ABSTRACT

LAYTON, JONATHAN M. Interplay between Host Genetics, Extrinsic Factors, and the Piglet Gut Microbiome during Lactation. (Under the direction of Dr. Christian Maltecca).

During piglets' early life stage, especially during weaning, changes in diet, environment, and social structures can impact their long-term health. This includes physiological alterations, vulnerability to diseases like *E. coli* and porcine circovirus type 2, and changes in the gut microbiome associated with diarrhea or successful weaning. Understanding the factors affecting gut dysbiosis during weaning is crucial due to economic and health risks. Furthermore, transmission of essential gut microbes from sow to piglets, pathogens like *Streptococcus suis*, and the influence of climate and genetic background on the microbiome warrant further investigation. The main objective of this dissertation was to utilize 16S rRNA gene sequencing to characterize the microbial composition of fecal samples from sows and piglets. The study aimed to identify factors such as the sow, pen environment, and gender that contribute to similarities and differences in the piglet microbiome during lactation, with a focus on taxonomic, environmental, and genetic factors. Results of the study demonstrated the host effects of sex and genomics, and the extrinsic effect of pen environment on piglet gut microbiome composition and diversity.

Interplay between Host Genetics, Extrinsic Factors, and the Piglet Gut Microbiome during Lactation

by
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BIOGRAPHY

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CHAPTER 1

Literature Review

INTRODUCTION

The microbiome continues to be a hot topic across many levels and fields of research due largely to its impact on human and livestock health. Consequently, there is an ever-growing need to develop methods for understanding how the interactions between these microorganisms, their environments, and their hosts. Chapter 1 of this thesis aims to provide a broadly encompassing overview of the work previously done to define the microbiome, develop methods for microbial investigation, and characterize the microbiome's impact on its hosts. While the first two sections are not species-specific, the literature review's final section examines the microbiome's unique impact on swine health and production. The context provided in this chapter will help frame the research questions, statistical methods, and conclusions in Chapter 2.

BRIEF HISTORY OF METHODS AND DEFINITIONS OF THE MICROBIOME

In 1986 L.R. Hegstrand and R.J. Hine established that conventionally housed rats had significantly higher quantities of histamine found in the hypothalamus than germ-free rats (Hegstrand and Hine, 1986). Thus, providing a foundation for continued research into what is now known as the gut-brain axis and further supporting the theory of host-microbe symbiosis. A modern definition of the microbiome was defined in 1988 as "*a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties. The…term refers to microorganisms…[and] their theatre of activity.*" (Whipps et al., 1988). These early studies set the stage for continued exploration of the microbiome and its potential impact on its host organism.

Since then, several attempts have been made to find a widely accepted definition of the term. In 2001, Nobel Prize winner Joshua Lederberg claimed to coin the microbiome as an "*ecological community of commensal, symbiotic, and pathogenic microorganisms*" (Lederberg and McCray, 2001) that are contained within an organism or some other environment. Later, Marchesi and Ravel (Marchesi and Ravel, 2015) expanded on the classic ecological-based definitions of Whipps and Lederberg to include all microorganisms and their accompanying genomes and the biotic and abiotic factors of some defined environment. In the same year, Schlaeppi and Bulgarelli (Schlaeppi and Bulgarelli, 2015) defined the microbiome as only the complete set of microbiota genomes. In 2020, a seminal paper by Berg and colleagues (Berg et al., 2020) restored Whipps' original definition. They made two clarifications to the definition, stating that the microbiome includes both the microorganisms and the related theatre of activity and that it is both dynamic and interactive.

METHODS FOR MICROBIOME INVESTIGATION

To understand the complex dynamics of the microbiome and its potential impact on the host and/or environment, researchers have formulated three guiding questions: which microbes are present (Lührmann et al., 2021), what are they doing (Davids et al., 2016), and how do they interact with each other (Comolli, 2014), their host (Niederwerder, 2017), and the environment (Beveridge et al., 1997). Numerous methods have been developed to answer these questions, including techniques for extracting (Fiedorová et al., 2019), identifying, classifying (Park and Won, 2018), and visualizing (Peeters et al., 2021) the microbiome. This section will focus on the methods most relevant to the second chapter, which aims to characterize factors affecting the early piglet microbiome.

Investigation of the microbiome usually begins with the extraction of microbial DNA from the environment or the host, often by swabbing the area of interest, such as the skin, gut, or oral cavity. The extracted DNA is then quantified and sequenced, with researchers often choosing from one of four primary sequencing methods: targeted sequencing, which often involves the 16S or ITS rRNA regions; shotgun sequencing; whole genome sequencing; and transcriptomic sequencing. Once the DNA of the microbes is sequenced, it is classified into taxonomic units.

To find which microbes are present statistical analysis will often begin with a description of which microbes are there and how many of each are present in the samples. Relative abundance analysis, microbial core identification (Fiedorová et al., 2019), and rare species detection are major goals. To explore the microbial community further, researchers will often determine within-sample community diversity by calculating alpha diversity metrics using one or several available approaches. These metrics aim to determine a communities richness, evenness, or both. Beta diversity statistics and metrics are used to test between-sample or between-community differences in composition, which encompasses most analyses aimed at determining just how different microbial communities are from each other. These statistical methods often include the calculation of microbial similarity or dissimilarity between samples and testing for their degree of difference. To begin answering what the microbes are doing and how they interact with each other and with their surroundings, researchers will begin to implement network analysis, differential abundance analysis, discriminant analysis, functional annotation analysis, and more.

Sequencing Technologies

Before sequencing, scientists relied upon traditional culture methods (TCMs) to characterize the microbes in any given environment. TCMs require bacteria to be enriched in media, isolated, and then phenotypically identified through biochemical and morphological characterization. These methods hold a significant bias towards bacteria that can be cultured in a laboratory, require long incubation periods, and only identify a small fraction of microbes in a community or sample. Then, in 1953, Watson and Crick pieced together the three-dimensional, double-helical structure of DNA. A few short decades later, in 1977, the introduction of the Sanger 'chain-termination' Method began the age of sequencing (Sanger et al., 1977). Scientists could now begin to forgo traditional biochemical and morphological characterization methods for DNA sequencing. While the technology was limited, it laid the groundwork for second and third-generation sequencing in 2007 and 2011, respectively.

Before discussing methods for modern microbial sequencing methods and classification, it is important to discuss the compositional nature of microbiome sequence data. Compositional data involves measuring each sample as a vector of non-zero positive values carrying relative information (Aitchison, 1994). Such data is typically represented as a percentage of an arbitrary sum. The microbial abundance data received from modern NGS sequencers can only relay a relative microbial abundance, as the instrument has a fixed capacity and can only capture a definite number of microbes in a given sample and cannot capture actual species abundance. A few of the implications this has on the methods used to sequence and analyze microbial data will be further discussed.

In the late 2000s, sequencing technology had advanced to allow for high throughput sequencing on NGS platforms, like Illumina, Ion Torrent, and BGI/MGI. This made it possible to

rapidly and inexpensively sequence targeted DNA and whole genomes using shotgun and metagenomic sequencing. Although the cost of these methods has significantly declined, sequencing smaller, highly conserved regions of the DNA is still more cost and time-effective for studies aiming to capture the entire microbial composition of numerous communities. For this reason, many studies, including the one discussed in Chapter 2, have chosen primers targeting highly conserved DNA regions across thousands of microbial species. Two of the most commonly sequenced regions are the 16S rRNA (Kozich et al., 2013) region and the ITS rRNA region, which primarily targets fungi. First proposed for phylogenetic analysis by Carl Woese in 1977, 16S rRNA has become the standard for deep taxonomic classification of microbiota in their natural habitats (Woese and Fox, 1977). The number and depth of species captured by 16S rRNA sequencing are directly linked to the primers used during the PCR stage, which has become known as "primer bias."

Classifying Microbial Communities

With that in mind, researchers have developed several methods for classifying compositional microbial communities into taxonomic categories within the limitations of NGS technology. Two of the main methods are operational taxonomic unit (OTU) and amplicon sequence variant (ASV) assignment. The OTU was initially defined in 1963 as a taxonomic grouping method based upon some computational degree of observed similarity (Sokal, 1963). Modern approaches to OTU clustering using molecular sequence information began to arise in the early to mid-2000s (Parkinson et al., 2002), with algorithms for taxonomic separation continuing to evolve. Three general methods have become dominant for OTU assignment. The first has come to be known as closed reference assignment, where sequences are assigned to a cluster based on how similar they are to sequences in a predetermined reference cluster. While

efficient, this method is not flexible to sequences dissimilar from the reference sequences. The second method, *de novo* assignment, uses algorithms to assign sequences to clusters without a reference. Mothur (Schloss et al., 2009), one of the more widely used programs for *de novo* sequence analysis, uses the OptiClust method (Westcott and Schloss, 2017). The algorithm improved two core problems involved with OTU assignment: computational cost and quality. It first places each sequence into an existing OTU or a single OTU or species. It will then iteratively reexamine each sequence and recalculate its clustering quality. The third general method for OTU assignment is a hybrid of both closed and open reference algorithms. This approach allows for sequence clustering based on a reference but is flexible enough to create unique OTUs for sequences that are dissimilar from the reference. However, while these are robust methods for dealing with sequencing errors (Edgar, 2013), OTU clustering does not allow for high-resolution taxonomic assignment (Tikhonov et al., 2015).

In 2017, Callahan and colleagues (Callahan et al., 2017) proposed a new method for classifying microbial sequence data called an amplicon sequence variant (ASV). Initially referred to as the "exact sequence variant" (ESV), this approach assigns each unique sequence as a unique species and stores it as such in a feature table. Unlike reference-based OTU assignment, ASVs do not require any prior sequence knowledge. ASV assignment differs from *de novo* OTU clustering in that it is not dependent on the similarity or dissimilarity of other sequences. So, by utilizing the latest next-generation sequencing technologies, ASVs can provide precise, tractable, and reproducible sequence assignment.

After sequences are assigned as either an OTU cluster or an ASV, researchers will often assign the sequence unit to a correlated microbial taxonomic unit. This allows for inferences about which taxa are present and how their previously known functions may impact the

microbiome, its host, and the environment. To facilitate this process, databases have been built that combine research from across the globe. These databases classify thousands of unique ASVs and OTUs using traditional microbiology techniques. Over the years, these databases have continued to evolve, with each classifier using a unique method for database curation and maintenance. The Greengenes classifier is built on a curated *de novo* tree construction method, using 16S rRNA sequences from publicly available sequence databanks (McDonald et al., 2012). While widely used, the Greenegenes database was last updated in May 2013. Since then, several new classifiers have been developed. Among them, Silva has emerged as the largest and most popular database for researchers using 16S rRNA sequences (Balvočiūtė and Huson, 2017).

Alpha Diversity Analysis

After microbiome sequences have been assigned, through either taxonomic assignment or with ASV/OTU classification, two overarching analyses are generally followed. The first is alpha diversity (Whittaker, 1960), which involves within-sample community diversity. Alpha diversity metrics are often viewed in light of the first primary question of microbiome research: who is there, and how many? For example, alpha diversity indices have been used to study the impact of diarrhea in neonatal piglets on microbial diversity (Han et al., 2019). There are several mathematical formulas used for within-sample microbial composition. All of them aim to describe the richness of a community, the evenness of a community, or both. Richness refers to the number of unique species in a community, while evenness describes abundance distribution across the unique species community. The most basic of these formulas is the observed richness measure, which is simply the number of unique taxa or observed OTU/ASVs (Moore, 2013). The Shannon index (*H*) (Shannon, 1948) measures both richness and evenness, which is the diversity

of species across samples while placing more weight on species richness. In other words, *H* measures how similar the relative abundance of different species is in a given community. Other methods that measure species diversity include Simpson's index (Simpson, 1949) and the Chao1 index (Chao, 1984). Simpson's (*D*) index measures both richness and evenness while providing more weight to species evenness than *H*. The Chao1 index (S_{Chao1}) is an estimator of species richness based on species abundance. Metrics like Faith's phylogenetic diversity (PD) (Faith, 1992) measure the branch length between different species on a designated phylogenetic tree. Longer branches represent greater evolutionary distances and more significant contributions to overall biodiversity. Therefore, PD considers not only the number of species present but also their evolutionary relatedness. Once these metrics are calculated, numerous and ever-evolving ways exist to make statistical inferences. One simple metric is a pairwise comparison between groups, often using an ANOVA test for significant differences (Erős et al., 2020). After characterizing how experimental factors affect microbial diversity, research will move into the next general category for analysis, beta diversity.

Beta Diversity Analysis

Beta diversity tests between community or sample differences in species diversity and composition (Knight et al., 2018) to determine how different microbial communities are from each other. Often, an index of dissimilarities is calculated between samples. Popular distance and similarity indices include Euclidean, Aitchison (Aitchison et al., 2000), Bray-Curtis (Bray and Curtis, 1957), Jaccard (Jaccard, 1901), and Unifrac distances (Lozupone and Knight, 2005), each representing a different approach to analysis. The Aitchison distance, which is just the Euclidean

distance after transformation with a centered-log ratio (clr), attempts to account for the compositionality of microbial information (Gloor et al., 2017). The Bray-Curtis distance is a metric that quantifies dissimilarity between two samples based on differences in the abundance of shared species.

On the other hand, the Jaccard index is a similarity measure for the pairwise comparison of two sets (i.e., samples) utilizing species' presence or absence (0,1). The differences between Bray-Curtis and Jaccard become negligible when the presence-absence iteration of Bray-Curtis is used (Tang et al., 2016). UniFrac is a metric that measures the degree of dissimilarity between two microbial communities by comparing their evolutionary relationships in a phylogenetic tree. It quantifies the fraction of branch length that is unique to one community versus the other. This measures the phylogenetic separation between the communities (Lozupone and Knight, 2005). Ordination methods, such as PCA (Pearson, 1901), NMDS (Kruskal, 1964), and PCOA (Gower, 1966) are used to reduce the dimensionality of the data in an attempt to represent the information visually or to conduct further testing. For example, PERMANOVA, or permutational analysis of variance (Anderson, 2001), is a non-parametric multivariate test used to determine if the centroids and variance of defined groups are significantly different from each other. Both alpha and beta diversity analysis can be conducted on various platforms.

Much of the time, statistical analysis can be concluded with alpha and beta diversity analysis to answer which microbes are in a community and the magnitude of their abundance, and the degree of difference in microbial composition between communities. Hypothesis testing shows how different experimental factors can affect those. However, as mentioned above, the molecular function of those microbes and communities requires further analysis beyond the scope of this thesis.

BIOLOGICAL IMPLICATIONS OF THE MICROBIOME

The Microbiome and Host as a Symbiotic Relationship

Early understandings of the symbiotic nature between microbes and between hosts and microbes often relied upon association studies. As far back as 1683, Antonie van Leeuwenhoek used his early microscopes to identify and describe oral and fecal microbiota (Leuwenhoek A, 1683). In the process of investigating the differences between his stools while in a healthy state and a diseased state, he found protozoa in only the diseased stools. Thus, becoming the first to have ever recorded the differences in the gut microbiota in a state of disease and of health (Leuwenhoek A, 1683). While Leeuwenhoek may have been able to observe a small fraction of the microbiome, he failed to investigate and understand why certain protozoa affected his stool and how those changes came about. Microbiome research currently employs molecular and cellular high-throughput measurement techniques to shed light on the functional role of microbes in a specific environment (Heintz-Buschart and Wilmes, 2018). However, this task is challenging, as it involves deciphering the microbial ecosystem's temporal, dynamic, and interactive nature.

Since Leeuwenhoek made his first discoveries, microbiome research has evolved into immunology, food science, biotechnology, plant pathology, and many more. While there have been studies venturing into the oral (Kernaghan et al., 2012), skin (Horak et al., 2019), and lung (McCumber et al., 2021) microbiomes, the majority of research in mammalian models have focused on the microbiome of the gastrointestinal tract due to its relative ease of accessibility and sample collection compared to the other body sites. In 1977 Savage (Savage, 1977) estimated a 10:1 ratio of bacteria to human cells (B/H). However, recent research indicates a ratio of bacteria to human cells of 1.3, with an uncertainty of 25% and a variation of 53% in males averaging 70

kg (Sender et al., 2016). While these recent estimates of B/H are several factors smaller than previously estimated, there has been no indication that the understanding of the degree of impact of the microbiome on the host should also be reconsidered. It has been estimated that the gene set of microbes contained by humans is roughly 150 times larger than the host's gene number (Qin et al., 2010). The understanding of how the microbiome gene pool, and accompanying transcripts and translations, transmit and evolves is under constant research and evaluation.

Vertical Transmission of the Microbiome

There are currently several common proposals for the transmission of the microbiome to a given host. Vertical transmission is the transfer of a microbiome from parent to child without combination with microbes in the surrounding environment (Roughgarden et al., 2018). Work has been done to break down vertical transmission into three main types. The first is strict vertical transmission, a direct transfer of some or all species of the parents' microbiome to the progeny through the gamete, via transovarial transmission, or through vegetative reproduction (Moran and Bennett, 2014). Thus, this resembles the mode of gene transfer from parent to offspring. Such restrictive transfer mechanisms tend to decrease the variation and diversity of the microbes that can first inoculate the progeny. Famously, in 1998 the pea aphid *Acyrthosiphon pisum* was identified as a carrier of the gammaproteobacterial *Buchnera aphidicola* (Moran and Bennett, 2014). The phloem sap that aphids feed on is balanced due to the micronutrients and amino acids made available from their symbiosis with *Buchnera*. Transferred through the intracellular infection of oocytes, the Aphid-*Buchnera* symbiosis remains one of the few accepted forms of strict vertical transmission. As a consequence of the host-restricted

transmission pathway, the genome of *Buchnera aphidicola* has been restricted to 616-642 kb or 507-574 protein-coding genes.

The more common and widely researched “intimate neighborhood transmission” (INT) or indirect vertical transmission is the direct transfer from parent to offspring without environmental inoculation. This process involves a period of aposymbiosis, during which the host is temporarily free of symbiotic organisms. In animals, aposymbiosis occurs during germ cell development and embryonic development. During birth, the newborn will break open the amniotic sac and come in contact with the maternal vaginal and fecal microbiome, initiating microbial inoculation (Roughgarden et al., 2018). An additional mode of INT includes breastfeeding, whereby the newborn's microbiome ingests the maternal colostrum, and milk is ingested. To test the theory of INT, one study from 2015 (Milani et al., 2015) isolated and sequenced individual strains of *Bifidobacterium breve* and *Bifidobacterium longum*. The study collected fecal and milk samples from four sets of mother-child pairs and corresponding fecal samples from 3-month-old infants. Strain isolation and targeted genome reconstruction revealed that those strains were directly passed from mother to child.

Furthermore, those strains persisted in the infant gut microbiome for at least 6 months after birth. Functionally, *Bifidobacterium breve* and *Bifidobacterium longum* have evolved the ability to metabolize specific types of glycans, such as human milk oligosaccharides (HMOs). Sela and colleagues (Sela et al., 2008) found that certain HMOs in human milk were present in both the mother and child and were temporally stable over time. A similar study investigated possible vertical transmission *Streptococcus suis* (*S. suis*) from dams to piglets during birth. Samples were collected from 43 piglets born to 8 dams. Dams samples were collected from the oral and vaginal cavities. The oropharynx and dorsal surface of piglets were collected from piglets that

were removed from the vagina using sterile technique. Results show that multiple unique serotypes of *S. suis* were traced from the oropharynx of the dam to the oropharynx of that dams piglet. Thus, indicating that the source of *S. suis* was directly transferred to the dorsal surface and oral cavity of piglets when the fetus came into contact with the *S. suis* from vaginal secretions and providing evidence for vertical transfer in swine (Amass et al., 1997).

Horizontal Transmission of the Microbiome

Horizontal transmission is defined as an acquisition of microbiota from the surrounding environment (Roughgarden et al., 2018). In a classic example, the light organ of the squid *Euprymna scolopes* is exclusively colonized through the horizontal transmission of *Vibrio fisheri*. At dawn, adult squid "reset" the quantity of *V. fisheri* by venting ~90% of the crypt (Lee and Ruby, 1994), thus providing an ample quantity of parent-derived *V. fisheri* in the environment. After the squid eggs hatch, the immature light organ of the young squid is inoculated by the *V. fisheri* in the surrounding environment. Horizontal transmission is also a prevalent source of infection in livestock populations, with many disease outbreaks occurring from aerosolized, water-born, and blood-borne bacteria. In dairy cattle, one such disease is Neospora caninum, which is a leading cause of abortion. Bergeron and colleagues (Bergeron et al., 2000) tested the rates at which N. caninum was transmitted vertically and horizontally to cattle herds. To classify a case of horizontal transmission, a seropositive cow had to have been born to a seronegative dam with a minimum of 2 seronegative sisters. Of the 23 herds studied, 7 cases of horizontal transmission were identified in 6 herds.

Holobiont and Hologenome Theory of Evolution

In 1991, Lynn Margulis proposed the term “holobiont”, which came to be known as “an individual host and its microbial community (Theis et al., 2016).” From holobiont naturally emerged a new term: hologenome, or the entirety of the host and microbial genomes of a holobiont (Zilber-Rosenberg and Rosenberg, 2008). The microbial symbionts involved in holobiosis and the microbial genomes of the hologenome can be transmitted through both horizontal and vertical transmission or some combination of the two. Past and present research challenges the concept that the host and microbiome can be identified as two units. The hologenome theory of evolution proposes that there is covariance between the host organism and its symbiotic microbiota community, which can lead to phenotypic variation and natural or artificial selection (Theis et al., 2016). Dynamic interactions between the host and microbes result in what can be viewed as a single functional or phenotypic change in the holobiont. To fully understand the holobiont, it must be mentioned that strict coevolution and vertical transmission do not necessarily have to occur. Although uncommon, there are examples of strict coevolution, like the example of the obligate symbiotic relationship between *Wigglesworthia glossinidia* and the tsetse fly. *W. glossinidia* is located intracellularly in the epithelial cells of the fly (Aksoy, 2000) to provide blood-deficient nutrients like B vitamins (Douglas, 2014). Phylogenetic research indicates *W. glossinidia* form distinct lineages in concordance with the host fly species in a monophyletic taxonomic organization (Chen et al., 1999). In most cases, however, the inoculation of the progeny's microbiome often involves some mixing of microbes from the parent and the environment. The degree to which that mixing occurs is still unknown for most organisms. Furthermore, establishing a co-speciation event in mammalian organisms is an exceedingly difficult task due to the size and complexity of their metagenome and their larger

genomic and environmental complexities (Alberdi et al., 2022). One model suggests, through a simulation study, that any co-phylogenetic patterns between gut symbionts and their hosts result from allopatric host speciation and the resulting change in the diet and habitat of the host (Groussin et al., 2020). So, from a selection standpoint, the phylogenetic composition of the microbiome can be modulated by the host genome, but there is little to no evidence at this point to draw definitive conclusions about host and metagenomes evolving concordantly.

MICROBIOME AS A FACTOR IN SWINE PRODUCTION

The concept of the holobiont and the hologenome has led to increased research into the role of microbiota in swine evolution, selection, and health. One crucial aspect of this research is understanding how the gut microbiome of swine is established and its functional role in various stages of their lives (Salmon et al., 2009; Morissette et al., 2018; Bergamaschi et al., 2020; Maltecca et al., 2020; Raskova Kafkova et al., 2021). Regardless of the transmission method, the gut microbiome of a piglet, and most other mammals, is established within the first few hours of life (Nowland et al., 2022). This suggests that the establishment of the gut microbiome can have lasting effects on swine health and productivity. As a result, a comprehensive understanding of the swine gut microbiome is crucial for developing strategies to support the selection and maintenance of swine health.

Early Establishment of the Swine Microbiome

The provision of sow milk and colostrum is crucial for the postpartum development of piglets, as it serves as their only source of vitamins, essential nutrition, growth factors,

hormones, enzymes, and immune proteins (Theil et al., 2014). Maternally transferred secretory dimeric IgA (SIgA) via milk until weaning provides passive lactogenic protection, which allows piglets the time and opportunity to develop their immune response (Salmon et al., 2009). A recent study published in 2021 (Raskova Kafkova et al., 2021), investigated the impact of secretory dimeric IgA (SIgA) on "germ-free antibody-free" piglets. The researchers obtained purified SIgA from human colostrum/milk, which was subsequently digested or deglycosylated to test its effects in various molecular forms. The results revealed that SIgA protects against *E. coli* O55 infection. Another study (Sugiharto et al., 2015) assessed the impact of various milk options, including bovine colostrum, milk replacer, and conventional sow milk, on the levels of enterotoxigenic *E. coli* in the gut tissue of 23-day-old piglets. The study found that bovine colostrum and sow milk had significantly lower enterotoxigenic *E. coli* ($P < 0.001$). Gyles (1994) reported that enterotoxigenic *E. coli* is the leading cause of postweaning diarrhea, contributing to 50% of piglet deaths globally every year. These findings highlight the importance of colostrum and milk in establishing a healthy microbiome in piglets and acting as a protective barrier against harmful bacteria during the early stages of a piglet's life cycle.

Sow milk is also high in fat and contains other vital nutrients such as carbohydrates and minerals while playing a crucial role in establishing probiotic gut microbes like *Lactobacillus reuteri*, *Lactobacillus mucosae,* and *Akkermansia muciniphila* (Chen et al., 2018). Morissette and colleagues (Morissette et al., 2018) researched piglets derived from eight Yorkshire x Landrace sows that were crossbred with Duroc boars. The study found a strong correlation between colostrum intake and weight gain. Using non-metric multidimensional scaling (NMDS) and blocked multiresponse permutation procedure, they discovered that piglets with low weight gain exhibited different ileal mucosa ($p = 0.097$) and colonic lumen ($p = 0.024$) compared to high

weight gaining piglets. Additionally, the study found that piglets with a higher proportion of *Actinobacillus porcinus* and *Lactobacillus amylovorus* had a greater tendency for weight gain.

While there is a growing body of research on the swine gut microbiome, fewer studies have investigated the effects of environmental factors such as climatic stress and housing conditions on the modulation of microbiome composition. Nonetheless, some studies do suggest a relationship between these factors and alterations in the gut microbial community. In 2021 Xia and colleagues (Xia et al., 2022) continued to evaluate the effects of heat stress (HS) on the swine gut (Lambert, 2009; Baumgard and Rhoads, 2013; Pearce et al., 2013; Le Sciellour et al., 2019) while emphasizing how HS impacted the microbiome of the gut and how such changes affect host physiology. Their research on 24 crossbred boars revealed that the presence of HS (33 ± 1 °C) was associated with changes in the microbial composition of swine, which in turn led to an imbalanced production of short-chain fatty acids. Moreover, the study found that the relative abundance of *Bifidobacterium*, *Flavonifractor*, and *Thiomonas* was lower during heat exposure, whereas the relative abundance of *Chlamydia*, *Actinobacillus*, and others was significantly higher. Upon conducting further analysis of the metabolite production in the gut, the researchers discovered that opportunistic pathogens such as *Chlamydia*, *Bacteroides*, and *Staphylococcus* can establish a presence when there is a reduction in the abundance of beneficial bacteria like *Bifidobacterium* and *Lactobacillus*, which contribute to gut health. Specifically, the study found that *Chlamydia* and *Bacteroides* are negatively associated with IL-8 and IL-12, while *Staphylococcus* showed a positive correlation with MDA, GSH, and IL-2 concentrations. These findings provide further evidence that intestinal dysbiosis resulting from HS is likely to be partially correlated with significant changes in the gut microbiome.

Impact of Host Genetics on the Swine Microbiome

While the sow's milk and environment are shown to have a large influence on the early gut microbiome, genetics can also play a large role in the establishment of both pathogenic and non-pathogenic bacteria. Several studies have indicated that host genetic differences between breeds of pigs can result in differences in the composition of their gut microbiomes (Xiao et al., 2018; Crespo-Piazuelo et al., 2019; Bergamaschi et al., 2020; Ma et al., 2022a; Wang et al., 2022). In 2019, (Crespo-Piazuelo et al., 2019) used a genome-wide association study (GWAS) to indicate that 54 single nucleotide polymorphisms (SNPs), out of a 45k SNP panel, in 17 distinct regions of the genome were significantly associated with the relative abundance of; *Akkermansia, CF231, Phascolarctobacterium, Prevotella, SMB53,* and *Streptococcus* in swine. Many of the 17 associated regions are known to be involved with regulating host immunity. In 2018, (Xiao et al., 2018), conducted a study to assess the microbial variations in different gut regions of two distinct swine breeds. The alpha diversity of Jinhua pigs was found to be greater in the duodenum, jejunum, and cecum compared to Landrace pigs, and there were significant differences in beta diversity in the jejunal and ileal microbiomes between the two. They hypothesize that these differences may be due to Jinhua's inclination to be a more obese breed than Landrace, and as such, a more diverse gut microbiome could assist with metabolism and fat deposition. These studies and others (Bergamaschi et al., 2020; Ma et al., 2022a; Wang et al., 2022) suggest that understanding the effects of host genetics on the gut microbiome could be crucial for the improvement of swine productivity and health. Further research will be needed to understand better the mechanisms and pathways by which the genetics of the host and its microbiome can modulate each other.

Role of the Gut Microbiome in Swine Health and Development

Today, the gut microbiota has been well established as having a major role in the health and metabolism of swine. A body of research is emerging that indicates that the gut microbiota is involved in the regulation of the metabolism of glucose (Ma et al., 2022b), amino acids (Torrallardona et al., 2003), carbohydrates (Wang et al., 2019), lipids (Wu et al., 2021), and more. Yin and colleagues (Yin et al., 2020) showed that piglets fed a protein-restricted diet had a significantly higher abundance of *Spirochaetales* and *Gammaproteobacteria*. The relative abundance of *Lactobacillaeles*, which aids in improved piglet immunity (Wegmann et al., 2015), decreased under a protein-restricted diet (Yin et al., 2020), indicating that a balanced BCAA diet may assist growth promotion and amino acid metabolism. This study emphasizes the intricate connections between diet, metabolism, microbiota composition, and physiology.

Additionally, the gut microbiota has been shown to play a critical role in the gut-brain axis of swine. Since 1986, the work of Hegstrand and Hine (Hegstrand and Hine, 1986) has been expanded, revised, and improved upon. Emerging research shows that gut microbiota can influence immune (Saavedra, Dattilo 2012) and endocrine (CITE) pathways. Enteroendocrine cells (EECs) are specialized cells that make up roughly 1% of the total population of epithelial cells of the GI tract. Positioned near the lumen, these cells can detect and respond to luminal nutrients and microbial metabolites. In response, EECs secrete peptide hormones to regulate enzyme secretion, digestion, motility, and dietary intake (Worthington et al., 2018). The peptides that EECs release on vagal afferent fibers transmit information through the vagus nerve to the brain and play a vital role in the bidirectional communication between the gut and the brain (Cani et al., 2013). One such receptor of EECs is Gpr41, which is a G protein-coupled receptor responsible for energy regulation and gut motility (Samuel et al., 2008). One study, using mice

models, found that both germ-free and gnotobiotic mice with a recessive Gpr41 gene, colonized as young adults with *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii* were leaner and weighed less than the WT mice with a complete gut microbiome. This is associated with decreased levels of short-chain fatty acids (SCFA) that cause a reduction in the circulating levels of peptide YY (PYY), which inhibits gut motility and throws off host energy balance (Samuel et al., 2008).

The microbiome can also have a role in measurable traits that impact production traits, such as feed efficiency, which is defined as body weight gain per unit of feed consumed (Patience et al., 2015). Industry professionals aim to maximize feed efficiency to reduce feed costs, which can account for as much as 70% of the total input cost in swine production (Mullan et al., 2011), and minimize total time to slaughter. To begin elucidating how the microbiome may affect feed efficiency, a study in 2020 (Bergamaschi et al., 2020) contrasted differences in the fecal microbiome composition of three swine breeds. They found that the microbiome composition is significantly correlated with differences in feed efficiency and provides a link between the microbiome and traits that have only ever been selected for with traditional breeding techniques. These findings, and others, highlight the importance of gut microbiome composition on piglet health and welfare during and after the weaning process.

Gut Microbiome in Dysbiosis

After farrowing, piglets typically undergo a rapid microbial shift through to weaning at an age between 19 to 22 days. Weaning removes the piglet from the sow and consequently causes an abrupt change in diet from the sow's highly digestible milk to a more complex plant-based

diet formulation. Piglets also must adapt to the socially significant separation from the sow and the introduction of piglets from mixed litters and farms in a new housing environment (Lallès et al., 2007). This leads to a variety of physiological changes, including changes in energy and protein metabolism (Yang et al., 2016), growth rate (Yang et al., 2017), and increased vulnerability to diseases such as *E. coli* (Fairbrother et al., 2005) and porcine circovirus type 2 (PCV2) (Segalés and Domingo, 2002). A study by Karasova and colleagues (Karasova et al., 2021) investigated the gut microbiome of piglets pre and post-weaning, comparing those healthy to piglets that developed diarrhea. The study found that piglets with a higher abundance of *Actinobacteria* before weaning were preconditioned for diarrhea. Conversely, healthy piglets with successful weaning had a higher abundance of *Prevotella*, while piglets with increased levels of *Fusobacterium* or *Anaerovibrio* were more likely to develop post-weaning diarrhea.

Given the increased risks of gut dysbiosis, there are significant economic implications for producers. As a result, prophylactic, metaphylactic, and therapeutic antimicrobials are used in large quantities to stem and prevent disease outbreaks (Apley et al., 2012). While often successful, the overuse of antibiotics in the industry has led to bacterial resistance. If these strains spread to other farms, environments, or hosts, there is a significant health risk to both human and swine populations. As an example, the Center for Disease Control (CDC) indicates that the generation of extended-spectrum beta-lactamase (ESBL) producing bacteria, such as Enterobacteriaceae, resulting from the use of antibiotics like ceftiofur and cefquinome, represents one of the most severe and critical threats of the current era (Centers for Disease Control and Prevention (U.S.), 2019). Additionally, antibiotic use can further exacerbate gut dysbiosis and promote *Clostridioides difficile* infections in swine (Post and Songer, 2004). Recent research suggests that finishing swine treated with lincomycin have increased the

abundance of multi-drug resistant, and often infectious (Poor et al., 2017), *Clostridium* and *Corynebacterium* while at the same time decreasing the abundance of *Treponema*, *Succinivibrio*, *Fibrobacter*, and *Cellulosilyticum* which play a critical role in dietary fiber digestion in later stages of swine growth (Jo et al., 2021).

In conclusion, chapter 1 has established that extensive research has been done regarding microbes' role as symbiotic partners with their hosts. The microbiome is well established as a significant factor in swine development, health, and productivity. Furthermore, the microbiome of piglets is established at birth by the sow through her vaginal and fecal microbiome and by the milk and colostrum during lactation. The piglet gut microbiome is further shaped by the horizontal transmission of microbes, including diseases, and their genomic makeup. While these factors have been shown to play essential roles in piglet health and productivity, few studies have been able to differentiate between their relative contributions in piglets of similar genetic makeup and management practices. To address this gap, chapter 2 of this thesis will attempt to answer the question of how well piglets resemble each other microbially and which factors may be causing similarities or differences in their microbiome. Finally, chapter 3 will conclude by indicating how the results found in chapter 2 fit into the larger story researchers are building to comprehensively understand the dynamic interactions between the microbiome and its host.

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CHAPTER 2

Analyzing the influence of host genetics and environmental factors on the early piglet gut microbiome

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ABSTRACT

Background

The piglet gut microbiome is essential for health and productivity post-weaning. We evaluated the effects of sow genetics, sow microbiome, piglet sex, and litter environment on the gut microbiome of young piglets in a commercial sow farm.

Methods

At a commercial sow farm, 16S rRNA sequences were obtained from the fecal swabs of 57 sows and 306 piglets, between five and fifteen days of age. To characterize microbial composition differences, Bray-Curtis (BC) distance metrics were used to compare piglets, piglets and sows, and sows in both related and unrelated animals. Farm-wide sow and piglet microbial clusters were identified using unsupervised k-means clustering, and taxonomic abundance differences between those clusters were analyzed. The influence of genomics on the sow and piglet microbiome were evaluated, as well as variations in alpha diversity by sex, microbial cluster, and litter environment.

Results

Bacteroides, *Ruminococcus torques*, *Alistipes*, and *Butyricimonas* were found to dominate the core microbiome of piglets. Clustering analysis based on microbial composition revealed three distinct clusters of piglets and sows, with piglet clusters showing significant differences in alpha diversity. Significant differences in genus abundance were observed between piglet cluster groups, with notable changes among *Streptococcus*, *Ruminococcus torques*, *Akkermansia*, and *Parvimonas*. In contrast, the microbial clusters of the sows differed in the abundance of *Blautia*,

Mogibacterium, *UCG-005*, *Lactobacillus*, *Peptoniphilus*, and *Megasphaera*. There were no indications that sex had any effect on piglet composition or diversity.

Conclusion

While piglet sex did not affect the composition or diversity of the gut microbiome, there was a strong microbial resemblance among piglets from the same litter, thus indicating a familial influence. These findings highlight the role of the litter and the pen environment in shaping the early microbial composition of commercial piglet communities.

INTRODUCTION

The early life stage of a piglet is crucial for long-term health outcomes, especially at weaning. Abrupt changes in diet, housing environment, and social structures often induce stress in piglets during the weaning period. Biological stresses associated with these changes can lead to physiological changes, including modulations in energy and protein metabolism (Yang et al., 2016); growth rate (Yang et al., 2017); and increased vulnerability to diseases such as *E. coli* (Fairbrother et al., 2005) and porcine circovirus type 2 (PCV2) (Segalés and Domingo, 2002). Previous research has shown that as piglets adjust to a new environment, so too does their gut microbiome (Pajarillo et al., 2014; Dou et al., 2017). For instance, certain bacteria, such as *Actinobacteria*, can precondition piglets for diarrhea. Conversely, healthy piglets with successful weaning had a higher abundance of *Prevotella*, while piglets with increased levels of *Fusobacterium* or *Anaerovibrio* were more likely to develop post-weaning diarrhea (Karasova et al., 2021). Given the increased risks of gut dysbiosis and health impacts, there are significant

economic incentives for producers to understand the factors and management practices that can exacerbate or alleviate gut dysbiosis during weaning.

Previous research aimed at identifying vertical and horizontal modes of transmission essential in the establishment of the early piglet microbiome. Studies have demonstrated that sow milk plays a large role in establishing essential probiotic gut microbes like *Lactobacillus reuteri*, *Lactobacillus mucosae*, and *Akkermansia muciniphila* (Chen et al., 2018). Many of these, like *Actinobacillus porcinus* and *Lactobacillus amylovorus*, result in a greater tendency for weight gain (Morissette et al., 2018). *Streptococcus suis* (S. suis) is a Gram-positive pathogen that is commonly found in the upper respiratory tract of swine. Over the last several decades the swine industry has been economically impacted (Neila-Ibáñez et al., 2021) by *S. suis* infections, which can occur through both vertical transmission (i.e., from mother to offspring via vaginal secretions) (Amass et al., 1997) and horizontal transmission (i.e., between pigs via aerosols) (Madsen et al., 2001).

Research has also shown that climate (Pearce et al., 2013; Le Sciellour et al., 2019; Xia et al., 2022) and genetic background (Xiao et al., 2018; Crespo-Piazuelo et al., 2019; Bergamaschi et al., 2020b; Wang et al., 2022) can shape the microbiome of piglets both before and after weaning. While these studies have characterized the composition, traced sources of microbial transmission, and studied the long-term effects on host morphology, little has been done to determine which factors have the greatest impact on the microbiome of large populations of piglets born to sows with similar genomics and diet. Therefore, our study aimed to identify the factors that contribute to similarities and differences in the microbiome of piglets during lactation, including the effects of the sow, pen environment, and gender. To achieve this goal, we utilized 16S rRNA gene sequencing to characterize the microbial composition of fecal samples

collected from sows and their piglets at a commercial sow farm. Our analyses focused on determining the degree of similarity between piglets and identifying taxonomic, environmental and genetic factors that contribute to these similarities.

METHODS

Experimental animals and sample collection

The Purdue University Animal Care and Use Committee approved all procedures involving live animals (Protocol #1912001990). Animal husbandry and use protocols were based on the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, 2020). The data used in this study were provided by a commercial sow farm in North Carolina, USA. Rectal samples were collected from 57 F1 Landrace x Large White sows. Their piglets were a 3-way cross with a proprietary sire line. Diet formulations provided to the sows are given in Table 1. After 54 and 43 piglets were lost as stillborn and mummies, respectively, 817 piglets remained for sampling. 306 rectal swabs were obtained at random from the piglets of 66 sows. There were 66 pens, located in 6 rooms among 3 buildings. Samples were collected between 5 and 15 days after farrowing on 5 days between July 6th and June 29th of 2021 at the Maple Hill Sow Farm in Maple Hill, NC. Immediately following sample collection all rectal swabs were stored in labeled tubes and placed on ice before being moved for long-term storage at -80 °C for further processing.

DNA isolation and 16S rRNA sequencing

DNA was isolated from each sample using the Qiagen DNeasy Power Soil Pro Kit following product protocol, with the addition of a 10-minute, 70° C incubation before homogenization. A BioSpec Mini-Beadbeater 16 was used to homogenize material at 3450 RPM for 3 1-minute rounds (Biospec, Bartlesville, OK).

Extracted DNA was quantified using a BioDrop Duo (BioDrop, Cambridge, UK), and 50 ng from each sample were aliquoted into 96-well plates for amplification and library preparation. MiSeq library preparation and 151x151 paired-end sequencing (Illumina, San Diego, CA) was performed by the Argonne National Laboratory Institute for Genomics and Systems Biology Next Generation Sequencing Core following current Earth Microbiome Project amplification and sequencing protocols. Primers used spanned the V4 region of the 16S rRNA gene (515F: GTGYCAGCMGCCGCGGTAA, 806R: GGACTACNVGGGTWTCTAAT) (Apprill et al., 2015; Caporaso et al., 2010; Parada et al., 2016).

DNA sequence data analysis

Sequences were demultiplexed using the demux plugin from QIIME2 (Bolyen et al., 2019). Primers were removed and sequences were filtered and trimmed by DADA2 (Callahan et al., 2016), using truncation parameters determined by the Figaro (Weinstein et al., 2019) software, and subsequently merged. An amplicon sequence variant (ASV) table was constructed and chimeric sequences were removed. The latest Silva v132 (Quast et al., 2013) taxonomic classifier was used to assign taxonomy at a species level. The ASV and taxonomic table were exported to R for further analysis.

Statistical analysis

Characterizing community composition in sows and piglets

To characterize the piglet gut microbiome composition at a farm wide level, we analyzed the relative abundance and core microbiome of 306 piglets and 58 sows using 16S rRNA sequencing data. Phyloseq was used to remove singletons and transform the ASV table to a relative abundance table. A core microbiome was established for both piglets and sows, with a detection and prevalence threshold of 0.002 and 0.2, respectively. Further, a between-community, compositional analysis in piglets and sows was conducted using a pairwise Bray-Curtis (BC) distance metric. The taxonomic feature table was first pruned to remove any non-present taxa and filtered to remove rare taxa at a detection and prevalence threshold of 0.05% and 5%, respectively.

Analysis of community similarity

To assess how microbial composition varied by sex and by familial relatedness among housing environments, the degree of similarity between piglets, between piglets and sows, and between sows was measured by calculating the mean pairwise BC distance within and between family groups. The weighted mean BC distance of each contrast (piglet-piglet, piglet-sow, sow-sow) was calculated at different levels of housing location (farm, building, room, pen). This allowed for the comparison of piglets to unrelated sows and piglets housed in the same or different rooms, as well as their own sow and littermates. The average distance between sows in the same and different rooms was also calculated. To compare the differences between the microbial and genetic similarity between sows, the mean scaled genetic relationship of all sows was also calculated at each housing level.

Alpha diversity analyses in piglet populations

To assess the impact of sex, litter (i.e., both pen environment and maternal effects), and several confounding variables on the microbial diversity of piglets, we used several linear mixed-effects models. To more evenly balance the model design piglets in litters smaller than 3 were removed from the alpha diversity analysis, which resulted in piglet sample size dropping from 306 to 253. The response, or alpha diversity metrics, included measures of observed richness, Simpson's diversity index (Simpson, 1949), and Shannon's diversity index (Shannon, 1948). All diversity metrics were calculated with the R package phyloseq::estimate_richness. All measures were calculated using an untransformed ASV table. Mixed effect models were fit using the lme4 software package in R, and the fit of each model with and without random effects was assessed through unrestricted maximum log-likelihood estimation. All models without random effects were fit with stats::lm. Type 3 effect analyses were performed on mixed effect models using the lme4::anova function in R, following the Kenward-Rogers degrees of freedom approximation and *MS*, *F*, and *P* values were determined. All effects were considered statistically significant at a p-value ≤ 0.05.

Table 2 presents a summary of the distribution of four variables in the study: sex, sample collection batches (CB), parity group (PG), and lactation stage (LS). CB refers to the three-week period in which samples were collected, with each week identified as a separate collection batch. PG denotes the parity groups of sows, which were categorized into parities 2, 3, 4, and 5 and up. LS represents two general stages of lactation when piglet samples were collected (5- 10 and 11-15 days after birth). Pen and litter identifications for piglets were based on the pen location of their related sow, which was a nested concatenation of building, room, row, and pen. All piglets in the same pen were identified with the sow and therefore shared the same sample collection

batch, parity group, and lactation stage. As a result of the nesting between piglet pen and the confounding factors, we used two models. In the base model (Model I), we tested the experimental effects of sex and litter on the microbial diversity of piglets individually from any confounding variables. In the second model (Model II), we added a single fixed confounding effect to the base model (Model I) iteratively to examine if the inclusion of the environmental variables of CB and LS or the biological variable of the sows PG resulted better explained variations in alpha diversity.

We fit a base model (Model I) to examine the effects of sex and litter on piglet alpha diversity.

$$\text{I) } y_{ijk} = \mu + S_i + L_j + e_{ijk}$$

Where y_{ijk} was a vector of alpha diversity measures; S_i is the fixed effect of sex (2 levels); L_j is the random effect of the *j*th litter (63 levels) [$L_j \sim N(0, \sigma^2_L)$]; e_{ijk} is the random residual error [$e \sim N(0, \sigma^2_e)$].

A second model (Model II) was fit to account for potential environmental covariates by adding a random experimental confounding effect to the base model (Model I), which had sex as a fixed effect and litter as a random effect. Assumptions about the distributions of litter and the random residual are maintained from model I.

$$\text{II) } y_{ijkl} = \mu + S_i + X_j + L_k + e_{ijkl}$$

Where X_j is one of three fixed experimental confounding effects:

- CB_j for the fixed effect of the *j*th sample collection batch (14/20 July, 22/29 June, 6 June; 3 levels)
- PG_j for the *j*th parity group (2,3,4,5+; 4 levels)
- LS_j for the *j*th lactation stage (5-10 & 11+ days; 2 levels)

Alpha diversity analyses in piglet populations

To test for the environmental and biological effects that may impact the alpha diversity of all 57 sows the following model (model III) was fit. Each effect was fit as fixed and all abbreviations were maintained from the above piglet models, as were assumptions about the distribution of the random residual error.

III) $y_{ijkl} = \mu + CB_i + PG_j + LS_k + e_{ijkl}$

Cluster analysis in piglets

To better understand differences in piglet microbiomes that may go beyond what explained by pen environment or sex, a third grouping factor was identified using k-means cluster analysis (R package stats::kmeans). This analysis was based on the BC distance matrix of piglets and the optimal number of clusters was determined using the silhouette method (R package factoextra::fviz_nbclust). Piglet groups were visualized in two-dimensional space as a PCA plot, as shown in Figure 13. Significant differences in variance between microbial piglet groups were determined with PERMANOVA (R package adonis::adonis2) at 1000 iterations, using a single factor model formula and the BC distance matrix.

Cluster analysis in sows

Similarly, the 57 sows were clustered by a larger factor to help encapsulate the potential effects of pen environment, fluctuations in diet and feed consumption, and other unmeasured environmental confounders. PCA of sow microbial clusters were represented in Figure 13B. Additionally, genomic information from all 66 sows of the sampled piglets was used to develop a new family unit that grouped related sows together. The average molecular relationship was

calculated using an expanded database of 1639 related sows. Sows within the studied population were genotyped using the PorcineSNP50K Bead Chip (50,703 SNPs; Illumina, San Diego, CA, USA). The additive molecular relationship matrix was calculated (R package AGHmatrix::Gmatrix) (Amadeu et al., 2016) following VanRaden's first method (VanRaden, 2008). The threshold for both missing SNPs and minor allele frequency was set to 0.05. Using the first two dimensions of PCA the elbow method indicated the optimal number of kmean clusters (R package factoextra::fviz_nbclust) (Kassambara and Mundt, 2020). Significant differences in variance between microbial and genetic sow groups were determined with PERMANOVA (R package adonis::adonis2) at 1000 iterations, using a single factor model formula and the BC distance matrix.

Alpha diversity analyses of piglet clusters

We refined the alpha diversity analyses to examine the impact of the piglet microbial clusters, which represented a new experimental grouping factor, on microbial diversity. The mixed linear model framework used for models I-III was maintained in the following models (IV). The fixed random effect of litter was substituted for the fixed effect of piglet microbial cluster. The new base model (IV) is represented as

$$\text{IV)}\ y_{ijk} = \mu + PC_j + e_{ijk}$$

Where y_{ijk} was a vector of alpha diversity measures; PC_j is the fixed effect of the *j*th piglet microbial cluster (3 levels); e_{ijk} is the random residual error [e ~$N(0, \sigma^2_e)$].

Alpha diversity analyses of sow clusters

With a new experimental factor for sows, another model (V) was fit to examine if the sow clusters had significant impact on sow alpha diversity. We used the same mixed model framework as described for models (II and IV), with sow clusters fit as a fixed effect.

$$\text{V) } y_{ijkl} = \mu + SC_k + e_{ijkl}$$

Where $SC_k$is the fixed effect of the *k*th sow cluster (3 levels).

Differential abundance in sow and piglet populations

We employed a negative binomial model to determine which ASVs contributed to the differences in both sows and piglet groups (R package DESEQ2). Each model was fit with a single effect of: piglet microbial cluster, piglet sex, or sow microbial cluster. Prior to analysis, taxa with counts lower than 1 were excluded and animals with missing data were removed. The ASV feature table was filtered at a detection and prevalence threshold of 0.005 and 0.25, respectively. Only ASVs with a normalized count of at least 10 in 2 or mores samples were included. The significance of differentially abundant ASVs between each level of the design factors were obtained by the Wald Chi-Squared Test ($P < 0.05$) using the mean of gene-wise dispersion estimates, and corrected for multiple testing using the Benjamini and Hochberg method.

RESULTS

We collected fecal samples from 306 piglets of 66 sows. Fecal samples were collected from 57 of the 66 sows. 50K SNP chip information was collected for all sows. Table 2 summarizes all experimental and confounding factors. The experimental (sex) and confounding

factors (CB, LS, and PG) each had varied distributions. Roughly ten percent more piglets were male than female. A similar number of piglet fecal samples were collected during all three collection weeks. Around fourteen percent more samples were collected from 2-week-old piglets than 3-week-old piglets. Sow parity ranged from a minimum of 2 to a maximum of 7, with a slight tendency for the sows to have more parities than fewer. 3 piglets did not have a sex label and 11 piglets were missing information about their lactation stage and sow parity, and were therefore removed from the dataset for further analyses.

Piglet community composition

Before analyzing the taxonomic differences between piglet groups, we conducted a farm-wide examination of the microbial composition in all 306 sampled piglets to gain insight into the overarching microbial structure between 5 and 15 days of age. Among the 27 phyla detected, just 5 accounted for >85% of the microbial individuals observed phyla: *Firmicutes* (56.1%), *Bacteroidota* (18.2%), *Proteobacteria* (6.2%), *Actinobacteriota* (3.6%), and *Spirochaetota* (2.8%), as indicated in Figure 1. In 20% of samples, the core microbiome of piglets consisted of 96 ASVs (0.2% detection). As shown in Figure 2, further analysis of the core microbiome revealed that 4 prominent phyla (*Firmicutes*, *Bacteroidota*, *Desulfobacterota*, and *Proteobacteria*) comprised ~90% of 9 total phyla, with *Firmicutes* (60.4%) being the most abundant phyla. Genus-level analysis of the core found that 4 genera (*Bacteroides* (7.3%), *Ruminococcus torques* (5.2%), *Alistipes* (4.2%), and *Butyricimonas* (4.2%)) combined to represent ~21% of all genera in the piglet population, as presented in Figure 3.

A similar analysis of the 57 sows revealed that 5 phyla accounted for approximately 92% of the total microbial composition, as reported in Figure 4. Further analysis showed that the core

microbiome of sows was primarily composed of *Firmicutes* and *Bacteroidota*, as depicted in Figure 5. Figure 6 illustrates that at the genus level, *UCG-005, Prevotellaceae NK3B31 group, Blautia*, and *UCG-002* were the most prevalent genera in sows.

Microbial and genomic similarity was assessed within and between communities using a pairwise BC distance metric, as reported in Figure 7. There were 4 primary goals for this analysis. The first was to test if related piglets (same pen) had a more similar microbial composition than unrelated piglets. As presented in Figure 7A, the overall average BC distance between piglets (Piglet-Piglet) was 0.78 (SD= 0.09). Weighted mean differences were calculated for piglets housed in different rooms (μ= 0.78, SD= 0.08), unrelated piglets in the same room (μ= 0.76, SD= 0.08), and related piglets in the same pen (μ= 0.68, SD= 0.11). The second goal was to determine if piglets had more similar microbial composition to their sow than an unrelated sow. Figure 7A reports the overall mean BC distance between piglets and sows (Piglet-Sow) was 0.93 (SD= 0.05). Weighted mean differences were calculated for piglets and sows in different room environments (μ= 0.93, sd= 0.03), piglets and unrelated sows in the same room (μ= 0.93, sd= 0.02), and piglets and their mother (μ= 0.92, sd= 0.05). A third goal was to determine piglets of the same sex had a more similar microbiome than piglets of a different sex. In Figure 7B, we provide the BC distances between female piglets (μ= 0.78, SD= 0.10), male piglets (μ= 0.77, SD= 0.09), and male-female piglet pairs (μ= 0.78, SD= 0.09).

Finally, we were interested if the microbiome or genomics of sows differed by housing environment. Figure 7C indicated the population wide mean BC distance between sows to be 0.55, with a sd of 0.11. Weighted mean differences were calculated for microbial distances between sows of different rooms (μ= 0.56, sd= 0.53, sd=0.08, and sows in the same room environment (μ= 0.53, sd= 0.10). Likewise, Figure 7D reports the population wide average

genomic relationship between sows as 0.22 (SD+0.08), along with the weighted mean genomic relationship between sows of different rooms (μ= 0.21, sd=0.08) and sows in the same room environment (μ=0.22, sd= 0.09).

Analysis of alpha diversity in piglets and sows

Models were first fit to test the experimental effects of piglet sex and pen environment on within community diversity. Sow models were also fit to test for the effects of confounding factors on sow microbial diversity.

Piglet model I:

The type 3 ANOVA results presented in Table 3 indicate that sex did not have a statistically significant effect on observed richness (F= 0.22, P= 0.64), Shannon's diversity (F= 0.47, P= 0.49), or Simpson's diversity (F= 2.31, P= 0.13). Further examination of the least squares means (LSMeans) estimate for sex in Figure 8A reveals that there was no significant difference between males and females for observed richness (*P* = 0.64, SE= 3.33), Shannon's diversity (*P*= 0.49, SE= 0.05), or Simpson's diversity (*P*= 0.13, SE= 0.01). In addition, random effect intercepts were obtained, as shown in Figure 8B. As reported in Table 4, the differences in log-likelihood (LogL) between the null model and model without the random effect of litter were: -15.5 for observed richness, -4.6 for Shannon's diversity, and -1.9 for Simpson's diversity.

Piglet model II:

The results from the type 3 ANOVA presented in Table 3 suggest that there was no significant impact of sex on observed richness, Shannon's diversity, or Simpson's diversity in any of the three confounding models tested (LS, CB, and PG). The F-values for the impact of the sex

effect on observed richness ranged from <0.001-0.45 with corresponding P-values ranging from 0.50-0.98. For the effect of sex on Shannon's diversity, F-values ranged from 0.01-0.52 with corresponding P-values between 0.47 and 0.93. Furthermore, there is no indication sex impact Simpson's diversity (*P*: 0.13 - 0.32; F: 1.0 - 2.35). Among the confounding variables, all three significantly impacted observed richness (*P*<0.01, F: 3.90-14.62), but did not significantly change Shannon (*P*: 0.20 – 0.39; F: 0.95-1.66) or Simpson (*P*: 0.50-0.72; F: 0.44-0.47) measures of alpha diversity.

Figures 9a, 10a, and 11a present LSMs for the fixed effects of sex and one of the three confounding effects. These do not indicate significant differences between male and female piglets across any of the three confounding models tested. Among observed richness measures, the LSMs of sexes were not significantly different (*P*: 0.50 - 0.98; SE: 3.30 - 3.33). Similarly for Shannon's diversity, P-values ranged from 0.47 to 0.93 (SE= 0.05). For Simpson's diversity, P-values ranged from 0.13 to 0.32 (SE= 0.01) for the differences between sexes. The LSM differences between lactation stages were significant for observed richness (*P* <0.01; SE= 4.85), but not for Shannon's diversity (*P*=0.20; SE= 0.07) or Simpson's diversity (*P*= 0.50; SE <0.01). The only significantly different collection batches were between the last week and the first two weeks for observed richness metrics (*P* <0.01). Only piglets born to sows in their second and piglet born to sows in their fourth parity had significantly different observed richness (*P* <0.01, SE= 7.27), with no differences between parities in Shannon's or Simpson's diversity indices.

Random effect intercepts are shown in Figure 9b, 10b and 11b. LogL differences between the null model and model without the random effect of litter ranged between 7.2 and 10.8 for observed richness, 3.9 and 5.3 for Shannon's diversity, and 1.5 and 2.9 for Simpson's diversity, as reported in Table 4.

Sow model III:

Table 5 depicts the type 3 ANOVA for sow model III in which LS, CB, and PG were all fit as fixed effects. There were no indications that LS had a significant effect on observed richness (F=0.30, *P*=0.59), Shannons's diversity (F=1.44, *P*=0.24), or Simpson's diversity (F=1.29, *P*=0.26). Furthermore, there were no indications that CB had a significant effect on observed richness (F=1.98, *P*=0.15), Shannon's diversity (F=1.36, *P*=0.27), or Simpson's diversity (F=1.17, *P*=0.32). Finally, PG did not significantly affect observed richness (F=2.02, *P*=0.12), Shannon's diversity (F=0.57, *P*=0.64), or Simpson's diversity (F=0.54, *P*=0.66). Figure 12 provides support for the absence of statistically significant differences ($P > 0.05$) in the LSMeans between the contrasting levels of CB, PG, and LS for any of the alpha diversity measures.

Clustering sows and piglets

The sex and litter effects in piglet populations, and the confounding effects in sow and piglet populations did not capture the full degree of separation in piglet and sow microbiomes. To account for any unexplained effects, both sows and piglets were clustered. Single factor PERMANOVA analysis revealed that piglet cluster group membership accounted for 2.8% of microbial variance and significantly partitioned sows ($P < 0.001$). Similarly, microbial cluster group membership accounted for 15.5% of microbial variance and significantly partitioned sows ($P < 0.001$). Piglets, in Figure 13A, and sows, in Figure 13B, were found to separate into three unique clusters, as shown in. Figure 13C indicates that piglets did not appear to cluster by sow or by sow cluster. Sows were too closely related to each other genetically for them to be separated into larger familial groups.

Alpha diversity testing with microbial cluster groups

Piglet model IV:

Linear models were used to investigate differences in alpha diversity among piglet clusters. Piglet grouping was included as the sole fixed effect. Type 3 ANOVA presented in Table 6 indicated that piglet clusters had a significant impact on observed richness ($P<0.001$, $F=44.33$), Shannon's diversity ($P<0.001$, $F=104.17$), and Simpson's diversity ($P<0.001$, $F=63.74$). Figure 14 shows that piglet cluster 1 had significantly different values for observed richness ($P<0.001$, SE: 4.7), Shannon's diversity ($P<0.001$, SE: 0.06), and Simpson's diversity ($P<0.001$, SE: 0.01) from that of cluster 2 and cluster 3. Piglet cluster groups 2 and 3 were not significantly different from each other for observed richness ($P=0.12$, SE: 3.5) and Simpson's diversity ($P=0.25$, SE: 0.01). However, the two clusters differed significantly in Shannon's diversity ($P<0.001$, SE=0.04).

Sows model V:

To evaluate the fixed effect of sow clusters on alpha diversity, a linear model was employed and the results were presented as ANOVA Table 7. Type 3 ANOVA analysis revealed that sow clusters had a significant impact on observed richness ($P=0.03$, $F=3.75$) and Simpson's diversity ($P=0.016$, $F=4.46$), while no significant effect was observed for Shannon's diversity ($P=0.10$, $F=2.37$). As shown in Figure 15, sow clusters 2 and 3 showed a significant difference in observed richness ($P=0.02$, SE=19.9), while sow cluster 1 was not significantly different from clusters 2 ($P=0.11$, SE=18.5) or 3 ($P=0.53$, SE=15.1). Moreover, no significant difference was observed between clusters 1 and 2 ($P=0.63$, SE=0.13), 1 and 3 ($P=0.09$, SE=0.11), or 2 and 3

(P=0.71, SE=0.14) for Shannon's diversity. Clusters 1 and 3 were found to be significantly different for Simpson's diversity (P=0.03, SE=0.01).

Differential abundance testing between sexes and between piglet microbial clusters

To further classify the taxonomic features between piglet and sow groups contrasts were attained between sexes, and between piglet microbial groups. Figure 16 depicts the results of differential abundance testing between males and females. This revealed six significantly different genera with an absolute log2 fold change of at least one. Of these, four belonged to the *Firmicutes* phylum and two to the *Bacteroidota*. *Ruminococcus torques* and *Bacteroides* represented four of the 12 differentially abundant ASVs. The two largest log2FoldChange differences in abundance were observed for *Parvimonas* and *Bacteroides*, with log2FoldChanges of 1.58 and 1.61, respectively

A total of 292 piglets were used to identify differentially abundant taxa between piglet clusters and visualized in Figure 17. After ASVs were filtered by a detection and prevalence threshold, 155 unique taxa were identified. After controlling for statistically significant ASVs ($P < 0.05$), a total of 13, 16, and 8 genera displayed significant differences with an absolute Log2FoldChange of at least one among piglets for 1 vs. 2, 1 vs. 3, and 2 vs. 3 clusters, respectively. Within these, 46% (6), 56% (9), and 88% (7) were of the *Firmicutes* phylum for 1v2, 1v3, and 2v3, respectively. The genera with the greatest absolute Log2FoldChange were *Streptococcus* (-4.49) followed by *Ruminococcus torques* (-2.90) for the 1v2 contrast. Meanwhile, the largest absolute Log2FoldChange was observed for *Ruminococcus torques* (-2.60) followed by *Akkermansiagroup* (-2.22) for the 1v3 contrast. Lastly, *Streptococcus* (2.35)

followed by *Parvimonas* (-2.12) showed the largest absolute Log2FoldChange for the 2v3 contrast.

Differential abundance testing between sow microbial clusters

Contrasts between sow clusters were also obtained and visualized in Figure 18, in an effort to further decompose the taxonomic features that cause differences between sows. The sow cluster abundance analysis identified 62, 59, and 45 significantly different genera with an absolute Log2FoldChange of at least one for 1v2, 1v3, and 2v3 clusters, respectively. Of these, 63% (39), 58% (34), and 58% (26) were of the *Firmicutes* phylum for 1v2, 1v3, and 2v3, respectively. The genera with the largest absolute Log2FoldChange in the 1v2 contrast were Blautia (-3.84) followed by *Mogibacterium* (-3.75) (Fig. 3). In the 1v3 contrast, *UCG*-005 (-3.51) followed by Lactobacillus (-3.43) showed the largest absolute Log2FoldChange (Fig. 3). Lastly, *Peptoniphilus* (4.78) followed by *Megasphaera* (-4.52) exhibited the largest absolute Log2FoldChange for the 2v3 contrast.

DISCUSSION

The objective of this study was to investigate the factors contributing to differences in piglet gut microbiomes shortly after birth. Previous research by Theil et al. (2014), Ma et al. (2022), and Xia et al. (2022) identified the sow, host genetics, and environment as main drivers of the gut microbiome, but their relative contributions remain unclear. In this study, we collected data from commercial three-way crossbred piglets and their LR x LW sows.

Findings indicated that piglet sex did not affect gut microbiome composition or diversity. Alpha diversity analysis (Observed, Shannon, Simpson) confirmed little variation in diversity between the sexes, as shown in Tables 3 and 4. This is consistent with previous research that

indicates castrated males do not have different gut microbiomes from co-housed females (Xiao et al., 2016). Given the young age of the piglets, the hormonal differences between males and females are negligible, which explains the similarities observed between un-castrated males and females.

It was also found that piglets from the same litter exhibited a stronger microbial resemblance compared to piglets from unrelated familial groups in different pens and rooms (Ruczizka et al., 2020). The influence of litter membership on microbial diversity was further supported by alpha diversity testing. Several factors contribute to the variation in microbiomes between litters, including genetic makeup, vertical transmission (from sow), and horizontal transmission (e.g., environmental, litter). Previous studies have shown the genetic influence breed on the microbiome in swine populations (Lu et al., 2018; Yang et al., 2018), with specific SNPs shown to significantly impact the microbiome (Bergamaschi et al., 2020a). . However, the present study, which analyzed genomic relationships among sows using 50K SNPs, revealed a genetically homogeneous population with limited variability. Consequently, drawing definitive conclusions regarding how piglet genetics influenced their microbiome was not feasible.

Vertical transmission from sow to piglet occurs during birth via placental and microbial transmission (Roughgarden et al., 2018) and through sow milk and colostrum (Sela et al., 2008; Milani et al., 2015). In this study, we simplified these transmission modes into a single main "sow effect." Initially, significant differences were observed between the microbiomes of piglets and their sows, regardless of familial relationship. However, variations in sow microbiomes could still impact their piglets. Factors such as lactation stage, collection batch, and parity did not significantly affect sow alpha diversity or composition. Instead, all interactive and main effects were consolidated into three distinct microbial sow groups. Although these sow clusters

exhibited differences in alpha diversity and composition, they did not separate based on the same factors that differentiated piglet groups. Additionally, the primary genera distinguishing piglet and sow groups were not the same. To further understand how individual sows affect the microbiome of their litters, similar studies analyzing microbial sequences obtained from the milk of the sows would provide valuable insights into unraveling the intricate relationship between sow and piglet microbiomes.

The key bacterial taxa associated with piglet microbial differences were primarily from the *Firmicutes* phylum and the *Streptococcus*, *Ruminococcus torques*, *Akkermansia*, and *Parvimonas* genera. Alterations in the abundance of *Firmicutes* have been significantly tied with alterations in feed behavior (He et al., 2022). While Streptococcus encompasses more than 50 recorded species, it is widely recognized for its pathogenic associations with diseases (Vecht et al., 1992; Chen et al., 2020). However, the majority of the species are simply common commensal organisms found in the respiratory tract (Gottschalk and Segura, 2019). Several ASVs were identified with the *R. torques* genus, none were classified to the species level. Limited research in humans suggests *R. torques* may be associated with gut dysbiosis (Wang et al., 2013). *Akkermansia muciniphila* has been found to be negatively associated with dysbiosis, such as ulcerative colitis and metabolic disorders, in various mammalian species (Derrien et al., 2017). *Parvimonas* are more highly expressed in swine with *S. suis* (Niazy et al., 2022) and is significantly associated with gut dysbiosis and cancer in human populations (Higashi et al.). It's important to note that these genus and compositional differences between piglet groups only capture differences at a single life stage. Furthermore, long-term information on phenotypic outcomes of the piglets was not collected. Future research should investigate how weaning

affects microbial separation in piglets and explore the potential long-term phenotypic differences and health outcomes resulting from these microbial groupings.

Assumptions about the effects of horizonal transmission were limited due to experimental design. Microbe transmission within the pen was absorbed by the litter effect, as well as the individual sow effect. Conducting microbial sampling of the physical pen slat could provide insights into microecological differences between pens and help draw conclusions about how those differences affect the piglet microbiome. None of the confounding factors examined had a significant impact on piglet microbial composition or alpha diversity.

CONCLUSIONS

The study found that sex had no significant impact on the composition or diversity of the piglet gut microbiome. However, a notable pattern emerged, revealing stronger microbial resemblance among piglets from the same litter, indicating a familial influence on the piglet microbiome. The core microbiome of piglets was predominantly composed of *Bacteroides*, *Ruminococcus torques*, *Alistipes*, and *Butyricimonas*. Differences between piglet populations were primarily characterized by differences in the abundance of *Streptococcus, Ruminococcus torques, Akkermansia,* and *Parvimonas* genera. These results provide valuable insights into the early microbial composition of commercial piglet communities, highlighting the significant influence of both familial factors and the pen environment on the microbiome.

Tables

Table 1. Ingredient composition for the standard diet for sows during the present study period

Sow Feed Formula	**Gestation Period**
Ingredients	**Percent of Feed Weight (%)**
Corn Fine Micron	71.380
Sow Blend	17.500
Soybean Meal	6.800
Limestone	1.550
Phosphate 21% Monocal	1.050
Salt	0.525
Fat	0.250
Lysine HCl 98%	0.230
Trace Mineral PC Sow 2.5#	0.125
Threonine	0.125
Choline Cl Liq 70%	0.100
MHA Dry 84%	0.090
MMobilize (flow agent)	0.075
Sodium Metabisulfite (Flow Agent)	0.075
SFD Sow/Nur Vit CP	0.075
Tri-Basic Copper Chloride	0.025
Tryptophan	0.025
Total	**100**

Sow Feed Formula	**Lactation Period**
Ingredients	**Percent of Feed Weight**
Corn Fine Micron	48.560
Soybean Meal	28.750
Bakery Meal	15.000
Fat	4.350
Limestone	1.200
Phosphate 21% Monocal	1.100
Dynamate	0.750
Salt	0.425
Lysine HCl 98%	0.255
Trace Mineral PC Sow 2.5#	0.125
Choline Cl Liq 70%	0.100
Threonine	0.090
MMobilize (flow agent)	0.075
SFD Sow/Nur Vit CP	0.075
MHA Dry 84%	0.070
Sodium Metabisulfite (Flow Agent)	0.050
Tri-Basic Copper Chloride	0.025
Total	**100**

Table 2. Summary of the frequency of piglets sampled at each level of different experimental and confounding factors

Factor	Level	n	%
Sex	F	132	43.14%
	M	171	55.88%
	Unknown	3	0.98%
CB	Week 1	86	28.10%
	Week 2	101	33.01%
	Week 3	119	38.89%
LS	Days 5-10	166	55.1%
	Days 11-15	124	41.2%
	Unknown	11	3.65%
PG	2	58	19.3%
	3	53	17.6%
	4	84	27.9%
	5+	95	31.6%
	Unknown	11	3.65%
Total		306	100%

CB= collection batch; LS = lactation stage; PG= parity group; Unknown= piglets missing information; n= number of piglets at each level; %= percent of total piglets represented in each level

Table 3. Analysis of Variance (ANOVA Type III) results for piglet models I-II

Model	Fixed effects	d.f.	Observed			Shannon			Simpson		
			MS	F	p	MS	F	p	MS	F	p
Model I	Sex	1	160.17	0.22	0.64	0.08	0.47	0.49	0.01	2.31	0.13
Model II	Sex	1	1.65	<0.001	0.96	<0.001	0.01	0.91	<0.001	1.10	0.30
LS	LS	2	10352.44	14.62	**<0.001**	0.27	1.66	0.20	<0.001	0.47	0.50
Model II	Sex	1	329.69	0.45	0.50	0.09	0.52	0.47	0.01	2.35	0.13
CB	CB	2	8257.55	11.20	**<0.001**	0.17	0.95	0.39	<0.001	0.49	0.61
Model II	Sex	1	0.64	<0.001	0.98	<0.001	0.01	0.93	<0.001	1.01	0.32
PG	PG	4	2776.87	3.90	**<0.01**	0.23	1.42	0.25	<0.001	0.44	0.72

Model I is a mixed linear effects model where the fixed effect of sex and the random effect of litter is tested against three alpha diversity metrics (Observed, Shannon, Simpson)

Model II is a mixed linear effects model where the fixed effect of sex, a single confounding variable (LS, CB, PG) and the random effect of litter is tested against three alpha diversity metrics (Observed, Shannon, Simpson)

d.f.= degree of freedom; MS= mean sum of square; LS = lactation stage; CB= collection batch; PG= parity group; L= random effect of Litter

P-values bolded where significance ($P < 0.05$) is found

Table 4. Log-likelihood results of models I and II

Model	Fixed effects	Random effects	Model	Observed	Shannon	Simpson
				LogL	LogL	LogL
Model I	Sex	Litter	Full	-1436.9	-178.3	495.1
			(-)Litter	**-1452.4**	**-182.9**	**493.2**
Model II LS	Sex + LS	Litter	Full	-1372.6	-162.2	486.4
			(-)Litter	**-1383.4**	**-167.9**	**483.5**
Model II CB	Sex + CB	Litter	Full	-1426.9	-177.3	495.6
			(-)Litter	**-1434.1**	**-181.2**	**494.1**
Model II PG	Sex + PG	Litter	Full	-1373.8	-160.9	486.8
			(-)Litter	**-1384.5**	**-166.2**	**484.0**

The log-likelihood of models I-II are first evaluated as a full linear mixed model. A single fixed or random effect is then literately removed while maintaining all other effects.

LogL= log-likelihood; LS = lactation stage; CB= collection batch; PG= parity group
LogL values bolded where removal of effect negatively affected model fit

Table 5. Analysis of Variance (ANOVA Type III), results for sow model III

Fixed effects	d.f.	Observed			Shannon			Simpson		
		MS	F	p	MS	F	p	MS	F	p
LS	1	784.55	0.30	0.59	0.19	1.44	0.24	<0.01	1.29	0.26
CB	2	5206.53	1.98	0.15	0.18	1.36	0.27	<0.01	1.17	0.32
PG	3	5313.17	2.02	0.12	0.08	0.57	0.64	<0.001	0.54	0.66
Residuals	49	2624.67			0.13			<0.01		

Sow model III is a linear effects model where fixed effects (LS, CB, PG) are tested against three alpha diversity metrics (Observed, Shannon, Simpson)

d.f.= degree of freedom; MS= mean sum of square; LS = lactation stage; CB= collection batch; PG= parity group; L= random effect of Litter

Table 6. Analysis of Variance (ANOVA Type III) results for piglet models IV

Model	Effect	d.f.	Observed			Shannon			Simpson		
			MS	F	p	MS	F	p	MS	F	p
Model IV	PC	2	34472.344	44.332	< **0.001**	12.347	104.174	< **0.001**	0.097	63.743	< **0.001**
	Residuals	298	777.589			0.119			0.002		

Model IV is a mixed linear effects model where the fixed effect of piglet cluster is tested against three alpha diversity metrics (Observed, Shannon, Simpson)

d.f.= degree of freedom; MS= mean sum of square; LS = lactation stage; CB= collection batch; PG= parity group; PC= piglet cluster
P-values bolded where significance ($P < 0.05$) is found

Table 7. Analysis of Variance (ANOVA Type III) results for sow model V

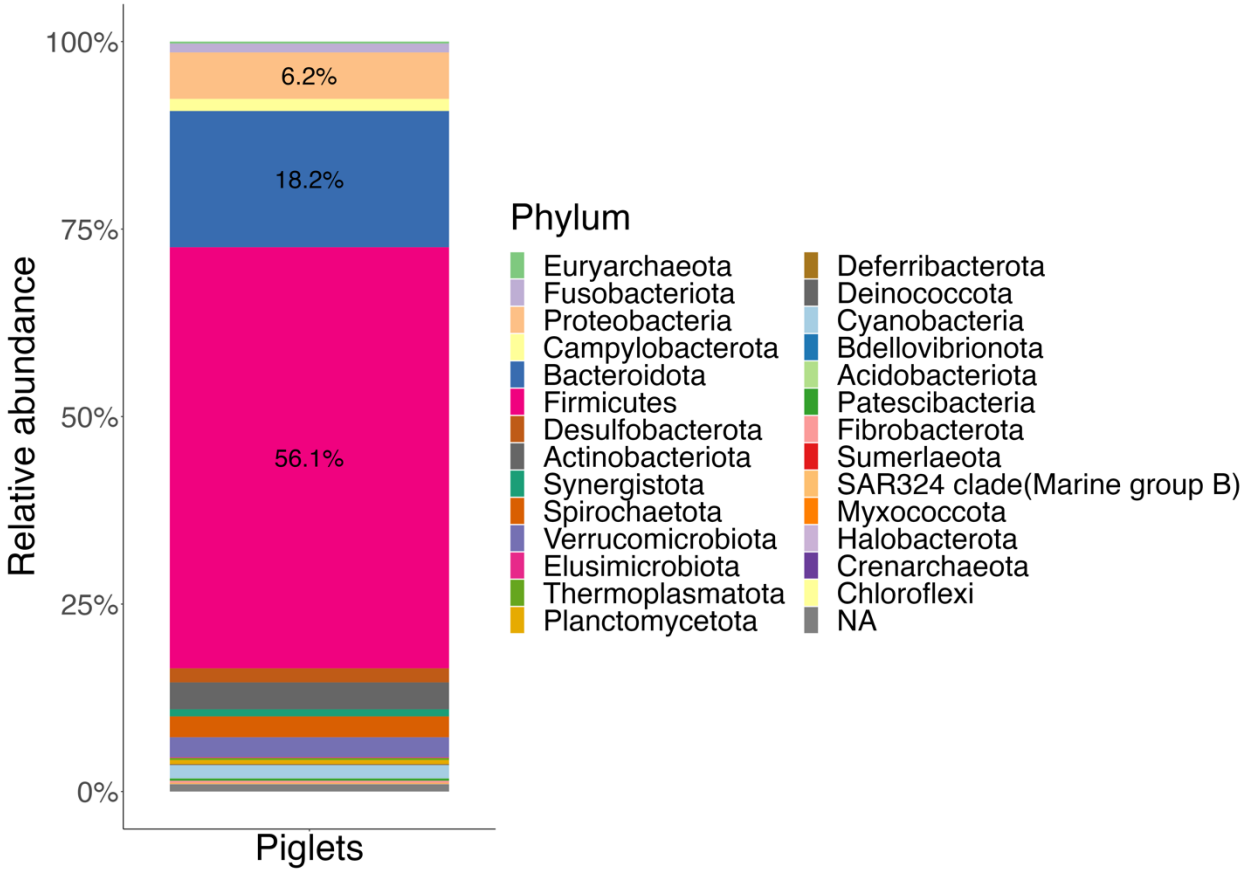
Model	Effect	d.f.	Observed			Shannon			Simpson		
			MS	F	p	MS	F	p	MS	F	p
Model V	SC	2	9510.023	3.750	**0.030**	0.291	2.365	0.104	0.004	4.462	**0.016**
	Residual	54	2535.778			0.123			0.001		

Model V is a mixed linear effects model where the fixed effect of sow cluster is tested against three alpha diversity metrics (Observed, Shannon, Simpson)

d.f.= degree of freedom; MS= mean sum of square; LS = lactation stage; CB= collection batch; PG= parity group; L= random effect of Litter; SC= sow cluster
* P<0.05; ** P<0.01; ***P<0.001
P-values bolded where significance ($P < 0.05$) is found

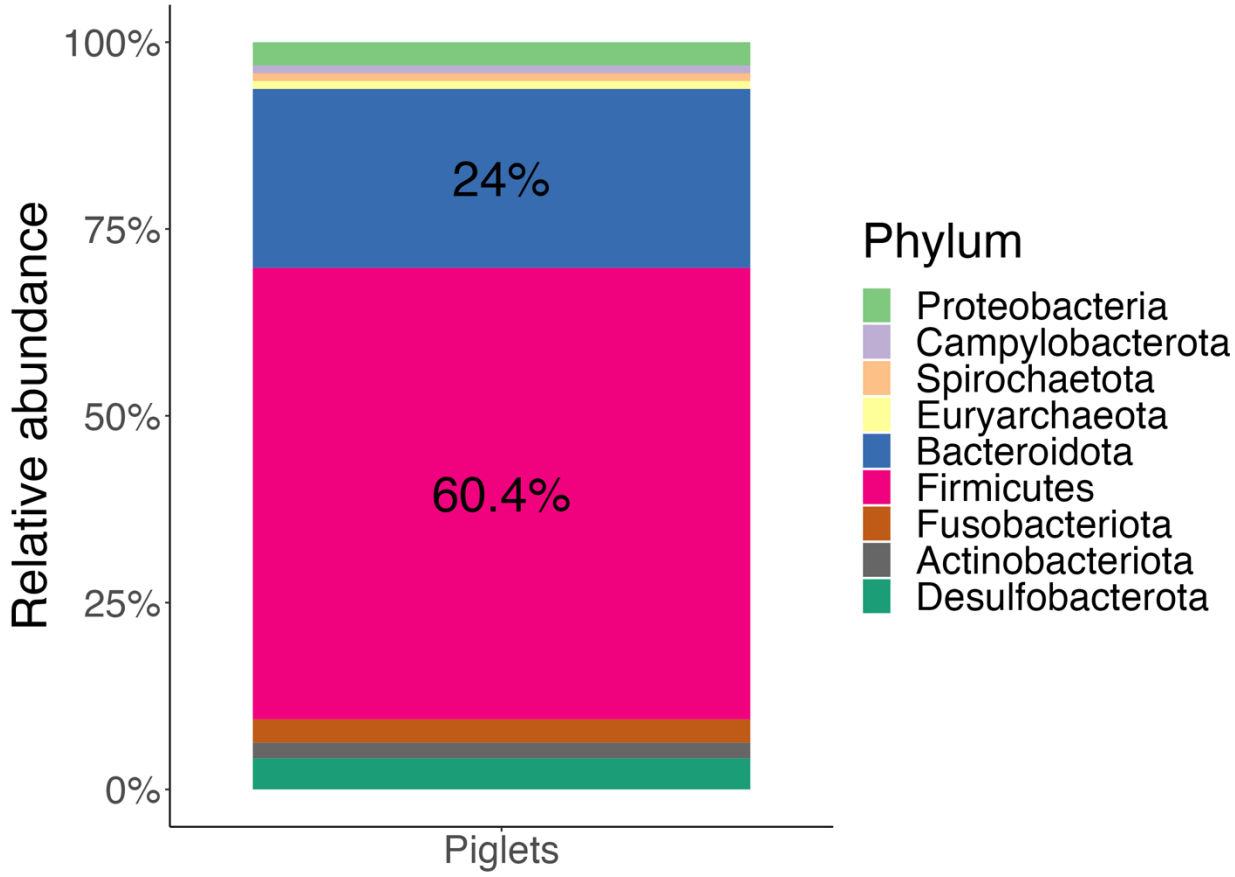
Figures

Figure 1.



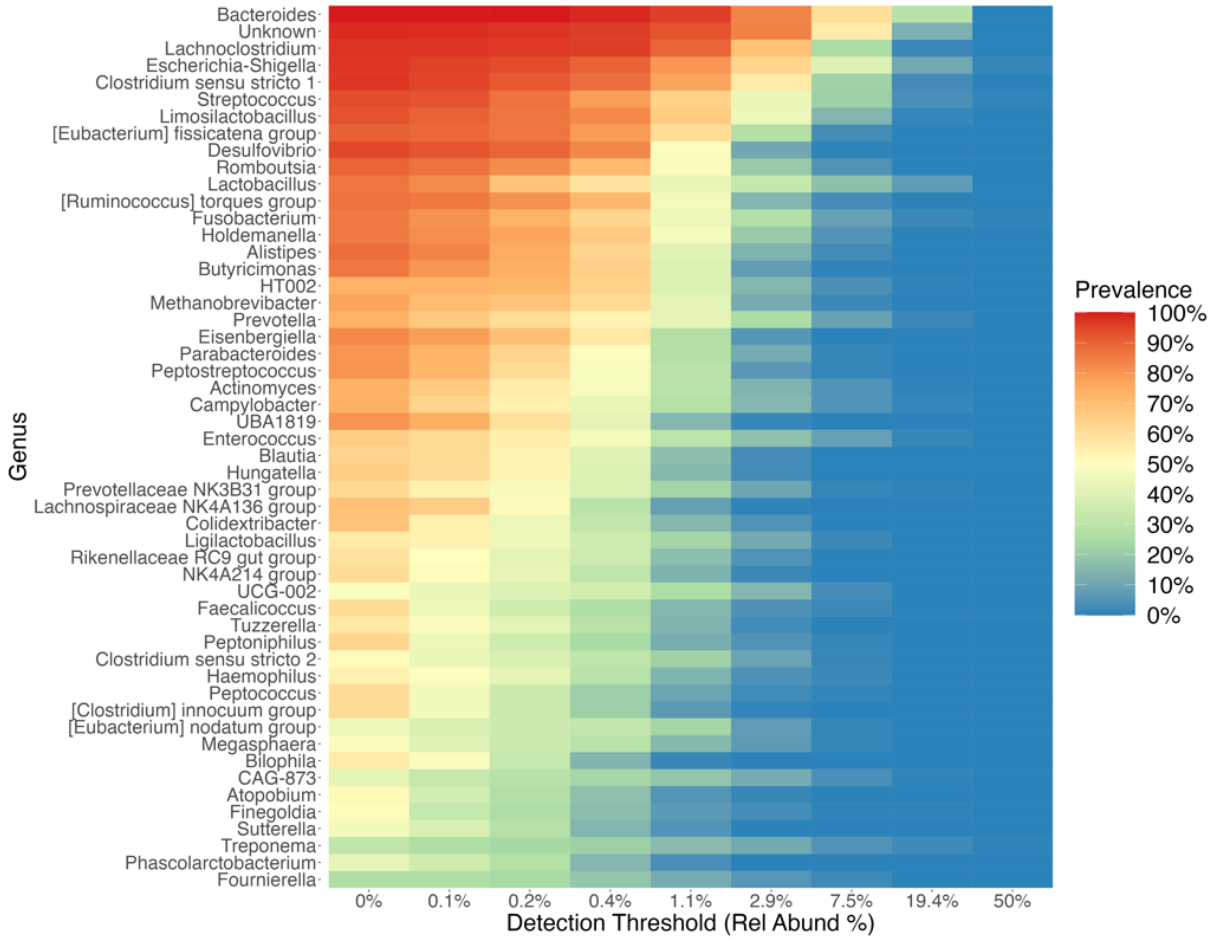
Relative abundance of microbiome taxa at the phylum level in a farm-wide population of piglets between five and ten days of age

Figure 2.



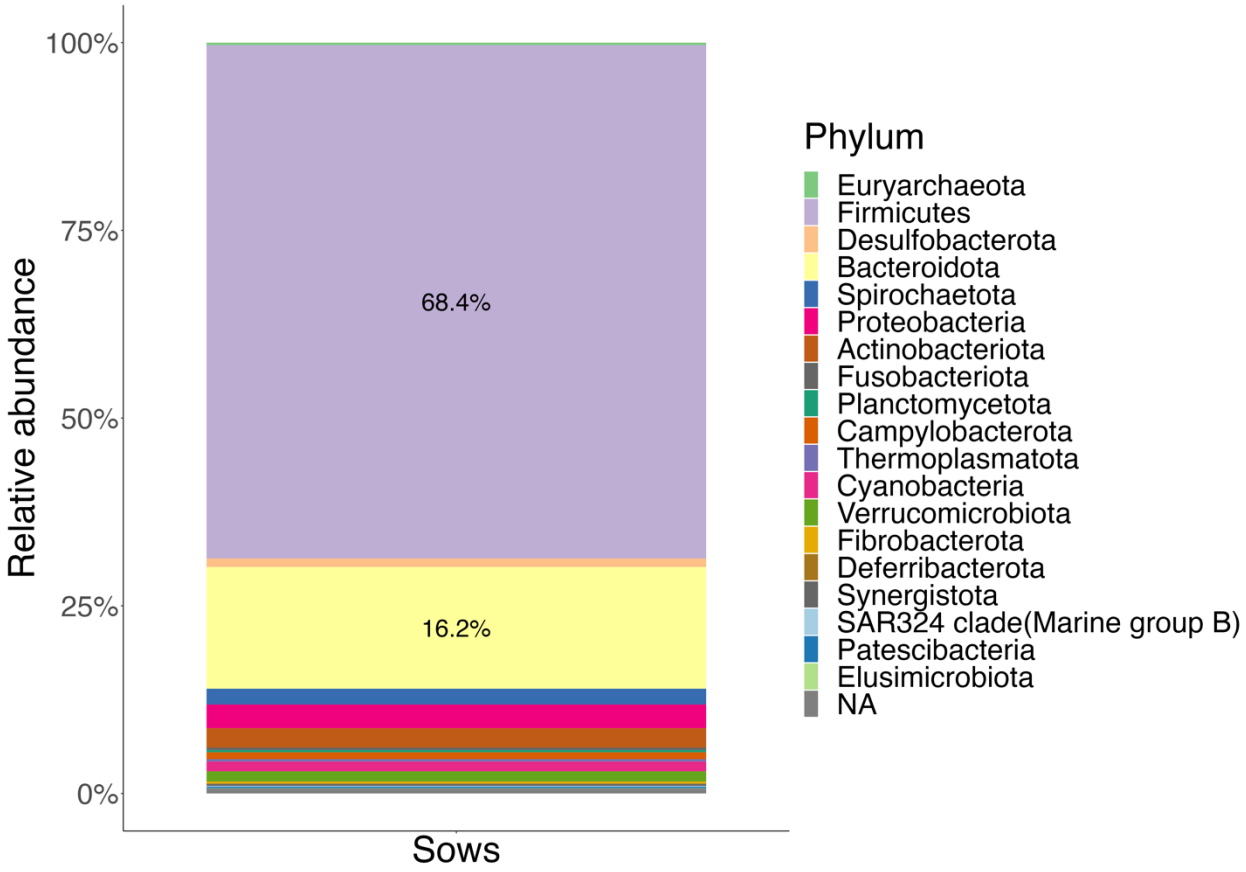
Relative abundance of core microbiome taxa at the phylum level in a farm-wide population of piglets between five and ten days of age

Figure 3.



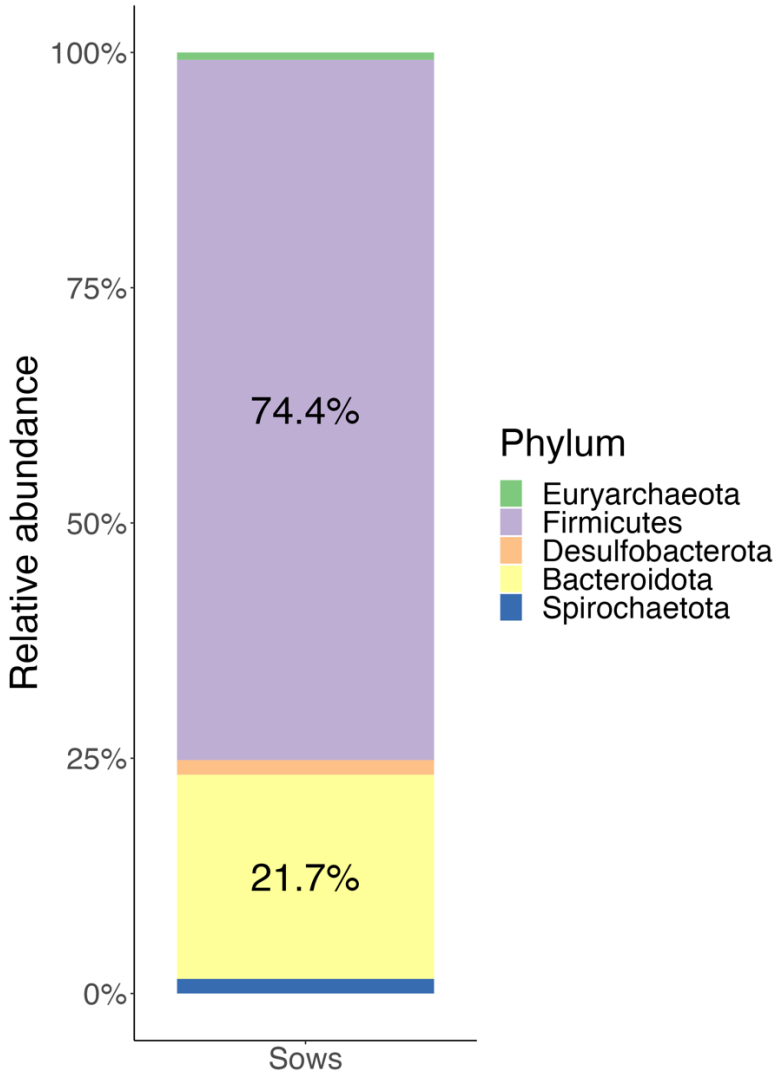
Heatmap indicating the microbial core bacterial genera across different detection thresholds in a farm-wide piglet population.

Figure 4.



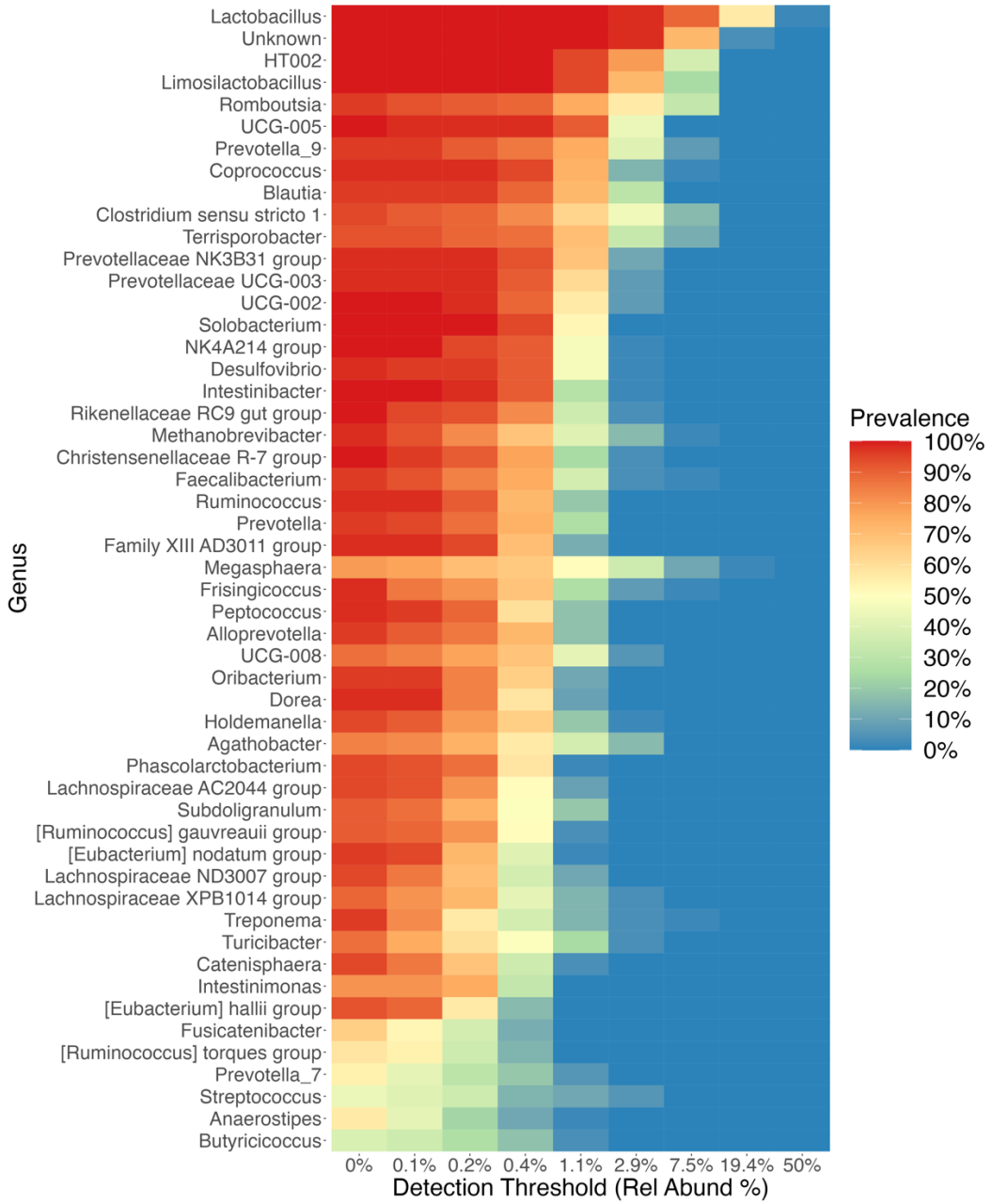
Relative abundance of core microbiome taxa at the phylum level in a farm-wide population of sows

Figure 5.



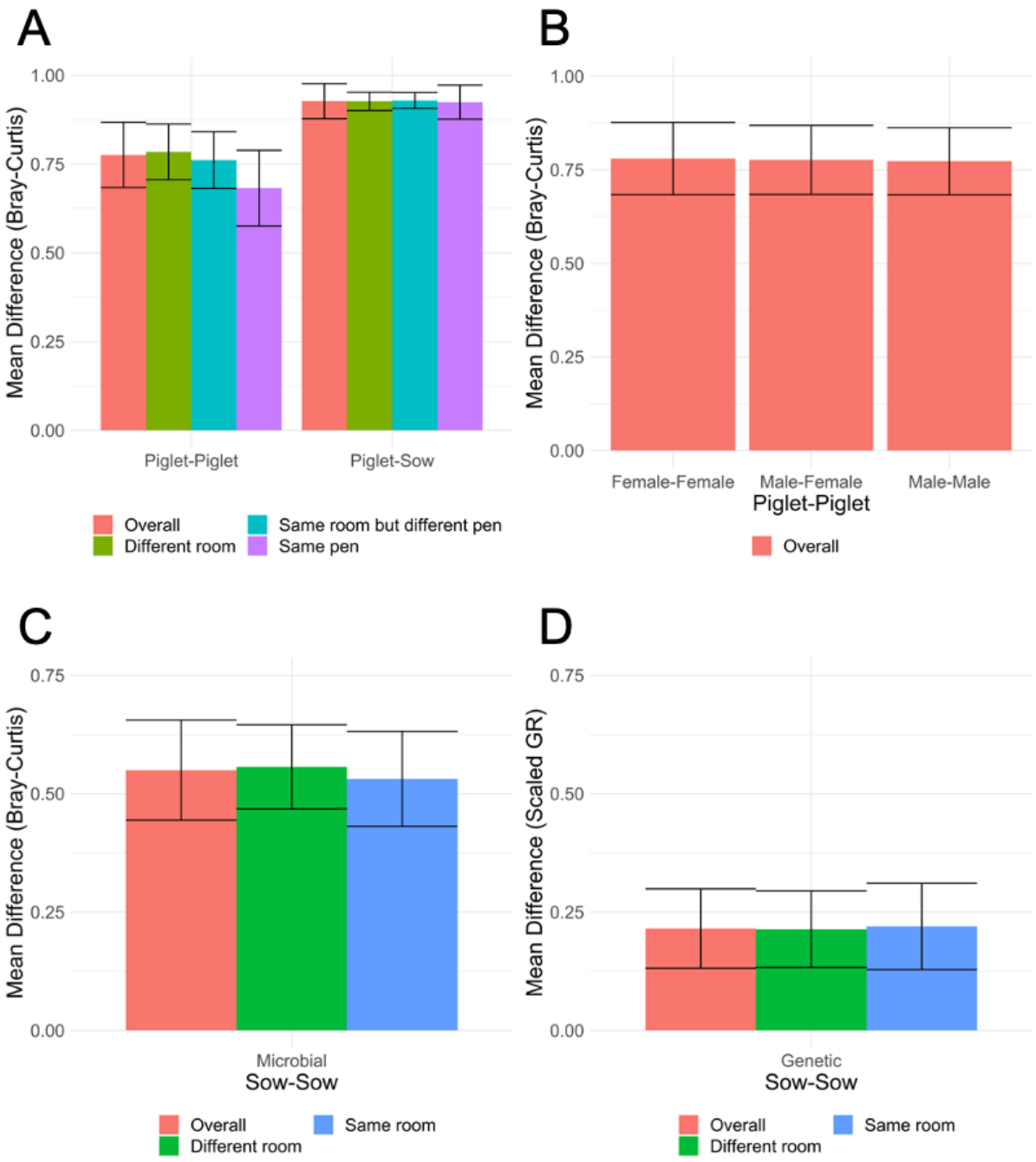
Relative abundance of core microbiome taxa at the phylum level in a farm-wide population of sow

Figure 6.



Heatmap indicating the microbial core bacterial genera across different detection thresholds in a farm-wide sow population.

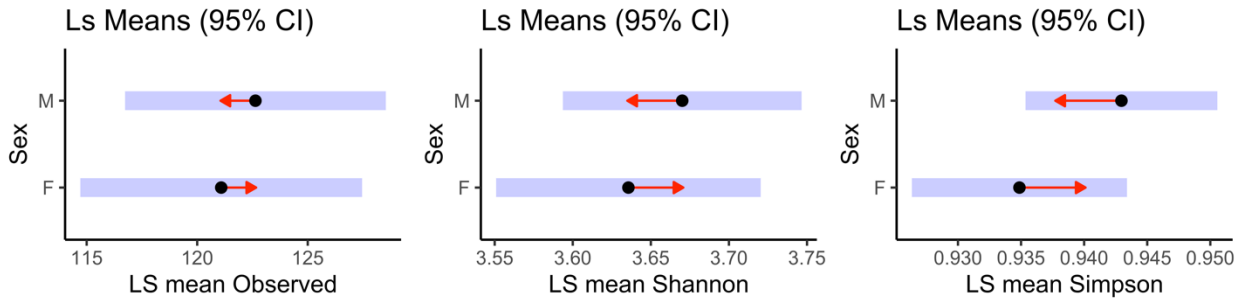
Figure 7.



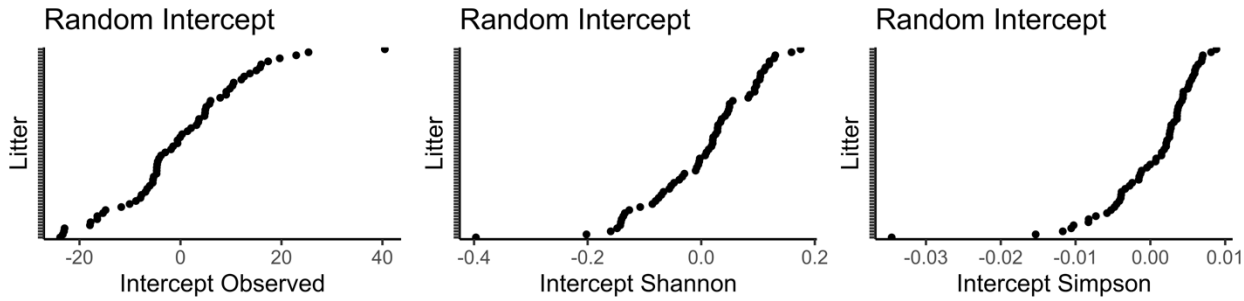
Microbial and genomic community similarity within (pen) and between (building, room) family groups. Panels (A-C) depicts the mean (± 95% CI) Bray-Curtis (BC) distance between family members (same pen) and between different families (different room or pen). 'Piglet' indicates five-to-fifteen-day old pigs. 'Sow' indicates the mothers of those piglets. Panel (D) depicts the mean scaled genomic relationship between sows at each housing level (overall, different rooms, and same room).

Figure 8.

A)



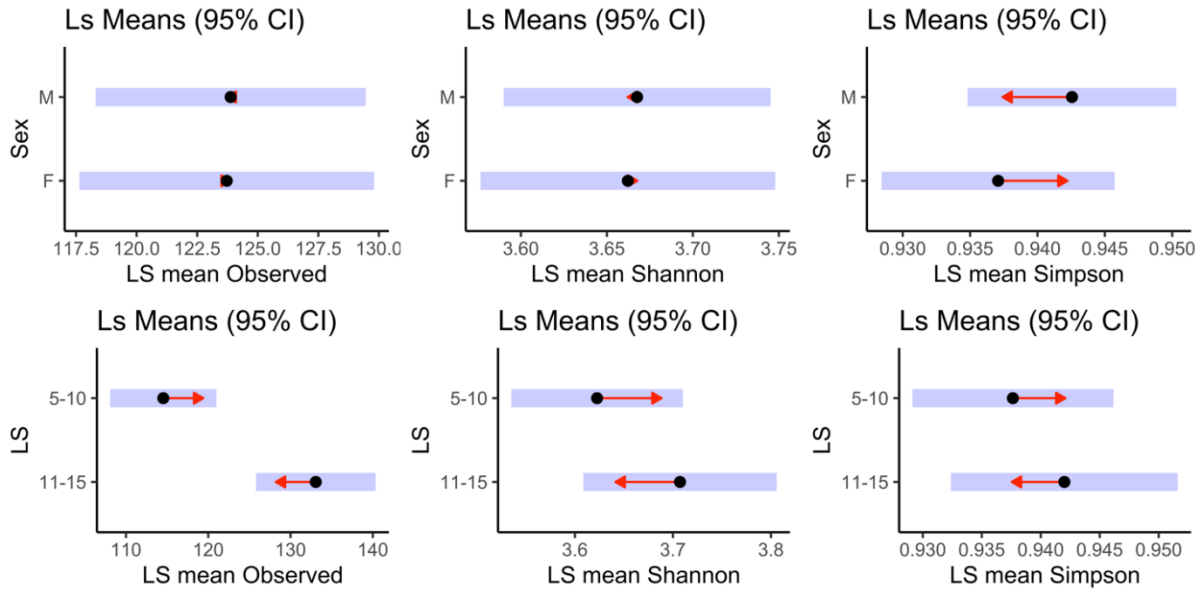
B)



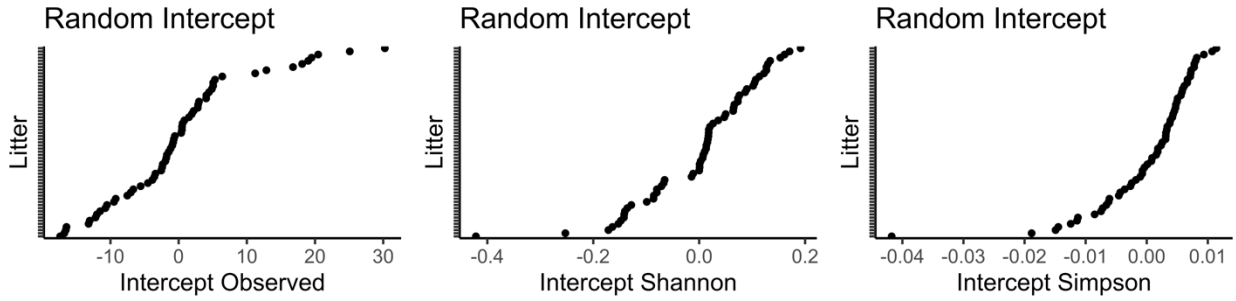
The least-squared means (95% CI) and conditional modes of piglet model I, with a fixed effect of sex and random effect of litter as predictors for alpha diversity measures (observed richness, Shannon's diversity, and Simpson's diversity). Panel (A) presents the least-squared mean (Ls Means) (95% CI) estimates for the fixed effect of sex. Red arrowed lines determine homogeneous groups, with overlapping red lines indicating that two groups are not significantly different, based on a post hoc Tukey test. Panel (B) depicts the conditional modes of the random effect of litter across all three alpha diversity measures.

Figure 9.

A)



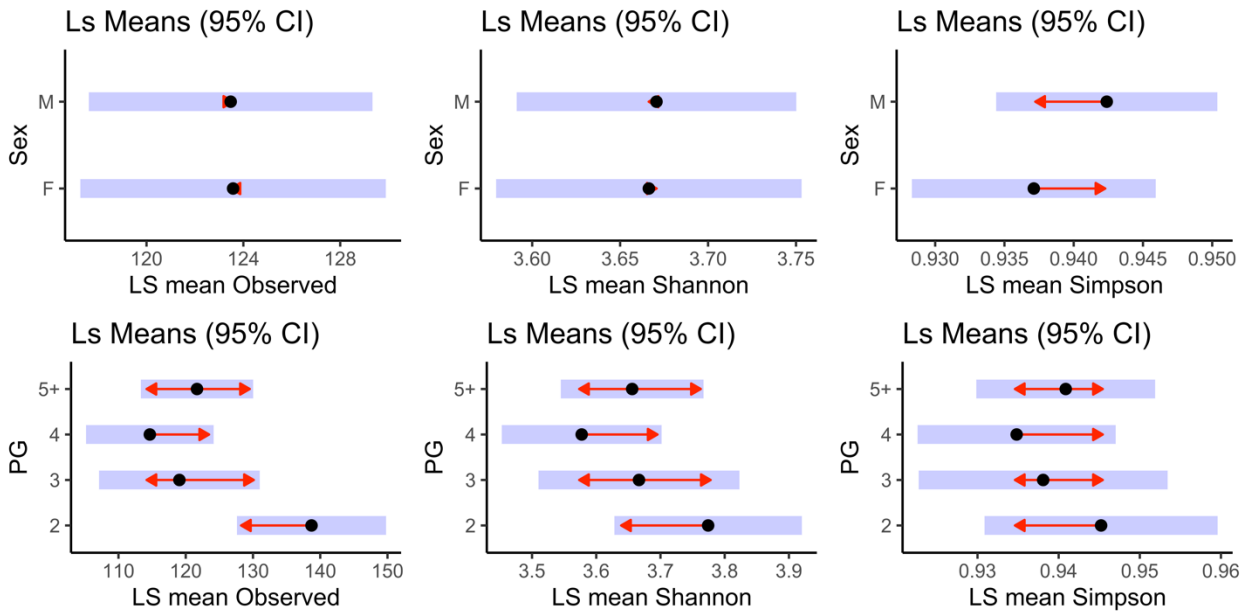
B)



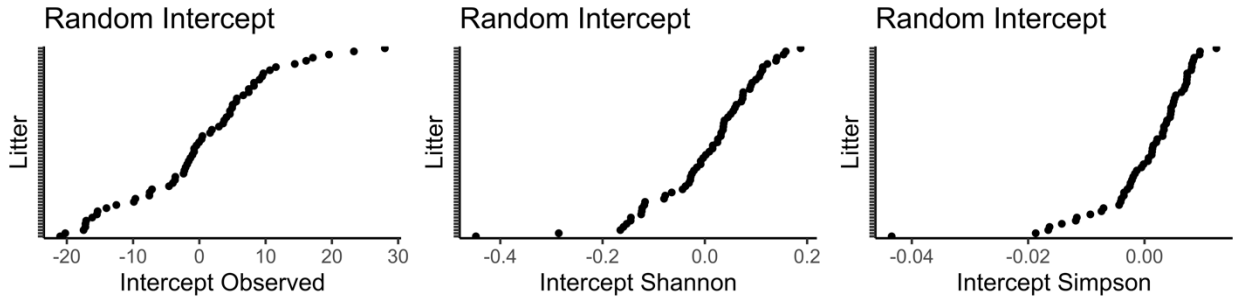
The least-squared means (95% CI) and conditional modes of piglet model II, with the fixed effects of sex and lactations stage (LS) and the random effect of litter as predictors for alpha diversity measures (observed richness, Shannon's diversity, and Simpson's diversity). Panel (A) presents the least-squared mean (Ls Means) (95% CI) estimates for the fixed effects of sex and LS. Red arrowed lines determine homogeneous groups, with overlapping red lines indicating that two groups are not significantly different, based on a post hoc Tukey test. Panel (B) depicts the conditional modes of the random effect of litter across all three alpha diversity measures.

Figure 10.

A)



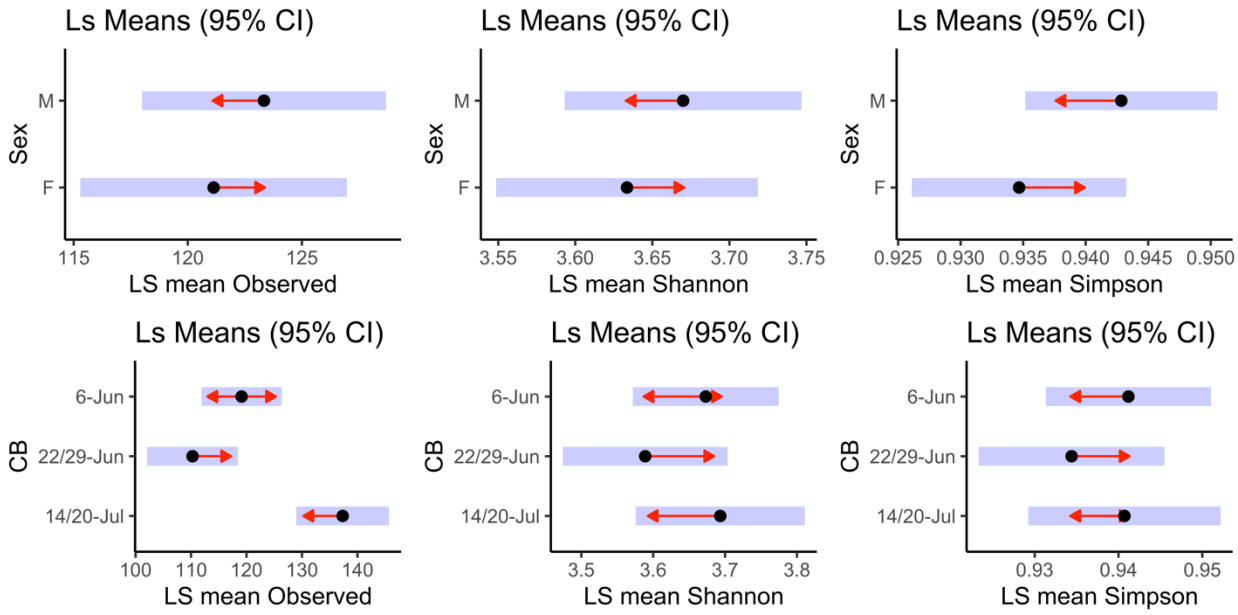
B)



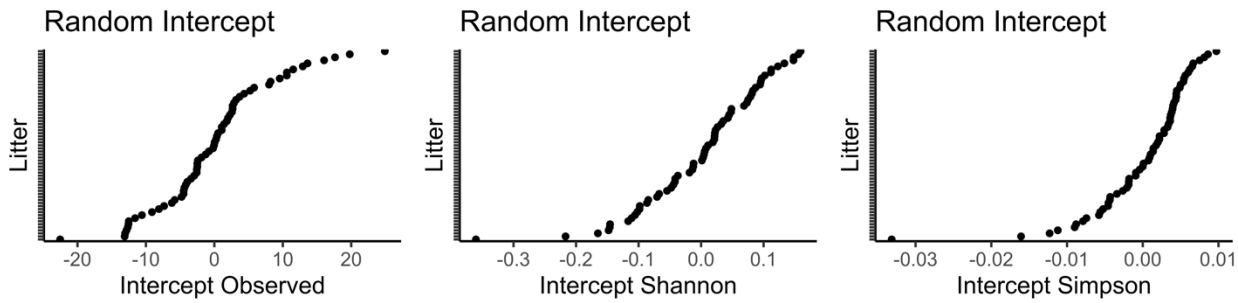
The least-squared means (95% CI) and conditional modes of piglet model II, with the fixed effects of sex and parity group (PG) and the random effect of litter as predictors for alpha diversity measures (observed richness, Shannon's diversity, and Simpson's diversity). Panel (A) presents the least-squared mean (Ls Means) (95% CI) estimates for the fixed effects of sex and PG. Red arrowed lines determine homogeneous groups, with overlapping red lines indicating that two groups are not significantly different, based on a post hoc Tukey test. Panel (B) depicts the conditional modes of the random effect of litter across all three alpha diversity measures.

Figure 11.

A)

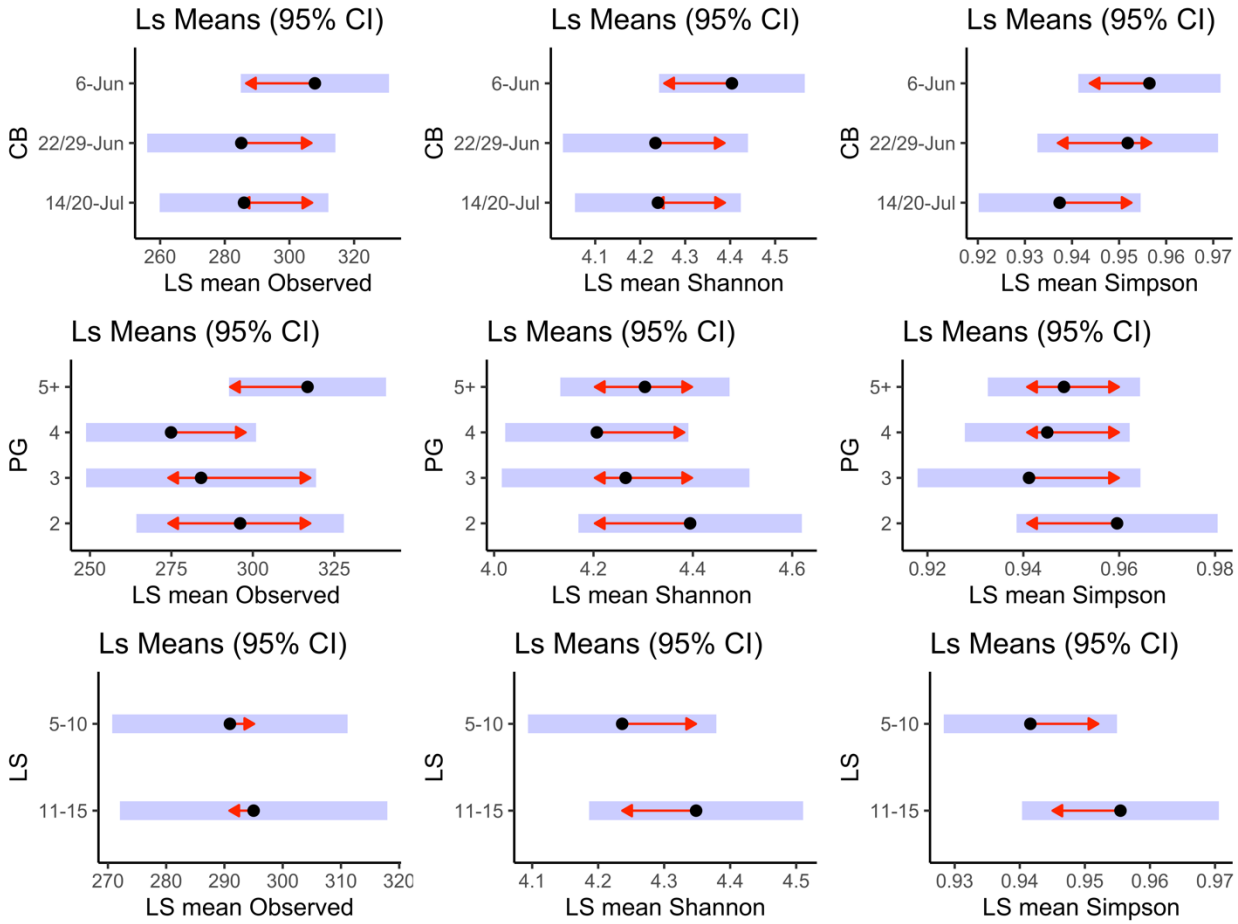


B)



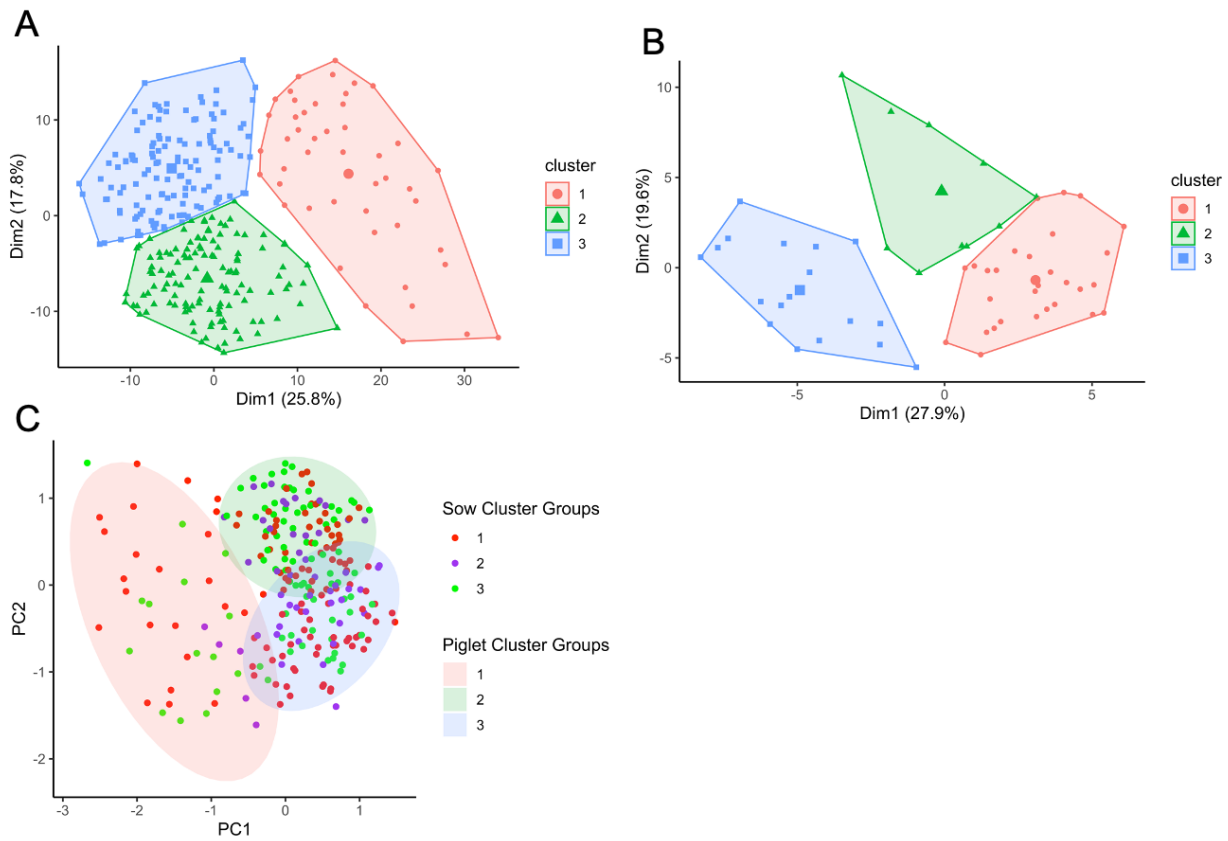
The least-squared means (95% CI) and conditional modes of piglet model II, with the fixed effects of sex and collection batch (CB) and the random effect of litter as predictors for alpha diversity measures (observed richness, Shannon's diversity, and Simpson's diversity). Panel (A) presents the least-squared mean (Ls Means) (95% CI) estimates for the fixed effects of sex and CB. Red arrowed lines determine homogeneous groups, with overlapping red lines indicating that two groups are not significantly different, based on a post hoc Tukey test. Panel (B) depicts the conditional modes of the random effect of litter across all three alpha diversity measures.

Figure 12.



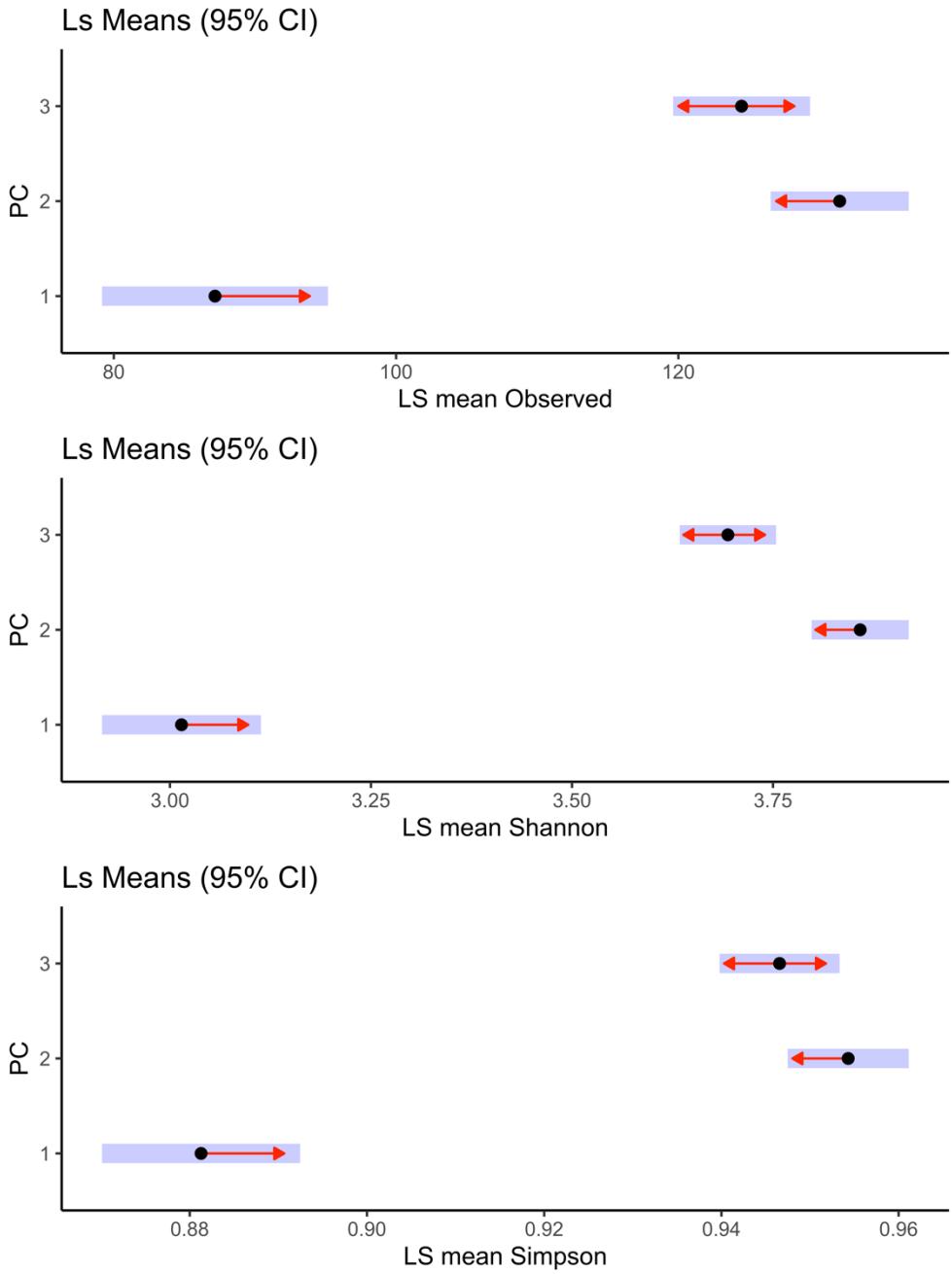
The least-squared mean (Ls Means) (95% CI) estimates of sow model III for the fixed effects of fixed effects of collection batch (CB), parity group (PG), and lactation stage (LS). Red arrowed lines determine homogeneous groups, with overlapping red lines indicating that two groups are not significantly different, based on a post hoc Tukey test.

Figure 13.



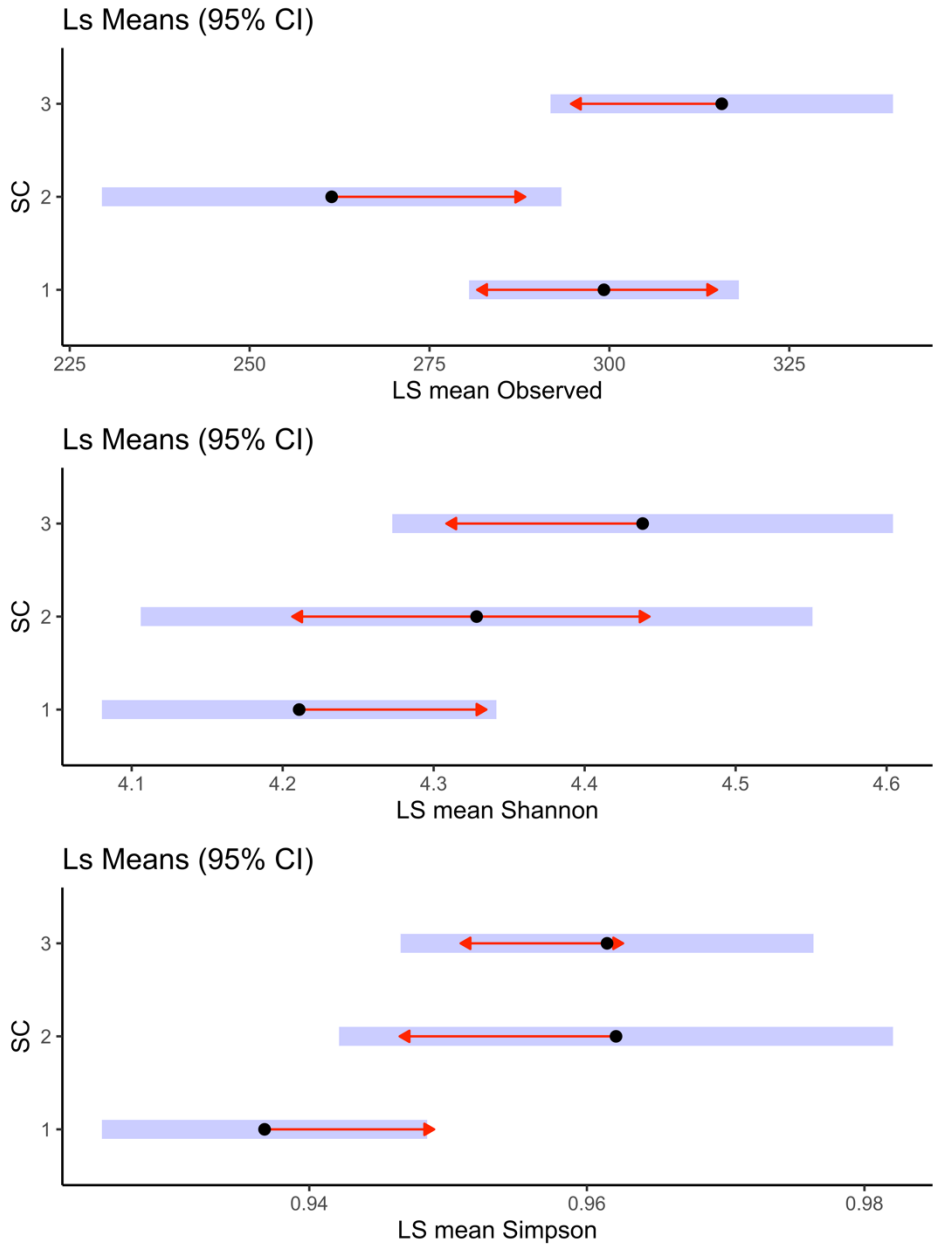
Variation between the gut microbiota communities of piglets (A and C) and sows (B). Panels (A and C) show the PCA plot of microbial clusters, using Bray-Curtis (BC) distances between piglets. Panel (B) shows the PCA plot of microbial clusters, using BC distance between sows. Panel (C) shows how well piglet clusters identify with sow clusters in PC space.

Figure 14.



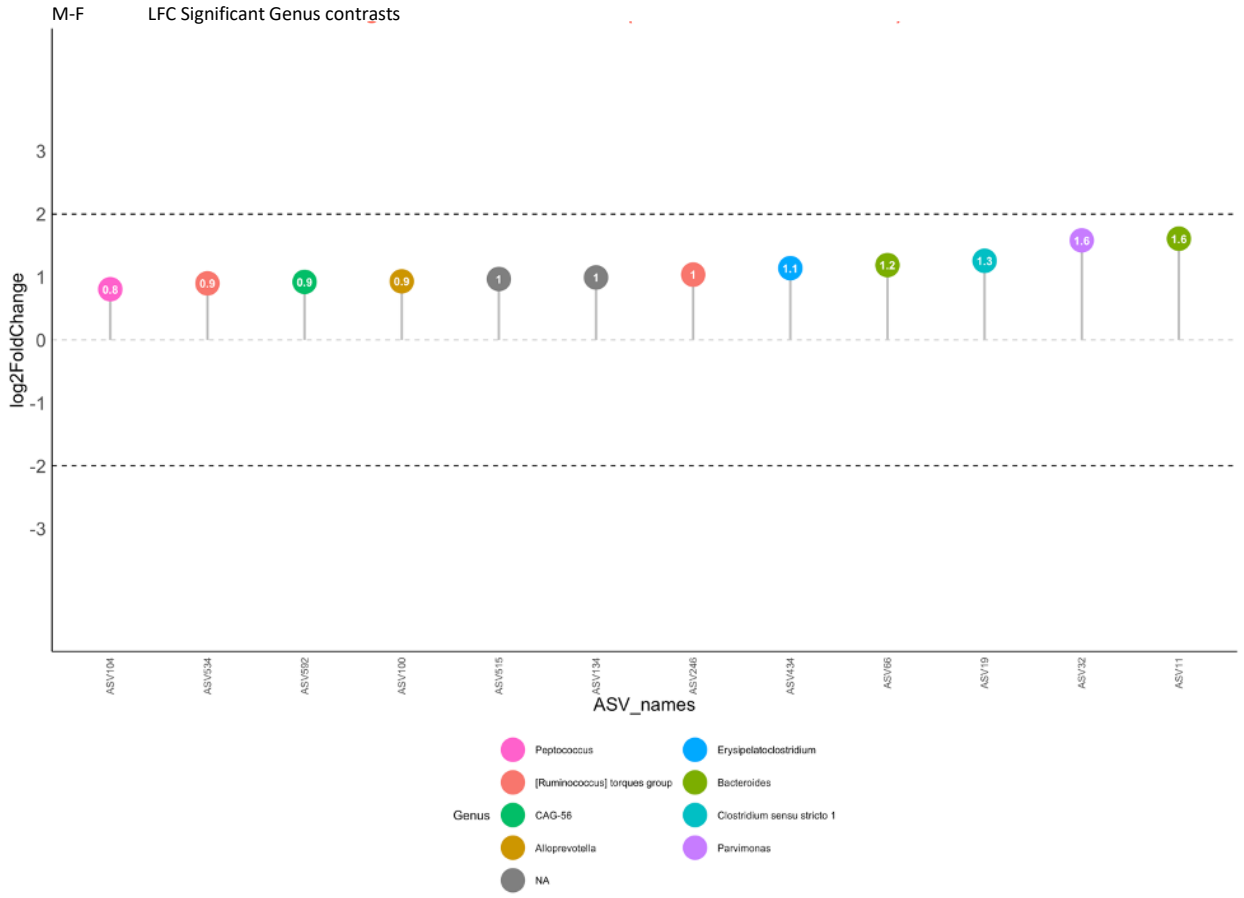
The least-squared mean (Ls Means) (95% CI) estimates of piglet model IV for the fixed effects of fixed effect of piglet cluster (PC) on alpha diversity metrics in piglet populations. Red arrowed lines determine homogeneous groups, with overlapping red lines indicating that two groups are not significantly different, based on a post hoc Tukey test.

Figure 15



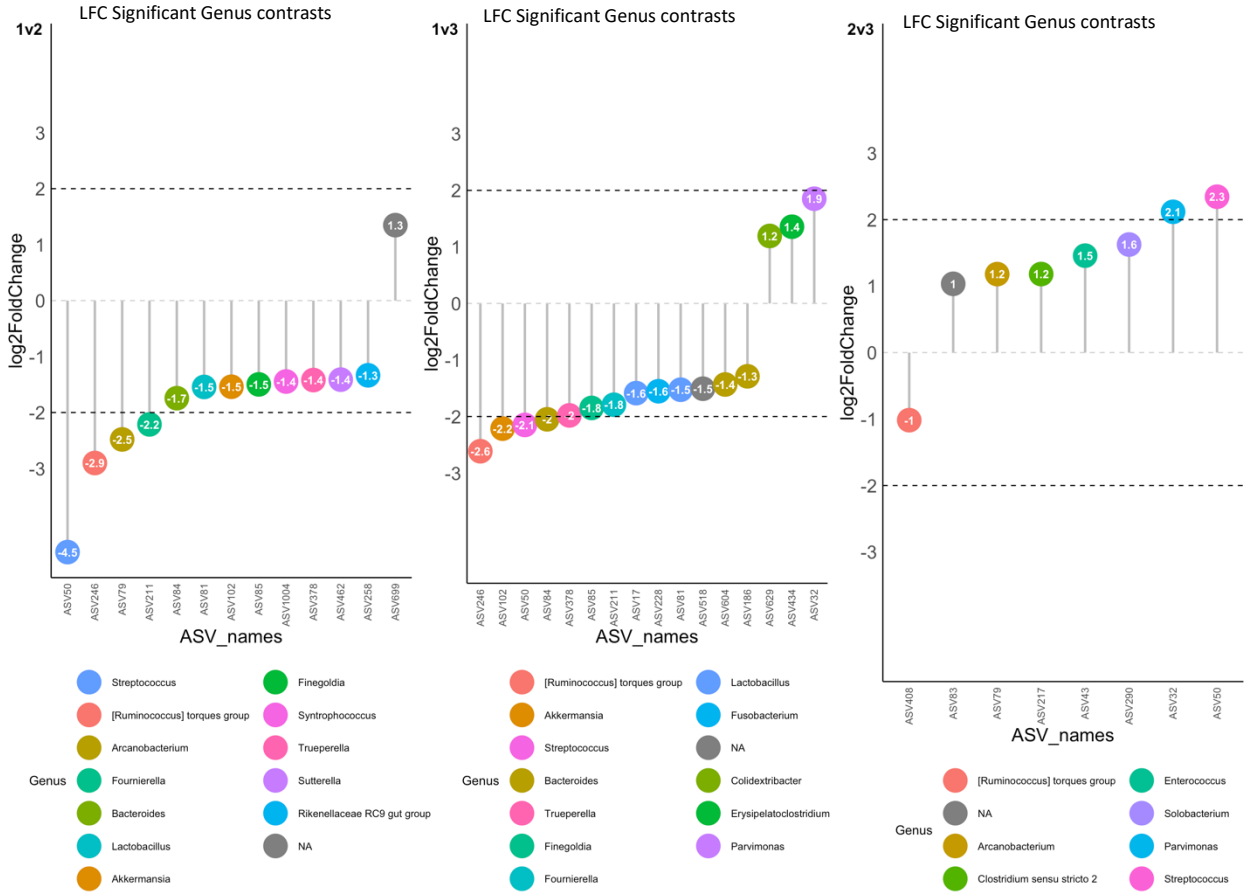
The least-squared mean (Ls Means) (95% CI) estimates of sow model V for the fixed effects of fixed effect of sow cluster (SC) on the alpha diversity metrics in sow populations. Red arrowed lines determine homogeneous groups, with overlapping red lines indicating that two groups are not significantly different, based on a post hoc Tukey test.

Figure 16.



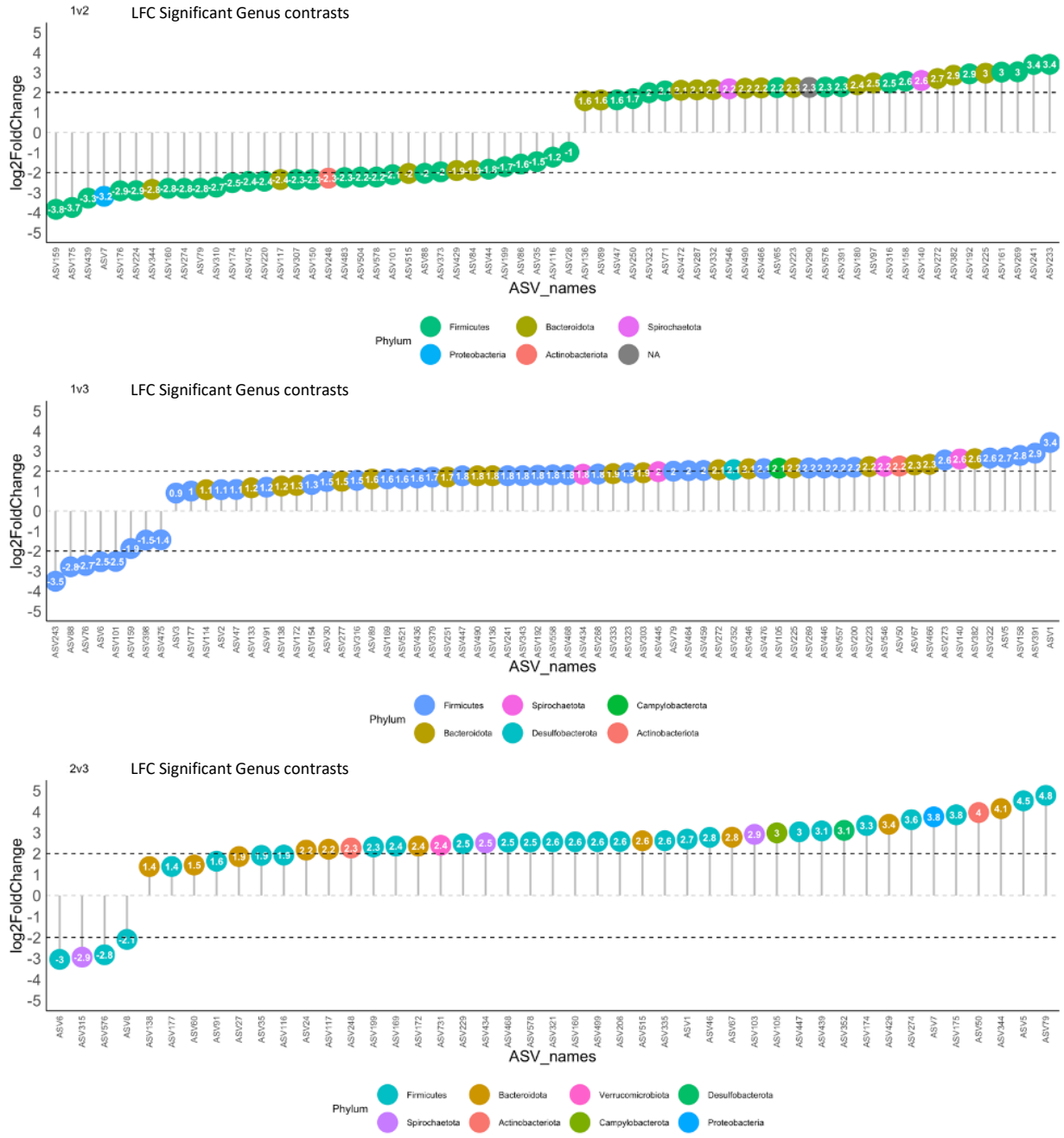
Genera differential abundance between male (M) and female (F) piglets. Results are expressed as Log2FoldChange (LFC).

Figure 17.



Genera differential abundance between piglet microbial clusters (1,2,3). Results are expressed as Log2FoldChange (LFC)

Figure 18.



Genera differential abundance between sow microbial clusters (1,2,3). Results are expressed as Log2FoldChange (LFC)

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CHAPTER 3

Thesis Conclusions

The research presented in this dissertation provides an important examination of the early piglet gut microbiome, and the features that cause microbial variability in a commercial production environment. Specifically, how swine gut microbiomes are developed and the factors driving how the microbiome is shaped early in a piglet's life. The primary focus of the first chapter involved examining the existing published literature concerning the definition of the microbiome, methods for investigating the microbiome, the biological effects of the microbiome, and how the microbiome is shown to affect swine production.

In the second chapter, we characterized the factors affecting differences and similarities in the microbiome of piglets during lactation, including the role of the sow, pen environment, and sex. Analysis of the effects of litter (pen environment) and room environment showed indication that piglets of the same family, sharing the same pen, show higher degrees of microbial similarities to each other than to unrelated piglets. Further, microbial diversity is at least partially explained by piglet litter membership. We did not see indications that the piglet microbiome was directly influence by the sow gut microbiome. However, piglets did separate into distinct groups, with clear differences in the abundance of unique genera. The sow populations were too closely related to begin drawing conclusions about the effects of piglet relationships on their microbiome. Analysis of the effects of sex on the microbiome reveal no significant impact on piglet microbiomes. We hope that future research will continue to develop the ideas presented here and continue to further our understanding of the crucial role the microbiome plays in piglet development and health.