MATERNAL VERSUS ENVIRONMENTAL CONTRIBUTIONS TO THE PIGLET PIONEER MICROBIOME

A Thesis

by

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ABSTRACT

The pioneer microbiome is the initial colonization of microbiological organisms that has lifelong implications for animal health and performance. The objective was to quantify maternal and environmental contributors to the piglet's pioneer microbiome. Piglets born from five gilts were individually identified, weighed, and selected for microbiome analysis. Environmental samples were collected from the farrowing crate prior to gilt introduction (Empty Crate) and after gilts were moved in (Full Crate). Maternal samples were collected from the birth canal during farrowing (Birth Canal) and colostrum was collected from each gilt during farrowing (Colostrum). The piglet's rectum was swabbed on days 0 (pre-suckle), 3, and 10 post-farrowing and at weaning (21.6 ± 1.0 days post-farrowing). Swabs and colostrum were stored in sterile tubes at -80°C until sequencing. Bacterial DNA extraction and genome sequencing targeted the V4 hypervariable region of the 16S rRNA gene. Statistical analyses were conducted using PROC GLM and PROC REG in SAS 9.4. Maternal and environmental sources did not differ for the phyla Firmicutes and Proteobacteria. Yet the piglet microbiome shifted from birth to weaning. The relative abundance of phylum Firmicutes was lower on day 3 compared to day 0 and at weaning but did not differ from day 10. Within the phylum Firmicutes, *Lactobacillus* and *Clostridium* genera were greater on day 3 compared to day 0, 10, and at weaning. The relative abundance of phylum Proteobacteria, and the relative abundance of genus *Escherichia* within this phylum, were greater on day 3 compared to day 0, 10, and at weaning. Multiple regression analyses indicated that Birth Canal

explained 51.6% of the variation observed in piglet day 0 microbiome and 6.5% of the variation in the piglet day 10 microbiome. The piglet day 0 microbiome explained 10.0% of the variation observed in day 3 microbiome and 15.6% of the day 10 microbiome. Finally, day 10 microbiome explained 58.6% of the variation observed in the piglet microbiome at weaning. The microbiome of Colostrum and the farrowing crate did not impact piglet microbiome. Results indicate the piglet pioneer microbiome is largely influenced by the microbiome of the birth canal and may be largely established by 10 days of age.

DEDICATION

I dedicate this thesis to my mother, Stacey Slovacek.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1. Introduction

Pork production is a major revenue source within the agricultural industry. In 2021, it generated $28.02 billion of gross cash receipts from hog sales and was estimated to support $57.20 billion of gross national product (Cook and Schulz, 2022). However, various swine health challenges may reduce producer profit potential by decreasing pig growth rates and efficiency and increasing morbidity and mortality. These impacts have been estimated to account for economic losses of $8.49 to $29.82 per pig marketed (Cornelison et al., 2018).

Modern commercial swine production practices have adapted to mitigate these risks. Raising pigs in climate controlled indoor facilities with proper ventilation, implementing strong biosecurity protocols, and utilizing specialized farm sites to cater to pig requirements at different phases of production promote proper pig performance, efficiency, and health (Maes et al., 2020). Despite these improvements, incidences of swine disease continue to trouble pork producers.

Perhaps most notably, the newborn piglet is at significant risk of disease challenges. The newborn piglet has an immature immune system, and bacterial infections resulting in diarrhea being one of the most common disease challenges on the breed-to-wean or farrow-to-finish swine farm (Konstantinov et al., 2006). Pre-weaning diarrhea resulting from disturbances in gastrointestinal microbial populations slows piglet growth and development, and if untreated, reduces litter survivability to weaning.

With clear economic and welfare implications of pig health in mind, the intent of this literature review is to understand the piglet gastrointestinal microbiome and its impact on swine performance and health in order to improve pork production systems and efficiency.

1.2. Microbiome Definition and Analysis

To begin, it is important to define "microbiome" and distinguish it from other commonly used terms. The "microbiota" is the microbial taxa associated with a host, whereas the "microbiome" is the compilation of microorganisms as well as their genomes and extrachromosomal elements located both inside and on the surface of a host (Ursell et al., 2012; Dominguez-Bello et al., 2019). These terms are nearly synonymous, yet the presence of and ability to precisely quantify the genome of the microbiome makes the distinction important.

Indeed, 16S rRNA gene sequencing is a relatively recent discovered practice that has become more popular to utilize in microbiome studies and more financially feasible. The 16S rRNA gene codes for the RNA component of the 30S subunit of a prokaryotic ribosome and is made up of ~1550 base pairs and nine hypervariable regions (V1 to V9, Clarridge, 2004), with V4 being the most commonly studied region. Gene sequencing methods target a small portion of the microbial DNA within the 16S rRNA gene, which allows for phylogeny determination and species divergence (Duchene et al., 2016). One or more hypervariable regions are amplified using broad-range primers that bind to a region and are sequenced, and the information gathered from the sequencing allows for the taxonomic composition and diversity to be reconstructed (Weinroth et al., 2022). The method essentially serves as a survey of which microbes are present and at what abundance and allows for diversity estimates and comparisons between samples. Additionally, this sequencing technology has vastly expanded knowledge about microbial species and the microbiome. Prior to now, most bacterial identification was completed using

culture-based methods, which limit the scope of microbial identification to only 2% of the microbial population (Amann et al., 1995). Due to this, most bacterial identified through culture methods were thought to be pathogenic. Limitations to 16S rRNA gene sequencing do exist. It cannot account for the activity or metabolic potential of a microbial community, may not differentiate between individual bacterial species or strains, or reflect a total microbial load (Weinroth et al., 2022).

There are several biological sites that have their own microbiome, such as the skin, nasal cavity, mouth, reproductive tract, and the gastrointestinal tract (GIT). Among these, the GIT microbiome has historically been the focus of the greatest research efforts. Indeed, studies determining the relationship between a host and the microbiota that inhabit it date back to the 1960's (Knecht et al., 2020). Until recently, these studies primarily focused on human health. The primary focus of this literature review will be the piglet GIT microbiome, yet there will be some mention of human and mice microbiomes where relevant, as the greatest emphasis in the scientific literature has historically been in these species.

1.3. The Pig Gastrointestinal Microbiome and Effects on Performance

The GIT is a versatile organ system that not only regulates nutrient and water absorption, but also serves as a barrier to keep harmful substances such as toxins and pathogens out of the body. The GIT also contains the greatest number and diversity of microorganisms of all body systems (Patil et al., 2020). The GIT microbiome is comprised of a substantial population of bacteria, archaea, fungi, and viruses (Borody and Khoruts, 2012). A normal, healthy GIT microbiome is crucial, as it influences countless body processes in gut health and function that positively relate to animal performance and feed efficiency. The three main duties of the GIT microbiota are protective, metabolic, and trophic roles (Guarner and Malagelada, 2003). More

specifically, it is involved in energy harvest, nutrient digestion, and intestinal health (Yang et al., 2017). Further, the GIT microbiome has important interactions with the host immune system to support proper immune function (Brown et al., 2013).

Immediately after birth, the GIT microbiota utilizes competitive exclusion to act as a protective barricade against harmful microorganisms, play a supportive role in the digestion and metabolism of colostrum and milk, and lastly aid in the growth of GIT barrier epithelial cells, which line the intestinal lumen and foster immune system homeostasis both early and later in life (Guarner and Malagelada, 2003). Importantly, this epithelial cell barrier is maintained by gut microbes that provide energy in the form of short-chain fatty acids, primarily butyrate, which allows for proper proliferation and differentiation (Sakata, 1987).

The piglet is susceptible to both preweaning and postweaning diarrhea, and this is one of the most common issues faced in swine production and presents a major economic cost due to losses in piglet performance. Piglet diarrhea is caused by uncontrolled proliferation of harmful strains of pathogenic bacteria such as *Escherichia coli*, which in turn creates shifts in GIT microbiota and immune function (Konstantinov et al., 2006). In addition to the increase in pathogenic *E. coli*, incidences of piglet diarrhea are also characterized by decreased abundance of beneficial bacteria such as *Lactobacillus sobrius*, *L. acidophilus*, and *L. reuteri* (Konstantinov et al., 2006). Further, Ding et al. (2019) noted that greater microbial richness (i.e., greater taxonomic diversity) in the jejunum and greater abundance of the phyla Firmicutes and Bacteroidetes in the colon are positively correlated with greater pre-weaning weight gain. At the genus level, greater abundance of *Selenomonas* and *Moraxella* in the ileum, as well as *Lactobacillus* in both the cecum and colon have also been positively correlated with greater piglet pre-weaning weight gain (Ding et al., 2019). Finally, a microbial marker correlated with

piglet diarrhea at and after weaning is the presence of species from the genus *Prevotella* in fecal microbiota. Multiple studies have identified greater abundance of *Prevotella* in the fecal microbiota profile of healthy piglets that did not have diarrhea after weaning (Karasova et al., 2021; Luise et al., 2021). Clearly, a healthy GIT microbiome is essential for early piglet health and development. Additionally, these data, while acknowledging only a small proportion of the total GIT microbial community, provide reference for noteworthy phyla and genera and justify both the positive and negative roles of bacterial presence in the young pig.

Typically, swine farms neutralize all bacteria and avoid microbiome considerations by sterilizing barns with disinfectants before introducing new groups of pigs. This strategy has advantages, as noted by Law et al. (2021), who reported that piglets born in disinfected farrowing environments had greater birthweights, weaning weights, and post-weaning growth compared to piglets born in non-disinfected environments. Yet these management practices also shift the pig's microbiome composition at weaning (Law et al., 2021). Indeed, with these management strategies to mute the environmental microbiome in mind, understanding the various bacteria associated with gut health as well as the origins of the piglet's microbiome becomes more important.

Genera *Lactobacillus* and *Prevotella* have been suggested as commensal bacteria, as within the piglet GIT microbiome greater abundance of *Lactobacillus* has been associated with increased piglet weight gain, while greater abundance of *Prevotella* being characterized as a microbial marker for piglets not displaying diarrhea (Ding et al., 2019; Karasova et al. 2021; Luise et al., 2021). Meanwhile, the presence of typically pathogenic bacteria *E. coli* and *Campylobacter* in the piglet GIT can have negative effects on GIT function. The incidence of *E. coli* infection has been linked to more frequent reason of severe piglet diarrhea, along with lower

abundances of Bacteroidetes being associated with metabolic dysfunction and post-weaning diarrhea (Sun and Kim, 2017; Ren et al., 2022). Further, *Campylobacter* species have been shown to yield toxins and trigger inflammation, causing reduced nutrient use efficiency (De Rodas et al., 2018).

Additionally complicating pig microbiome considerations are recent genetic selection decisions made within the swine industry that have altered litter composition characteristics, thereby inadvertently resulting in GIT immaturity and decreased piglet viability. The swine industry values enhanced sow prolificacy to cater to the growing demand for efficient pork production. Accordingly, average litter size has increased, yet there are inverse relationships between litter size with litter survival, piglet birth weight, and piglet weaning weight (Ding et al., 2019). Further, increased litter sizes have also increased the proportion of low birthweight (LBW) pigs born per litter (Martineau and Badouard, 2009). This is because the uterine capacity of the sow to adequately supply the fetuses with nutrients has not increased at the same rate as litter size, which can cause fetuses to suffer from intrauterine growth restriction (IUGR; Foxcroft et al., 2006). Fetuses that suffer from IUGR typically are born with an underdeveloped GIT (among other organs; Amdi et al., 2013), which impairs the maturation of the GIT and the colonization of the microbiome, and in turn leads to a loss in performance, efficiency, and decreased survival rates (Wang et al., 2005).

Low birthweight piglets are defined as those having a birthweight below the 10th percentile of the mean birthweight of the litter, or a birthweight greater than two standard deviations below the mean birthweight (Cooper, 1975). In two separate studies, Li et al. (2018; 2019) compared the GIT microbiome between LBW piglets and normal birth weight (NBW) piglets through the first five weeks of life. At 21 days of age, the LBW piglets hosted lesser

abundance of typically beneficial genera *Lactobacillus, Streptococcus* and *Prevotella* and greater abundance of typically harmful genera *Campylobacter* and *Bacteriodes* when compared to NBW piglets. Further, these LBW pigs had decreased fecal metabolites associated with fatty acid metabolism, which can cause reduced epithelial cell barrier function.

Gaukroger et al. (2020) determined the interaction between piglet birthweight (LBW or NBW) and growth rate through 56 days of age in a 2 × 2 factorial experiment. Within birth weight classes, pigs were assigned to a post hoc growth category based on average daily gain (ADG) below ("poor" growth) or above ("good" growth) the ADG of their birthweight class (LBW: 0.28 kg/d; NBW: 0.37 kg/d). The NBW piglets showed a greater number of observational taxonomic units (OTUs, used to classify groups of closely related individuals) on day 21, with NBW piglets also showing greater abundance of both *Ruminococcaceae UCG-005* and *UCG-014* genera at day 21, yet then reduced abundance by day 32. While there was no significant difference in observed OTUs between ADG classes, there was however a greater abundance of the genera *Lactobacillus* at day 4, unclassified *Prevotellaceae* at day 8, and *Ruminococceae* at day 14 in the "good" ADG group compared to the "poor" ADG group. There was also a three-way interaction between microbiota composition, birthweight class and ADG class, as NBW "good" piglets showed greater abundance of *Ruminococcaceae UCG-005* genera compared to NBW "poor" at day 14. Overall, it is clear that GIT microbiome composition differences present between both birthweight and growth classes indicates that GIT microbiome composition has the ability to affect early life performance and efficiency of piglets.

1.4. Procurement of the Microbiome

The longstanding belief is that the microbiome originates during parturition when the neonate travels through the birth canal from a "sterile" environment into the microbially

populous and diverse environment of the outside world (Palmer et al., 2007). However, that has become a more contested theory recently. Indeed, evidence suggesting intrauterine gut microbiome colonization has been reported in human infants (Collado et al., 2016) and calves (Guzman et al., 2020). In swine, Nowland et al. (2021a) reported the presence of bacteria within the spiral colon of stillborn piglets, suggesting GIT colonization during the final stages of the pre-natal period in an animal that had no opportunity for environmental interaction. Ultimately, there is evidence that both environmental and biological sources play at least some role in the acquisition of the pioneer piglet microbiome, and these will be discussed independently below.

1.4.1. Environmental Influence

Environmental influence on the microbiome has been shown repeatedly in humans. For example, children who are raised on farms with rich environmental microbial diversity have reduced incidences of asthma, hay fever and allergies (Braun-Fahrlander et al., 1999; Schroder et al., 2015). Subjection to more microbial exposure allows for improved responses from both the innate and adaptive immune system responses (Von Mutius and Vercelli, 2010). Within swine research, greater focus has considered how the pre-weaning environment may influence the piglet's microbiome. Swine projects have considered multiple environmental factors such as rearing environment (high hygiene isolators versus outdoor versus indoor), soil exposure, maternal fecal exposure, and even disinfection practice differences.

A foundational study in environmental contributions of the piglet microbiome considered the effects of rearing piglets in high hygiene isolators vs. being housed indoors vs. being housed outdoors. The high hygiene isolators used in the study were specific pathogen free, positive pressure units supplied with a high efficiency particulate air (HEPA) filter (Schmidt et al., 2011). Results of this showed differences in microbial succession and stabilization within piglets in

different environments. Indeed, the microbiome of piglets raised within the high hygiene isolators was disturbed, indicating that continuous environmental microbial exposure must be present in order for proper development of the pioneer microbiome to occur (Schmidt et al., 2011). Similar results were reported by Mulder et al., (2011), who recorded delayed gut closure in piglets raised in isolators as compared to piglets raised in an indoor or outdoor environment.

Under conditions more relevant to commercial swine production, Law et al. (2021) compared different disinfection methods in the farrowing environment and the consequences on both the sow and piglet microbiomes. In the study, three Landrace × Yorkshire sows farrowed in a disinfected environment (hot water power washing plus disinfectant [D]), while three litters were farrowed in a nondisinfected environment (hot water power washing only [Nde]). The D environment yielded reduced quantitative PCR copy numbers, potentially indicating reduced microbial load, which would be expected following disinfection. Although, disinfection method did not impact the sow gut, skin, vaginal, milk or oral microbiome, it did however have an effect on both the nasal and gut microbiomes of the piglets. Bacterial diversity was greater in the fecal and nasal cavities of piglets in the Nde treatment group on the day of farrowing, day 0. These results suggest that the microbial population of an environment directly impacts the early in life establishment of the piglet microbiome. More recently, Nowland et al. (2021b) showed that removing sow fecal matter from the farrowing crate twice daily for the first ten days after parturition altered microbiota colonization as well as boosted piglet survival and growth, when compared to leaving the maternal feces in the farrowing crate.

Clearly, an animal's interaction with their environment impacts their microbial acquisition. Yet neonatal animals also interact with other animals, and this is particularly true in the piglet that is born in a litter. To compare genetic versus environmental effects on gut

community stability and overall makeup, Thompson et al. (2008) split littermate pigs into different rearing environments. Over the course of three trials, 35 male piglets were obtained at 3 days of age from a total of five different litters sourced from two different commercial swine operations, and then continuously housed in pairs according to size. Unrelated piglets were paired together wherever possible based off size similarity. The cohabitation effect was not observable early in life and sibling pigs were more similar. However, by day 36 the cohabitation effect was extremely strong as non-sibling cohabiting pigs were similar in regard to microbiome composition, meanwhile sibling pigs raised separately were not, thus, suggesting at least partial influence of environmental effects on the microbiome (Thompson et al., 2008).

With American large-scale production in mind, the relevance of some of these topics (mainly outdoor rearing environment and soil exposure) is reduced: the modern farrowing environment promotes biosecurity and limits environmental access. When considering the current, modern production practices and the environment in which piglets are raised, it is likely that the constant proximity to or contact with the sow's skin and feces has at least some influence on the developing piglet microbiome.

1.4.2. Genetic or Maternal Influence

Host genetics influence the developed GIT microbiome, as the microbial community of human twins has been shown to be more similar than that of genetically differing individuals (Zoetendal et al., 2001), and monozygotic (identical) twins are more similar than dizygotic (fraternal) twins (Goodrich et al., 2014). Studies have also considered the differences between infants born vaginally versus via caesarean section. Studies comparing the method of delivery method on the microbiome have shown that infants delivered vaginally are initially colonized by bacterial populations that closely resemble that of the mother's vaginal microbiome, while those

delivered by caesarean section have lower richness and diversity and are more similar to that of the mother's skin instead (Dominguez-Bello et al., 2010; Groer et al., 2014).

Pajarillo et al. (2014) conducted a study to determine the effect of host genetics (i.e., breed) on the piglet microbiome. Pregnant sows of the three main American breeds used in production (Duroc, Yorkshire and Landrace) farrowed in the same farrowing environment and were fed the same rations. Landrace and Yorkshire piglets had similar microbiomes while Duroc piglets were the most different, having the greater abundances of *Catenibacterium, Phascolarctobacterium* and *Subdoligranulum* at the genus level. These findings provide strong evidence that host genetics play at least some contributory role in the pioneer piglet microbiome.

Postnatal maternal factors also contribute to the development of the offspring microbiome, as suggested by microbiome differences recorded between breast-fed and formula-fed human infants (Harmsen et al., 2000). These postnatal maternal factors may be of greater importance in pigs compared to humans, owing to differences between species between placental type (hemochorial in humans vs. epitheliochorial in swine) and the relative value of colostrum. Colostrum is the first milk produced by the sow that is provided within the first 24 hours after parturition and provides the piglet with energy and immune cells (Le Dividich et al., 2005). Maternal milk has been identified as a critical postnatal factor for establishing an appropriate pioneer microbiome (Morissette et al., 2018), as colostrum and milk contains a wide variety of bacteria and prebiotic compounds (Bian et al., 2016). As previously discussed, the recent industry trend of selecting genetic lines to boost prolificacy has resulted in decreased average piglet birthweight. The piglet's ability to acquire colostrum is influenced by birthweight (Milligan et al., 2002), and with the greater abundance of low birthweight pigs, colostrum acquisition has become an increasing concern for not only microbiome procurement but also

future health and performance implications. Morissette et al. (2018) showed that colostrum/milk intake during the first two weeks after birth strongly influenced microbiome development, with piglets with greater weight gain (i.e., greater milk consumption) displaying greater levels of genera *Bacteroides* and *Ruminoccocacae*, and reduced levels of *Actinobacillus porcinus* and *Lactobacillus amylovorus* when compared to piglets with lower weight gain (i.e., decreased milk consumption). It has even been documented that domestic animals that are raised on a formula milk replacer instead of biological milk develop reduced intestinal microbiota diversity, as well as an increased susceptibility to disease (Inman et al., 2010; Iozzo and Sanguinetti, 2018). Interestingly, sows that were fed the probiotic *Enterococcus faecium* NCIMB10415 (SF68) during gestation showed modified fecal microbiomes, and microbiome differences in piglets reared by these sows were also present, suggesting a responsive link between the maternal and neonatal microbiomes (Starke et al., 2013).

To elucidate the prenatal and postnatal maternal contributions to the offspring microbiome, Bian et al. (2016) conducted a cross-fostering study: Yorkshire and Meishan sows had half of their piglets removed and fostered onto the opposite breed, immediately after birth prior to suckling. All piglets had a relatively similar bacterial community at birth, with the main exception being that Meishan pigs displayed higher levels of bacteria within the *Lactobacillus* genus and lower levels of *Escherichia-Shigella* (typically harmful bacteria). The impact of host genetics was greater than the impact of the nurse sow during the suckling period, as more of the microbiome differentiation was explained by maternal host genetics than the nurse sow at 14 days post-farrowing. Yet, the differences in sow's milk were still influential. The main nutrient constituents of sow's milk are lactose, protein and fat, and variation in all nutrients impacted the piglet GIT microbiota. Interestingly, the milk lactose concentration had the greatest contribution

to the piglet GIT microbiota (74.5%) and may be the main driving factor behind the nursing effect. These data are supported by Chen et al. (2018), who noted that the piglet's microbiome more closely resembles the sow's microbiome with greater time nursing the sow, suggesting amplified impact of maternal contributions over time.

Notably, weaning disrupts the piglet's microbiome. Common management practices associated with piglet weaning, such as moving the animal into a new environment and changing the course of nutrients (i.e., sow milk vs. solid feed) diminishes the impact of both the biological sow and the nurse sow (Bian et al., 2016). These data suggest that the maternal influence on the piglet microbiome (and therefore piglet health and development) is of greatest concern early during the pre-weaning period.

1.5. Conclusion

The piglet's pioneer microbiome has important implications for piglet health, pig growth performance, and producer profit. There are multiple contributing sources to this microbiome, including the environment of piglet farrowing and rearing, and the sow's reproductive tract, colostrum and milk, skin, and feces. The proof-of-concept studies discussed in this literature review demonstrate this. Yet the percent contributions of the environmental and maternal factors to the piglet's microbiome remain to be answered and understanding these will be necessary to shape future genetic selection decisions or management changes to further enhance the health, performance, and profitability of swine production.

CHAPTER II

MATERNAL VERSUS ENVIRONMENTAL CONTRIBUTIONS TO THE PIGLET PIONEER MICROBIOME

2.1. Introduction

The gastrointestinal (GIT) microbiome impacts countless body processes in gut health and function and has major influence on swine health and performance. Specifically, the GIT microbiome is involved in energy harvest and nutrient digestion and promotes intestinal health by fostering important interactions with the host immune system (Brown et al., 2013; Yang et al., 2017). A healthy microbiome is associated with proper piglet development, while early-in-life dysbiosis can lead to pre-weaning diarrhea that reduces piglet growth and elevates litter mortality (Konstantinov et al., 2016).

The piglet pioneer microbiome, which is the initial microbial colonization and has long-term influence on host health and performance, receives inputs from both maternal and environmental factors. Schmidt et al. (2011) demonstrated that raising pigs in different rearing environments (i.e., completely indoors vs. outdoor access) creates variation in microbial succession and stabilization. Further, farrowing crate disinfection methods impact piglet nasal and gut microbiome composition (Law et al., 2021). Environmental contributions to the piglet microbiome are logical, as the animal is inundated with microbes through interactions with their surroundings.

Yet the environment in which a pig is raised does not wholly explain an animal's microbiome; genetics also make a sizable contribution, both between and within breed. Pajarillo et al. (2014) compared the GIT microbiome of piglets born from Duroc, Landrace, and Yorkshire

dams in a common farrowing environment and under similar feeding and management. Despite identical environmental exposure, the Duroc piglet microbiome was significantly different from that of Landrace and Yorkshire piglets. Similarly, in a cross-fostering study, Bian et al. (2016) identified the maternal contribution as a host genetic effect: the microbiome of piglets that were removed from their biological sow and fostered onto a nurse sow immediately after birth showed greater similarity to the biological sow than the nurse sow at 14 days of age.

It is apparent that the piglet's pioneer microbiome is influenced by both maternal and environmental factors. However, the exact proportion of environmental versus maternal contributions to the piglet pioneer microbiome remains unknown, and this ambiguity impedes accurate genetic selection or targeted management protocols to improve the microbiome, and therefore piglet health and performance. The objective of this study was to explain the percent contributions from maternal and environmental sources to better characterize and understand the piglet pioneer microbiome.

2.2. Materials and Methods

This study was conducted at the O.D. Butler, Jr. Animal Science Teaching, Research and Extension Complex Swine Center (ASTREC) at Texas A&M University in College Station, TX and performed under protocol approved by the Institutional Animal Care and use Committee of Texas A&M University (IACUC 2022-0043).

2.2.1. Experimental Animals and Management

Five gilts were bred at the Texas A&M University swine center in December 2021 and farrowed in early April 2022. Gilts were a Landrace x Yorkshire x Duroc composite and bred to pooled Duroc semen. Gilts were group housed on solid-concrete floors prior to breeding and throughout gestation and were vaccinated with a combination killed *Escherichia coli* bacterin

and *Clostridium perfringens* Type-C bacterin-toxoid (LitterGuard LT-C; Zoetis, Parsippany, NJ) at 5 weeks and 3 weeks prior to anticipated farrowing date based on farm health management protocols. Gilts were moved into the ASTREC farrowing barn at 109.6 ± 0.5 days in gestation and housed in industry-standard farrowing crates providing 0.6 x 2.1 m gilt space and 0.5 x 2.1 m piglet space with unrestricted access to a water nipple and a self-feeder. Prior to gilt introduction, all spaces of the farrowing barn were power washed with hot water and then treated with a broad-spectrum (gram-positive and gram-negative) bactericidal detergent disinfectant (Tek Troll II; ABC Compounding Co., Inc., Atlanta, GA) that is commonly used in the swine production industry. One farrowing crate was left empty between gilts to minimize opportunity for organic material spread between litters and to allow personnel to catch piglets and collect samples without stepping into the occupied farrowing crate. Gilts were provided 2.3 kg per day of a gestation diet during pregnancy and transitioned to *ad libitum* access to a lactation diet after farrowing. Both diets were formulated to meet or exceed nutritional requirements for gilts relative to the respective physiological state (NRC, 2012).

Continuous supervision of the farrowing room commenced on day 113 of gestation and continued until all gilts had farrowed. Human supervisors were trained in farrowing barn biosecurity procedures prior to participation and wore clean clothing and disposable plastic boot covers and gloves while in the farrowing barn. All piglets were individually identified with an ear notch and weighed immediately after birth and then returned to the farrowing crate. Piglets were only touched at sampling and processing times, while handlers wore gloves. Piglets processing was completed by one person after day 3 sampling, and the individual wore gloves and changed gloves between litters to avoid cross contamination. Piglet processing included iron administration, needle teeth clipping, tail docking, and castration of all male piglets. Causes and

timing of piglet mortality was recorded when occurring. Piglet weight at weaning was recorded at 21.6 ± 1.0 days post farrowing.

2.2.2. Sample Collection for Microbiome Analysis

Sterile swabs were used to collect the microbiome of environmental and maternal sources. The farrowing crate was sampled after disinfection and prior to gilt introduction (Empty Crate) and again after gilts were moved into the farrowing crate on day 113 of gestation (Full Crate). Both Empty Crate and Full Crate samples were collected utilizing a standardized twirling technique in three locations: approximately 0.2 m from the back of the farrowing crate in the gilt's dunging space and approximately 1.0 m from the back of the crate in the middle of the piglet creep spaces. The gilt's gastrointestinal tract was sampled at day 113 of gestation by inserting the swab approximately 2.5 cm into the rectum. Colostrum was collected from each gilt during farrowing (48.6 ± 17.0 minutes after birth of the first piglet) from a representative sample of teats into a single sterile collection cup (Colostrum). The Birth Canal was also sampled during farrowing (68.2 ± 19.4 minutes after birth of the first piglet). One person wearing a sterile glove drenched with obstetric lubricant (O B Lube; Centaur Animal Health, Olathe, KS) held the sterile swab and gently drug it along the vaginal wall until reaching the pelvic opening. Five piglets per litter weighing greater than 1,200 grams at birth were randomly selected for repeated rectal sampling on days 0 (prior to suckling), 3, and 10 post-farrowing and at weaning (21.6 ± 1.0 days post-farrowing). Samples were collected by inserting the swab just past the rectum. The same piglets were sampled on each day. All swabs were collected in duplicate, and swabs and colostrum were stored in sterile microcentrifuge tubes at -80°C until gene sequencing was performed.

2.2.3. DNA Extraction and 16S rRNA Gene Sequencing

Swab samples were sent to FERA Diagnostics and Biologicals Corp. (College Station, TX) for DNA extraction and 16S rRNA gene amplicon sequencing. Samples were transferred to a 96-well plate and DNA extraction was performed using Mag-Bind® Universal Pathogen 96 Kit (Omega Bio-Tek, Norcross, GA) according to the manufacturer's instructions. The 16S amplicons were amplified by PCR for individual metagenomic DNA samples according to previously described methodology (Bicalho et al., 2017). The V4 hypervariable region of bacterial/archaeal 16S rRNA gene were amplified with 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers using methods optimized for the Illumina MiSeq platform (Caporaso et al., 2012).

2.2.4. Statistical Analysis

Phyla and genera constituting less than 2% relative abundance were classified as "Other". Samples from the dam's rectum were collected but were removed from further analysis due to high correlation of relative abundance with Full Crate (r=0.99). Differences in microbial relative abundance at phylum and genus level at the environmental and biological sampling locations was characterized using PROC GLM in SAS 9.4 (SAS Institute, Cary, NC). Multiple regression of the piglet's microbiome at each day of age was performed using PROC REG of SAS 9.4 in a forward stepwise manner using $p<0.99$ as the selection entry criteria. All environmental (Empty Crate and Full Crate) and only chronologically relevant biological variables were included in these models. The Day 0 (pre-suckle) piglet microbiome model variables included Empty Crate, Full Crate and Birth Canal. The model variables of piglets at older ages included the prior terms

plus Colostrum and the piglet preceding ages. Significance was defined as $p < 0.05$ and tendencies at $p < 0.10$.

2.3. Results and Discussion

Litter performance summary statistics are provided in Table 1. Swabbed piglets and non-swabbed littermates were similar in birth weight (1,463.9 g vs. 1,389 g, respectively, SEM: 51.9 g; p=0.31) and pre-weaning survival (87.5% vs. 90.6%, respectively, SEM: 5.6%; p=0.69) indicating that the piglets randomly selected to be swabbed were representative of their litters.

Phyla relative abundance in piglet and non-piglet samples are provided in Table 2. Most bacteria in the piglet GIT are classified into five phyla: Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Spirochaetes, of which Firmicutes and Bacteroidetes typically account for nearly 90% (Kim et al., 2011). In the present study, phylum Firmicutes displayed the greatest abundance in non-piglet samples (Empty Crate, Full Crate, Birth Canal, and Colostrum) as well as all piglet samples, except for the Day 3 piglet where phylum Proteobacteria had the greatest abundance (54.8% vs 35.8%, $p<0.0001$). Further, phyla Firmicutes and Bacteroidetes combine to make up at least 75% relative abundance in all piglet and non-piglet samples, aside from the Day 3 piglet which only recorded 35.8% and 5.5% relative abundance from phyla Firmicutes and Bacteroidetes, respectively. The Day 3 piglet displayed the lowest abundance of phyla Firmicutes (35.8%, $p < 0.0001$) and Actinobacteria (0.3%, $p<0.05$) compared to all other time points. Further, the abundance of Fusobacteria at Day 3 (3.5%) was greater than at Day 0 (p=0.04) and tended to be greater than Day 21 (p=0.06). The phyla elevated in the Day 3 piglet are known to be associated with neonatal piglet diarrhea (Hermann-Bank et al., 2015). These data, combined with data by Ding et al. (2019), who noted a positive correlation between abundances of phyla Firmicutes and Bacteroidetes with improved piglet pre-weaning weight

gain, would typically suggest increased sickness and decreased performance in the Day 3 piglet, however no symptoms of diarrhea or a loss of performance was observed in piglets at this timepoint.

Table 3 provides the genera relative abundance of piglet and non-piglet samples. Indeed, the Day 3 piglet showed elevated abundances of bacteria from the genera *Escherichia* (38.1%, $p<0.0001$), *Clostridium* (17.1%, $p<0.0001$) compared to other time points. Both genera are typically associated with decreased intestinal stability and increased prevalence of scours (Yaeger et al., 2002; Yang et al., 2019). The Day 3 piglet contained other bacterial markers known to be associated with incidences of piglet diarrhea (Yang et al., 2019), including decreased abundance of *Prevotella* (0.1%, $p<0.001$), *Bacteroides* (5.3%, $p<0.001$), *Ruminococcus* (0.8%, $p<0.0001$), *Lactobacillus* (0.4%, $p<0.05$), and *Treponema* (0.0%, $p<0.05$). Many of these bacteria, including *Ruminococcus* and *Lactobacillus*, are considered beneficial genera to the intestinal microbiome and play roles supporting proper gut function and health (Monteiro et al., 2022).

Multiple regression analysis of the Piglet Day 0 (pre-suckle) microbiome is shown in Table 4. Notably, the Birth Canal explains 51.64% ($p<0.0001$) of the variation in the Piglet Day 0 microbiome, while the Empty Crate and Full Crate explains only 2.14% ($P=0.0628$) and 0.00% ($p=0.9717$), respectively. Yet, this leaves 46.22% of variation in the Piglet Day 0 microbiome unexplained. Previously, Law et al. (2021) showed that farrowing crate disinfection method had no effect on the sow gut, skin, vaginal, milk, and oral microbiome, which may explain why there is a lack of variation explained by environmental contributors to the Piglet Day 0 microbiome. With over half of the Piglet Day 0 microbiome being explained by the Birth Canal, this suggest strong maternal contribution to the Piglet Day 0 microbiome, and also aligns with prior research

that showed neonates delivered naturally are initially colonized by bacterial populations that closely resemble that of the mother's vaginal microbiome (Dominguez-Bello et al., 2010, Groer et al., 2014).

Table 5 shows the multiple regression analysis of the Piglet Day 3 microbiome. Interestingly, only 15.51% of the variation in Piglet Day 3 microbiome is explained by other factors, leaving 84.49% of the variation unexplained. The biggest known contributor is from the Piglet Day 0 (9.95%, p=0.0041) and the smallest known contributor to the Piglet Day 3 microbiome is Colostrum (0.10%, p=0.7698). This was surprising, as this contradicts prior research that suggests colostrum is a key contributor to the piglet pioneer microbiome (Morissette et al., 2018). Overall, there was little known contributions from other maternal or environmental sources. The Full Crate was a statistically significant contributor to the Piglet Day 3 microbiome, yet the contribution was marginal. (2.60%, p=0.0041). Other factors were not statistically significant in the analyses, including the Birth Canal (2.62%, p=0.1297), Empty Crate (0.24%, p=0.6443), and Colostrum (0.10%, p=0.7698). Combined, non-piglet factors explained only 5.56% of explained variation. The vast amount of unexplained variation in the Piglet Day 3 microbiome relative to other time points provides evidence that the piglet microbiome is most unique at Day 3 than at any other time points prior to weaning. Based on these data, we hypothesize the cause of this to be that the neonatal piglet is subject to extreme microbial exposure throughout the first 72 hours of life, and that its naïve and underdeveloped immune system is essentially overloaded with microbial information. This is supported by the phyla and genera relative abundance results of the Piglet Day 3 microbiome. Consequently, this created severe shifts in the microbiome composition in the first days of life.

The multiple regression analysis of the Piglet Day 10 microbiome is shown in Table 6. Here, the Piglet Day 0 microbiome accounts for the most variation (15.60%, p=0.0003), while the Day 3 microbiome was not significantly associated and explained only 0.49% of the variation in the Piglet Day 10 microbiome (p=0.4898). In total, 23.16% of the Piglet Day 10 is explained by known contributors, leaving 77.84% of the variation unexplained. The Birth Canal explains 6.54% (p=0.0130) of the variation in the Piglet Day 10 microbiome, again suggesting evidence of maternal contributions. Colostrum (0.40%, p=0.5361), Full Crate (0.11%, p=0.7403), and Empty Crate (0.03%, p=0.8743) did not have an impact on the Piglet Day 10 microbiome. The significant associations between the Birth Canal and Day 0 microbiome with the Day 10 microbiome, combined with the lack of correlation between the Day 3 and Day 10 timepoints, suggest that the piglet is born with a maternally derived baseline microbiome that it can revert back to following early-in-life microbial challenges. Greater research will be needed to effectively categorize the timing and sources of the difficult to explain deviation from this baseline microbiome to fully understand genetic and environmental interactions on piglet health and development.

Table 7 shows the multiple regression analysis of the Piglet Day 21 microbiome. In total, 63.18% of the Piglet Day 21 microbiome is explained by known contributors which is greater compared to other time points, this suggests as the piglet progresses towards weaning beyond day 10, their microbiome becomes more established. Notably, the Piglet Day 10 microbiome explains 58.62% (p<0.0001) of the variation in the Piglet Day 21 microbiome. Hence, the piglet pre-weaning microbiome may be largely established beyond Day 10. There was negligible impact of the main environmental contributors, the Empty Crate (0.04%, p=0.7693) and Full Crate (0.04%, p=0.7775), on the Piglet Day 21 microbiome, and these data combined with prior

time point results suggest no significant contributions were made on the piglet pioneer microbiome through the studied environmental contributions.

In summary, the piglet pioneer microbiome shifted from birth to weaning. The Piglet Day 21 microbiome may be largely established by Day 10. The Piglet Day 3 microbiome showed the greatest deviation from piglet microbiomes at other time points, as it showed a greater abundance of typically harmful phyla Fusobacteria and Proteobacteria, and a reduced abundance of beneficial phyla Firmicutes and Bacteroidetes. These collective characteristics are typically associated with neonatal piglet diarrhea and decreased piglet performance (Hermann-Bank et al., 2015; Ding et al., 2019). Piglet diarrhea was not observed in the present study, yet future experiments may consider the microbiome in relation to the timing and severity of scours, when occurring, to better map the piglet's shifting early-in-life microbiome. Maternal contribution to the piglet pioneer microbiome was evident, as the Birth Canal largely influenced the Day 0 Piglet and maintained a significant influence throughout the suckling phase. Interestingly, Colostrum and environmental contributions from the farrowing crate environment did not impact the piglet pioneer microbiome ($p>0.10$). Law et al. (2021) showed that the piglet microbiome changes due to differences in farrowing crate disinfection methods. The lack of environmental contributions in these results could be attributed to all 5 litters being raised in the same farrowing crate environment, where all crates were hot-water power washed and disinfected with a broad-spectrum disinfectant. Additionally, our results identifying significant contribution from the Birth Canal and no impact from Colostrum corroborate with the findings from the Bian et al. (2016), who utilized cross-fostering to demonstrate that more microbiome differentiation in piglets at 14 days of age was explained by maternal host genetics than by nurse sows.

2.4. Conclusion

It is evident that there are clear shifts in the piglet pioneer microbiome prior to weaning, and although our results showed significant maternal influence on the piglet microbiome from the birth canal, and no environmental influence from the farrowing crate environment, this does not align with all prior research, as varying degrees of both maternal and environmental impact on the pioneer piglet microbiome have been reported. The findings from this study should help better understand the development and characterization of the pioneer piglet microbiome, but large unexplained variation at certain time points warrant future studies. These future studies should include characterizing an ideal piglet microbiome at weaning to achieve improved efficiency, health, and performance, as well as how to genetically select for and achieve said microbiome. Finally, long term associations and effects of the piglet microbiome beyond weaning and into the growing, finishing and breeding phases of production will be necessary in order to achieve proper industry efficiency and profitability.

CHAPTER III

TABLES

Table 1. Summary statistics of litter characteristics

Trait	Mean ± Standard Deviation
Gestation length, days	116 ± 1.1
Total number born	13.4 ± 2.9
Number born alive	12.8 ± 2.8
Number of stillborn pigs	0
Number of mummified fetuses	0.6 ± 0.8
Number weaned	11.4 ± 2.2
Mean piglet birth weight, g	1,426 ± 292
Mean piglet weaning weight, kg	5.7 ± 1.5

Table 2. Phyla relative abundance percentage in piglet and non-piglet samples

Phylum	Piglet Samples					Non-Piglet Samples				
	Day 0	Day 3	Day 10	Day 21	S.E.M.	Empty Crate	Full Crate	Birth Canal	Colostrum	S.E.M.
Firmicutes	58.4	35.8	42.2	51.3	3.0	70.0	80.7	77.5	74.8	7.1
Proteobacteria	15.3	54.8	13.2	11.4	2.7	7.5	2.7	9.0	7.3	6.4
Bacteroidetes	21.3	5.5	35.9	26.5	1.8	11.6	12.0	9.5	10.1	4.3
Actinobacteria	1.8	0.3	2.8	1.7	0.5	7.7	2.0	2.2	5.0	1.1
Spirochaetes	1.0	0.0	2.1	4.7	0.6	1.0	1.7	0.4	0.5	1.5
Fusobacteria	0.9	3.5	2.9	1.1	0.9	0.1	0.0	0.3	0.1	2.2

Table 3. Genera relative abundance percentage in piglet and non-piglet samples

Genus	Piglet Samples					Non-Piglet Samples				
	Day 0	Day 3	Day 10	Day 21	S.E.M.	Empty Crate	Full Crate	Birth Canal	Colostrum	S.E.M.
Escherichia	7.6	38.1	5.2	2.0	2.1	0.2	0.1	1.3	1.5	4.9
Lactobacillus	14.1	0.4	5.9	4.2	1.5	20.0	55.7	25.7	15.3	3.6
Bacteroides	7.9	5.3	27.1	14.4	1.7	3.3	2.8	2.7	2.7	4.0
Clostridium	5.3	17.1	5.0	6.4	1.4	10.5	2.8	4.8	5.8	3.3
Blautia	5.7	1.8	10.1	9.1	0.8	2.2	3.4	6.3	2.5	1.8
Prevotella	9.0	0.1	4.7	5.2	0.8	4.3	4.8	4.1	4.4	2.0
Streptococcus	7.0	7.1	2.2	0.6	1.2	1.2	2.0	9.4	4.6	2.8
Serratia	2.9	13.5	2.1	0.8	0.7	0.1	0.0	0.5	0.6	1.7
Ruminococcus	4.3	0.8	6.1	6.2	0.4	2.0	2.6	5.1	1.7	1.0
Oscillospira	2.2	0.1	2.4	4.9	0.4	1.1	1.6	2.0	0.9	0.9
Treponema	1.1	0.0	2.2	5.3	0.7	1.0	1.8	0.4	0.5	1.7
Fusobacterium	0.9	3.5	2.9	1.2	0.9	0.1	0.0	0.3	0.1	2.2
Staphylococcus	1.1	1.3	0.1	0.1	1.0	3.3	0.1	1.7	27.6	2.4
Campylobacter	0.3	0.1	2.1	3.2	0.4	0.3	0.3	0.3	0.1	1.0
Enterococcus	0.5	4.5	0.4	0.1	0.6	0.1	0.1	0.2	0.1	1.3
Turicibacter	1.1	0.0	0.1	1.3	0.3	7.6	1.3	2.0	2.4	0.6

Table 4. Multiple regression analysis showing amount of variation explained in piglet microbiome on day 0

Multiple Regression Analysis (Piglet Day 0)			
Variable	Partial R-Square	Model R-Square	P Value
Birth Canal	0.5164	0.5164	< 0.0001
Empty Crate	0.0214	0.5378	0.0628
Full Crate	0.0000	0.5378	0.9717

Table 5. Multiple regression analysis showing amount of variation explained in piglet microbiome on day 3

Multiple Regression Analysis (Piglet Day 3)			
Variable	Partial R-Square	Model R-Square	P Value
Piglet Day 0	0.0995	0.0995	0.0041
Full Crate	0.0260	0.1254	0.1533
Birth Canal	0.0262	0.1516	0.1297
Empty Crate	0.0024	0.1541	0.6443
Colostrum	0.0010	0.1551	0.7698

Table 6. Multiple regression analysis showing amount of variation explained in piglet microbiome on day 10

Multiple Regression Analysis (Piglet Day 10)			
Variable	Partial R-Square	Model R-Square	P Value
Piglet Day 0	0.1560	0.1560	0.0003
Birth Canal	0.0654	0.2214	0.0130
Piglet Day 3	0.0049	0.2263	0.4898
Colostrum	0.0040	0.2302	0.5361
Full Crate	0.0011	0.2314	0.7403
Empty Crate	0.0003	0.2316	0.8743

Table 7. Multiple regression analysis showing amount of variation explained in piglet microbiome on day 21

Multiple Regression Analysis (Piglet Day 21)			
Variable	Partial R-Square	Model R-Square	P Value
Piglet Day 10	0.5862	0.5862	< 0.0001
Piglet Day 3	0.0215	0.6077	0.0434
Birth Canal	0.0126	0.6203	0.1162
Colostrum	0.0060	0.6263	0.2810
Piglet Day 0	0.0047	0.6310	0.3419
Empty Crate	0.0004	0.6314	0.7693
Full Crate	0.0004	0.6318	0.7775

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