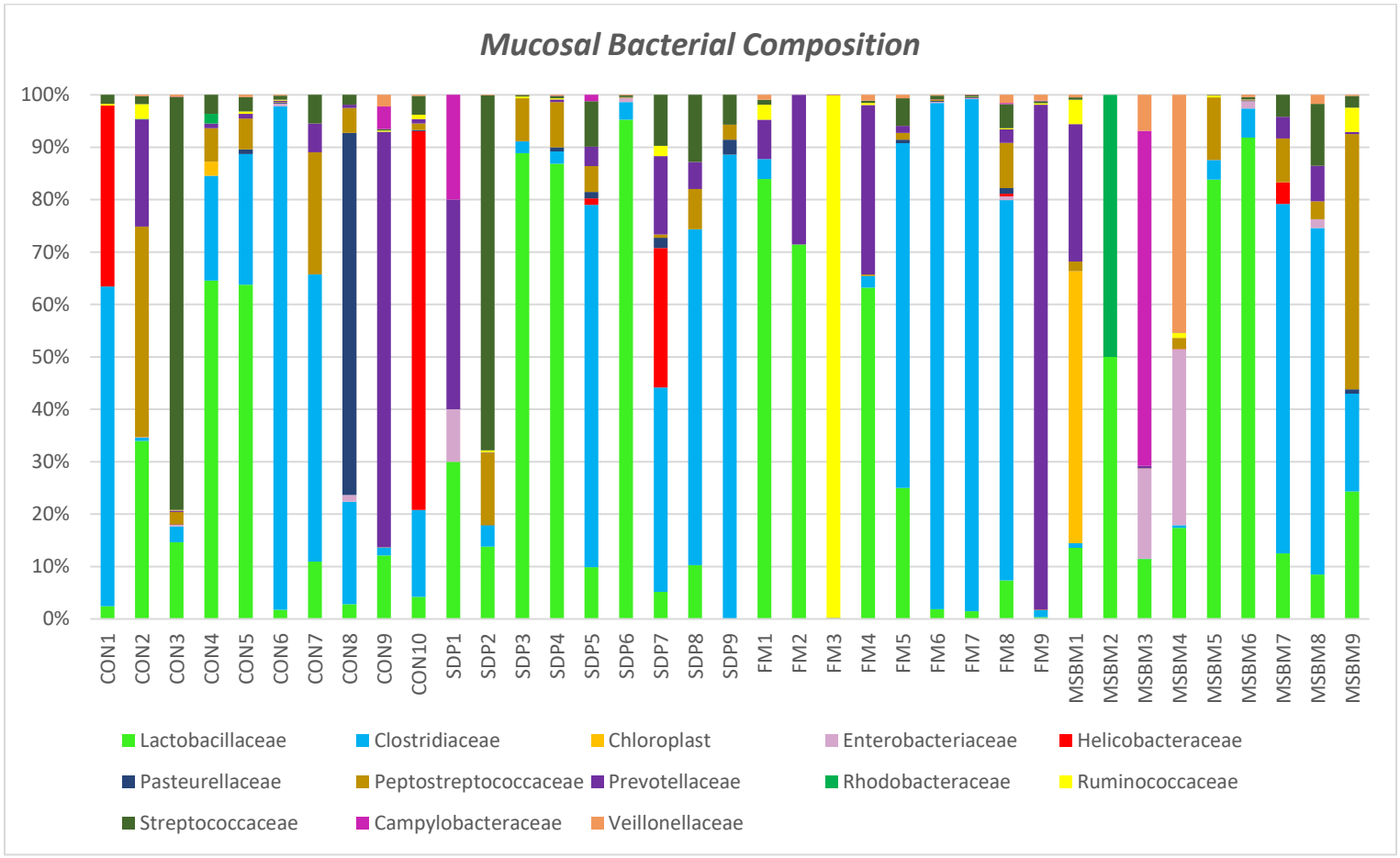


FIGURE 3.2 BACTERIAL COMPOSITION PROFILES OF BACTERIA FOUND IN ILEAL MUCOSAL SAMPLES



3.9.1 Captions

Figure 1 Relative abundance of bacterial families found in ileal luminal samples isolated from 21d post-weaned pigs fed diets containing corn, SBM, whey (25%), FM (7.5%), and SDP (6.5%) in the CON diet with decreasing inclusion levels of corn, SBM, whey (10%), FM (5%), and SDP (3%) within Phase I (d7) and Phase II (d21), respectively. MSBM replaced FM in the MSBM+SDP diet (7.5 and 5.0%), SDP in MSBM+FM diet (12 and 8%), and both FM and SDP in MSBM diet (20 and 15%) in Phase 1 and Phase II, respectively. Bacterial composition was determined by 16S rRNA gene pyrosequencing.

Figure 2 Bacterial family composition profiles of bacteria found in ileal mucosal samples isolated from 21d post-weaned pigs fed diets containing corn, SBM, whey (25%), FM (7.5%), and SDP (6.5%) in the CON diet with decreasing inclusion levels of corn, SBM, whey (10%), FM (5%), and SDP (3%) within Phase I (d7) and Phase II (d21), respectively. MSBM replaced FM in the MSBM+SDP diet (7.5 and 5.0%), SDP in MSBM+FM diet (12 and 8%), and both FM and SDP in MSBM diet (20 and 15%) in Phase 1 and Phase II, respectively. Bacterial composition was determined by 16S rRNA gene pyrosequencing.

Chapter 4

4.0 FINAL DISCUSSION

The primary focus of this thesis was to assess the effects of specialty protein sources on nursery pig microbiota at 21d post-weaning to identify a potential mode of action associated with gut health. Trials were conducted under the parameters of 1) simple diets (without complex high-quality ingredients, such as spray dried plasma) that investigated the ileal luminal bacterial composition and 2) complex diets (with the inclusion of specialty ingredients, such as whey, spray-dried animal plasma, FM) that investigated the ileal luminal and mucosal bacterial compositions. We hypothesized that the bacterial populations would reflect the growth performance reported by (Sinn *et al.*, 2016) and (Koepke *et al.*, 2017) in each trial; furthermore, it was hypothesized that bacterial composition, and its influence on gut health, was dependent on ileal location-based ecosystems (i.e. lumen or mucosa).

The influence of diet on the microbiota has been demonstrated to influence luminal populations in both simple and complex diets. The purpose of investigating simple diets containing only a primary protein source is to isolate the ingredient's effect on bacterial composition, as opposed to complex diets with multiple protein sources capable of providing substrate to non-test ingredients. Although no effect of treatment was detected on performance in either trial, (Sinn *et al.*, 2016) reported increased diarrhea incidence in simple CON diet. Thus, at a phenotypic performance level all treatment groups, except the simple NEG diet, are equal, but based on bacterial composition, it is evident that varying levels of subclinical gastrointestinal disturbance are occurring. If

data from simple diets adequately represents the effect of ingredient on bacterial populations, high prevalence of *Lactobacillaceae* may act as an indicator of gut health. Animals fed simple NEG, FM, and MSBM diets seem to demonstrate a progressively higher percentage of individuals with bacterial populations prevalent in *Lactobacillaceae* (63%, 77%, and 50%, respectively). These results were repeated in complex luminal sample diets as the positive CON, $MSBM_{+FM}$, and MSBM alone all demonstrated animals with high prevalence of *Lactobacillaceae* (100%, 66%, and 100%, respectively), which in complex diets aligns well with performance data.

A decreased in the number of animals containing a high prevalence of *Lactobacillaceae* in their bacterial profiles suggest that the test ingredient is not providing a substrate optimally utilized by *Lactobacillaceae*. Thus, data suggests that the presence of a dominant *Lactobacillaceae* profile has the potential to drastically reduce pathogenic populations. For instance, in simple diets that contained high levels of conventional SBM, 37% of samples contained bacterial populations with predominant families other than *Lactobacillaceae* and demonstrated increased diarrhea as compared to other treatment groups. Furthermore, in animals fed complex MSBM diets a high prevalence of *Lactobacillaceae* was observed in all samples. A recommendation for a reduction and/or removal of conventional soybean meal in nursery diets is strongly suggested to reduce subclinical gastrointestinal disruption until metabolic maturity has been reached in these young animals.

The high occurrence of *Lactobacillaceae* in both luminal and mucosal samples can be attributed to the high levels of carbohydrates included in dietary treatment groups, in the form of corn, SBM, and/or MSBM, from both simple and complex diets. Non-starch polysaccharides, mono-, di-, and oligosaccharides all comprise large structural components of SBM and other plant-based ingredients (Choct, 1997). SBM, and/or MSBM, was included in all treatment diets, suggesting that the high *Lactobacillaceae* in a majority of luminal samples across treatments in both simple and complex diets is due to high inclusion of plant-based ingredients. However, many bacteria colonizing the non-ruminant gastrointestinal tract can utilize carbohydrates as their metabolic substrate, thus a high prevalence of *Lactobacillaceae* suggests it has substrate priority over other taxonomic groups (Pudlo *et al.*, 2015). Furthermore, some have suggested that the presence of *Lactobacillaceae* may alter and/or mandate the substrate utilization of bacteria sharing the same ecological niche.

Bacteria that proliferate in the absence of *Lactobacillaceae* can utilize alternate substrates. For example, certain bacteria known to be carbohydrate fermenters can also use amino acids. These flexible amino acid producing bacteria are commonly associated with gastrointestinal disruption (Pudlo *et al.*, 2015). The presence of abundant *Lactobacillaceae spp*. may reduce these family-level taxa until they return to utilizing carbohydrates. This suggested mechanism explains the observed variation in some of the FM-fed animals, whose bacterial profiles were comprised of high *Prevotellaceae, Ruminococcaceae, Pasteurellaceae, Clostridiaceae,* and/or *Campylobacteraceae* in some

samples; while others, on the same treatment, demonstrated prevalence of *Lactobacillaceae*. This trend was evident in both luminal (P2, P3, FM7, and FM9) and mucosal (FM3 and FM5-FM9) samples.

Continued investigations could proceed in a variety of ways to identify consistent presence of prominent bacteria and identify functional correlations. An investigation into primarily metabolic substrates utilized by the prominent OTU identified could be conducted via culturing techniques and/or function via metagenomic analysis. Relatively new technology exists in the form of "Multifunction Bioreactor Systems" designed for intestinal modeling of the mammalian gut (Zhou *et al.*, 2018). This technology has the potential to replicate the select facets of the intestinal tract to investigate bacterial ecosystems in response to select nutrients and/or stress environments. A trial utilizing this technology may help to target more of an ecological understanding of interspecies and host relationships.

In conclusion, the ileal microbiota is highly reactive to changes in dietary composition; however, predicting these changes may be more complex (i.e. altering substrate utilization, niche autoregulation, etc.) than previously anticipated. Dietary impact on luminal populations has been confidently established; however, mucosal profiles are comprised of a highly interconnective network that is predominantly resistant to dietary changes. Mucosal populations may be a good reflection of gut health, but sampling and processing techniques are still not practical and/or efficient. Furthermore, based on luminal and mucosal results from trial 2, luminal samples are unable to predict

mucosal bacterial profiles even at the taxonomic family level. Finally, the data from these studies suggest that plant-based diets that are low in conventional soybean meal and FM, will encourage the development of a strong *Lactobacillaceae* population in luminal and mucosal populations as an indicator of gut health.

6.0 SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE S1 *COMPOSITION AND NUTRIENT CONTENT OF EXPERIMENTAL DIETS (AS-FED BASIS; REPLICATED FROM SINN ET AL.* (2016)).

	Phase I[1]			Phase II[1]			Phase III[1]
Item	NEG	POS	MSBM	NEG	POS	MSBM	
Ingredient (%)							
Corn	33.60	39.8	38.3	44.8	50.6	49.6	60.8
Soybean meal	37.30	25.0	25.0	40.7	30.7	30.7	34.2
Fish meal	-	7.49	-	-	4.99	-	-
MSBM	-	-	7.48	-	-	4.99	-
Whey powder	25.00	25.0	25.0	9.98	9.98	9.98	-
Soybean oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Calcium phosphate	1.22	0.35	1.25	1.55	0.95	1.55	1.60
Limestone	0.85	0.47	0.87	0.92	0.70	0.95	1.05
Sodium chloride	0.30	0.30	0.30	0.35	0.35	0.35	0.35
L-Lysine-HCL	0.11	0.05	0.21	0.14	0.15	0.25	0.30
DL-Methionine	0.14	0.11	0.07	0.13	0.12	0.10	0.14
L-Threonine	0.04	0.04	0.03	0.06	0.08	0.07	0.13
L-Tryptophan	-	0.04	0.02	-	0.03	0.02	-
L-Valine	-	-	0.04	-	-	0.04	-
Trace mineral premix[2]	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin premix[3]	0.05	0.05	0.05	0.05	0.05	0.05	0.25
Titanium dioxide	0.20	0.20	0.20	0.20	0.20	0.20	-
Analyzed composition (%)							
Dry matter	90.90	91.4	90.8	89.9	89.9	89.8	89.9
CP	24.30	23.9	23.7	23.9	23.0	22.5	22.7
Ash	6.71	6.70	6.80	6.56	6.07	6.32	5.76
Crude fat	2.44	2.95	1.41	1.97	2.64	1.79	2.43
Crude fiber	1.71	1.65	1.83	2.61	2.59	2.70	3.21
Lysine	1.60	1.48	1.53	1.51	1.57	1.50	1.44
Formulated content							
Metabolizable energy (MJ/kg)	13.90	14.2	14.3	13.8	14.0	14.1	13.8

Lysine:metabolizable energy (g/MJ)	0.97	0.95	0.94	0.98	0.96	0.96	0.90

[1]Experimental diets were fed from d 0 to 7 (Phase I) and from d 8 to 21 (Phase II) post-weaning. All pigs received a common Phase III diet d 22 to 35. A mixture of acids were used for dietary acidifier (KEMIN, Des Moines, Iowa, USA), which was included at 0.2% in NEG, POS, and MSBM diets to create NEGA, POSA, and MSBMA diets in Phase I and II.

[2]Provided per kg of the complete diet: 165 mg Zn as zinc sulfate, 165 mg Fe as ferrous sulfate, 43.5 mg Mn as manganese sulfate, 16.5 mg Cu as basic copper chloride, 0.36 mg I as ethylenediamine dihydroiodide, and 0.3 mg of Se as sodium selenite.

[3]Provided per kg of the complete diet: 11,002 IU vitamin A supplement, 1,651 IU vitamin D_3 supplement, 55.1 IU vitamin E supplement, 0.044 mg vitamin B_{12} supplement, 4.4 mg menadione as menadione dimethylpyrimidinol bisulfite, 9.91 mg riboflavin supplement, 60.6 mg D-pantothenic acid as D-calcium pantothenate, 55.1 mg niacin supplement, 1.1 mg folic acid, 3.3 mg pyridoxine as pyridoxine hydrochloride, 3.3 mg thiamine as thiamine mononitrate, and 0.171 mg biotin.

SUPPLEMENTAL TABLE S2 *NUTRIENT CONTENT OF THE MICROBIALLY-ENHANCED SOYBEAN MEAL (MSBM) AND MENHADEN FISHMEAL (FM), (AS-FED BASIS; REPLICATED FROM* SINN *ET AL.* (2016)).

	Ingredient	
Item	MSBM	Fishmeal
Gross energy (kcal/kg)	4,688	4,577
Dry matter (%)	95.80	92.90
CP (%)	58.40	63.20
Ash (%)	7.60	20.90
Crude fat (%)	0	9.22
Crude fiber (%)	5.74	0.39
Indispensable amino acids (%)		
Arginine	3.95	3.91
Histidine	1.53	1.53
Isoleucine	2.85	2.62
Leucine	4.74	4.56
Lysine	3.68	5.13
Methionine	0.83	1.79
Phenylalanine	2.95	2.35
Threonine	2.36	2.67
Valine	2.95	3.00
Dispensable amino acids (%)		
Alanine	2.63	4.01
Aspartate	6.57	5.80
Cysteine	0.85	0.52
Glutamate	9.89	8.66
Glycine	2.57	4.56
Proline	3.04	3.04
Serine	2.68	2.47
Tyrosine	2.06	1.99
Total amino acids	56.10	58.60

[1]Ingredients provided by Prairie AquaTech (Brookings, South Dakota, USA).

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