Investigation of the microbiomes of sow skin, milk and piglet hind gut

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Abstract

There is a lack of information on the microbiome of sow skin, sow milk and piglet faeces from New Zealand production systems. Therefore, we have a current lack of data on the bacterial populations present and the potential pathways of transmission to colonise the gut of neonatal pigs. Knowledge of these aspects would allow the identification of common probiotics, pathogens and commensal bacteria in pigs and provide information to help identify the pathways of colonisation for bacteria within the gastrointestinal tract of neonatal pigs. Samples were collected from 21 sows (N = 17 milk samples and N = 20 udder skin swabs) and 63 piglets (N = 63 piglet faecal samples) within 72 hours of parturition, on a commercial farrow-to-finish indoor pig farm. The DNA extracted from these samples underwent metabarcoding analysis using the greengenes 16S database and Kraken2 pipeline. The number of reads varied by different samples, even from within the same pen. For example, the milk sample of sow 676 only had 25,000 reads whilst the piglets suckling from this sow had between 125,000 – 160,000 reads. Piglet samples were dominated by populations of *Firmicutes* and *Bacteroidetes* bacteria, whilst the sow udder skin and milk samples were dominated by populations of *Firmicutes* and *Proteobacteria* bacteria. Milk samples displayed a greater presence of lesser common phyla compared to sow skin or piglet faeces, but these phyla are present at relatively low levels of reads, such as *Cyanonbacteria*, *Chloroflexi* and *Acidobacteria*. There were no reads for *Escherichia coli* bacteria present in any samples taken for this study which is an unusual but incredibly positive finding. *Clostridium*, a similar pathogenic bacteria in neonates, was found abundant in piglet faecal samples so we cannot be sure if *E. coli* did not exist on-farm at all due to upkept disinfection protocols or if the piglets had not yet picked it up. Probiotic bacteria were identified across the piglet faecal samples, namely *Pediococcus* and *Lactococcus*, including species of the phyla which are known to increase production and performance, as well as providing a protective function against *E. coli* bacteria. As probiotic bacteria are typically given as feed additives pre-weaning due to benefits aiding in gut microbiota development, these piglets seem to already have a good basis of these bacteria. The research was successful in what was set out to be completed, despite any limitations that were discovered, and resulted in future recommendations to continue characterising important microbiomes in the New Zealand pork industry, as well as their interactions and pathways of transmission to neonatal piglets.

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List of Abbreviations

spp. – species

MAG – metagenome-assembled genomes

GIT – gastrointestinal tract

ADG – average daily gain

TLR – toll-like receptors

IL-10 – Interleukin-10

IL-1 – Interleukin-1

mRNA – messenger ribonucleic acid

rRNA – ribosomal ribonucleic acid

PRR – pattern recognition receptors

SCFA – short-chain fatty acid

TNF-a – tumour necrosis factor alpha

HPGA – hypothalamic-pituitary-gut-adrenal

EH – enriched housing

CH – conventional housing

N – number of

D – disinfected

NDe – non-disinfected

AMR – anti-microbial resistance

BCFA – branched-chain fatty acid

PWD – piglet wasting disease

DNA – deoxyribonucleic acid

RNA – ribonucleic acid

IgG, IgA, IgM – immunoglobulins G, A and M

IGF-I and IGF-II – insulin-like growth factor 1 and 2

TGF-B – transforming growth factor B

EGF – epidermal growth factor

Na^+, K^+ – sodium and potassium ions

ASD – animal status declaration

MRSA - methicillin-resistant strain

Chapter 1. General Introduction:

There is a lack of information on the microbiome of sow skin, sow milk and piglet faeces from New Zealand production systems. Therefore, we have a current lack of data on the bacterial populations present and the potential pathways of transmission to colonise the gut of neonatal pigs. Knowledge of these aspects would allow the identification of common probiotics, pathogens and commensal bacteria in pigs and provide information to help identify the pathways of colonisation for bacteria within the gastrointestinal tract of neonatal pigs. Furthermore, this information would provide pathways to develop technologies which can advance on-farm diagnosis or identification of bacterial populations including common pathogens in the case of an outbreak.

The term 'gut microbiota' encompasses all commensal, symbiotic and pathogenic organisms present in the gastrointestinal tract of an individual organism – including bacteria, fungi, archaea, viruses and protists (Patil et al., 2020). These microbes have crucial roles in the developmental, metabolic and physiological processes which affect the hosts health and productivity (Chen et al., 2022; Kim and Isaacson, 2015).

Exposure to microbial populations in early-life is vital for the development of a diverse, stable and resilient gut microbiota in adult-life (post-weaning) (Patil et al., 2020). There are two main exposure time periods for piglets. Firstly, as a neonate, when the gut is considered sterile and is then quickly populated after birth; then secondly at the time of weaning, when there is an abrupt change in diet from the sows milk to a complex diet, which leads to a further change in the relative abundances of microbial populations relative to changing necessary biological functions. Although a portion of the initial microbiome remains following any changes, it is often diluted within the new population dynamics of the gut (Buddington et al., 2010). During these first weeks of life, the piglet's immune system is still immature which may allow pathogenic microbial populations to colonise in their gut (Lallès et al., 2007). For this research, the importance of initial piglet gut microbiota colonisation is not in question, but instead it is exploring the possible microbial colonisation pathways of the gastrointestinal tract of neonatal piglets. This research will focus on sow milk and sow skin as possible sources of microbial transmission. These microbiomes of the sow skin, sow milk and the subsequent

piglet faeces have not yet been characterised in New Zealand production systems, so we lack data on presence of bacterial communities as well as the pathways of maternal transmission.

Suckled milk has been recognised as a source of commensal and (potentially) probiotic bacteria for neonates (Jost et al., 2014). Identifying common bacterial species present in breast milk and in infant faeces would indicate a vertical route of transmission from the maternal gastrointestinal tract to the neonate (Chen et al., 2022). It has also been reported that along with sow milk, the vaginal tract and areolar skin of the sow can host a diverse microbial population and are important sources of bacteria for the infant gastrointestinal tract (Liu et al., 2019).

Gut microbiota plays a crucial role in the health of a piglet and hence their productive performance, specifically in terms of energy production for host, fermentative production of metabolites and the capacity to resist potential pathogens (Chen et al., 2022). It has also been reported there are specific time points within the first 24 days of life, where changes to the gut microbiota composition have a significant effect on performance, as does birthweight of piglets (Gaukroger et al., 2020). The most influential time-point determined was the initiation of weaning, where intake of solid feed first occurs and the piglet diet changes from milk-based to a cereal-based feed (Gaukroger et al., 2020; Lallès et al., 2007).

This research has been conducted to begin the process of characterising the various important microbiomes present in New Zealand pork production systems and identify possible routes of maternal microbial transmission for the neonatal piglet gut microbiota. Genomic technology has advanced greatly, providing an accessible option for sequencing and on-farm diagnosis of pathogenic bacterial presence. This has led to more studies commonly sequencing both the genome and metagenome of samples using a 16S rRNA metabarcoding sequencing method. In this study, 100 samples were taken from sows (milk and udder skin swabs) and piglet faeces, within 72 hours of parturition. 16S metabarcoding and Illumina sequencing were performed on the samples to characterise the relative abundances of microbial populations present in the piglet gut as well as sources of maternal transmission.

Overall, this research provides an initial insight into the bacterial compositions of various microbiomes of the New Zealand pork production systems as well as the origins of microbial populations present in piglet gastrointestinal tracts.

Chapter 2. Literature Review:

2.1. Introduction:

This literature review will provide context relating to the proposed research programme and includes all background information found prior to the research being carried out. It describes how characterising the piglet gut is crucial to begin to then identify pathways of greatest bacterial transmission from mother to neonatal piglets as well as how the bacterial composition of the piglet gut microbiota affects the productive performance of that individual. It provides context specifically around the general metagenomic findings of pigs, an overview of relevant farm management practises and elements of swine lactation, all of which all differentially affect the microbial composition of the piglet gut, and therefore have differential effects on the productive performance of the piglets.

2.2. Overview of Swine Metagenomics

2.2.1. An Overview of Metagenomics:

Metagenomics is a method for studying the entire isolated nucleotide sequence from organisms within a bulk sample. These data can then be analysed, regarding structure and function specifically. It is a process derived from conventional microbial genomics which typically uses a purified culture of a specific community of micro-organisms, however, this method does not require a pure cultured sample for sequencing to occur (Kunin et al., 2008). Because of this difference, the use of metagenomics allows the study of genomes from microbiomes which cannot be readily cultured. In the mammalian gut, most bacteria are difficult to culture due to the requirement for an anaerobic environment. Additionally, we may not be able to reproduce the required environmental conditions of the gut or the symbiotic relationships which occur there. Because of this only around 1% of the gut microbiota is cultivatable (Holman and Chenier, 2015).

In this literature review, metagenomics is defined as the process of applying shotgun sequencing or metabarcoding to DNA which has been obtained from an environmental sample directly (no culturing) or a series of related samples. Shotgun sequencing allows us to

read all DNA within a sample and can simultaneously identify and profile a variety of microbiomes including bacteria, viruses and fungi. This is in contrast to alternative methods such as, 16S rRNA sequencing, created by Carl Woese and George Fox (Fox and Woese, 1977), which will only detect a small portion of the gut microbiota that shotgun sequencing is able to uncover (Durazzi et al., 2021).

The assembling of complete genomes from samples which are not pure cultures requires an organism-specific clone from environmental-DNA libraries, or recovery from the environmental-DNA databases, to find target-organism-specific sequences (called 'contigs') which overlap with the sequenced genomes from the samples. In terms of organising the metagenomic sequenced data from the shotgun sequencing, several processes such as clustering, binning, gene annotation and gene prediction are utilised (National Research Council, 2008).

Laboratory-grown microbial cultures tend to experience a cultivational bottleneck which, before the use of metagenomics, skewed the diversity in microbial communities. The use of metagenomics provides an unbiased view of the richness and diversity present in a microbial community, but also of the functional potential these communities have (Hugenholtz and Tyson, 2008).

New concepts and methods for metagenomics are continually being developed to further allow such a nuanced view of microbes and increase the ability to capture micro-diversity and variation among strains of the same species.

2.2.2. Overview of Swine Gut and Metagenomics:

The gastrointestinal tract of swine is thought to be sterile prior to birth, and then rapidly colonised with bacteria from the sow, the piglets' diet and the rearing environment (Lallès et al., 2007). There are also major differences between the swine gut microbiota pre- and post-weaning, driven mainly by the dietary shift from sow's milk to a solid, cereal-based diet which are less digestible than the sow's milk. During the abrupt and simultaneous dietary, environmental and social change that pigs experience at weaning, they are susceptible to

disease challenge (Boma et al., 2014). The piglets' immune system is still relatively immature at this time, and the removal of milk antibodies, exposure to different organisms and changes to digestive tract morphology compound this (Bomba et al., 2014; Stokes et al., 2004; Lallès et al., 2007). The rigors of weaning present an opportunity for pathogenic bacteria to colonise in the gastrointestinal tract, but as the pigs age their gut microbiota becomes more developed, stable and resistant to pathogens (Lallès et al., 2007).

Gastrointestinal microbiota are incredibly vital for a healthy animal. Colonic bacteria metabolise any undigested carbohydrates into short-chain fatty acids (SCFA) which can then be absorbed and utilised by the animal, hence the intestinal bacteria help to recover nutrients which otherwise would be lost in excreta. Commensal microbiota are symbiotic or considered beneficial and provide resistance to colonisation by pathogens. This occurs through competition for nutrients and binding sites on the intestinal epithelium; and altering the local environment to produce volatile fatty acids (VFAs), modified bile acids and antimicrobial compounds (Holman and Chenier, 2015). It is important to note that the gut microbiota is not static and can be altered by a range of factors. Shifts in the microbiota are typically associated with changes in animal health and growth performance. Bergamaschi et al. (2020) investigated how the difference in microbiota composition impacts production traits, and concluded there is a relatively significant influence of host genetics on the structure and composition of the gut microbiome. The availability of sequenced genomes and their functional information for most of the pig gut microbiome is highly limited, so more and more studies are being conducted to construct expanded gene catalogues of the pig gut microbiome.

Many bacterial strains, as well as viruses and fungi, are difficult to culture and isolate in order to sequence genomically, due to their varying growth and nutrient requirements that are typically unknown (Renwick et al., 2021). Previous studies have been able to assemble strain-level genomes from short metagenome-assembled genomes, called MAGs, that were derived from pig samples (with the majority being faecal) at different times of their lives (Holman et al., 2022).

2.2.3. Swine Gut Microbiomes at Different Ages:

Generally, the development of the gut microbiome is influenced by the genetic variation of the host, as well as environmental factors and stochastic (random) events. At weaning, piglets will transition from lactose to plant carbohydrates as their main source of dietary carbohydrate; with the dietary shift also inducing a shift of the gastrointestinal microbiome (Wang et al., 2019). In pigs especially, this transition is abrupt which in turn induces a highly accelerated succession of new microbial communities in the weaner piglets' microbiomes (Frese et al., 2015). The gut microbiota also has an important role in facilitating adaptation of weaner piglets to fibrous feed and help minimise the risk of any pathogen colonisation post-weaning (Molist et al., 2014). Diet and age appear to be the most significant influences to shaping the communities in the piglet gut microbiota, and any litter effects appear to be transient. The age of the piglet specifically alters the gastrointestinal physiology and their immune and metabolic functions during weaning, changes which occur rapidly due to the abrupt transition from sows' milk to solid feed (Wang et al., 2019).

A study by Holman et al. (2022) collected faecal samples from piglets at 7 intervals between the ages of 7 days and 140 days old. Using shotgun metagenomic sequencing, 1,150 de-replicated metagenome-assembled genomes (MAGs) were produced, with a >90% completion level and with a <5% contamination level. These MAGs were used to compare the changing piglet gut microbiota from pre-weaning to post-weaning. Weaning was found to have an associated decrease in the abundance of 69 MAGs, and those recognised as *Escherichia coli* (E*. coli*) in particular, as well as an associated increase in 140 MAGs – those including *Clostridium* species (*spp*.) and *Oliverpabstia intenstinalis* (Holman et al., 2022). Around 82% of the MAGs from this study were matched to species which do not have any already cultured representatives, indicating the majority of a pigs' gut microbiota is poorly characterised, but also shows the potential use of MAGs in adding context to a gut microbiome. Similarly, another study carried out a metagenomic reconstruction of weaner piglets' gut microbiome and found that around 70% of their determined metagenomic bins could be identified at a genus or species level (Wang et al., 2019). This further emphasises a need for metagenomic and culture-based studies to characterise taxa. The study by Wang et

al. (2019) also agreed with previous studies (Kim and Isaacson, 2015; Tan et al., 2018; Crespo-Piazuelo et al., 2018; Yang et al., 2016) in that the most predominant phyla in the pig-gut microbiome are *Firmicutes* and *Bacteroidetes*, with these two representing about 90% of the microbiome at phylum-level both pre-weaning and post-weaning.

Microbial communities in the GI tract of piglets from 1-21 days old remained relatively stable, with highly abundant populations of *Bacteroidaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Lactobacillaceae* and *Enterobacteriaceae spp*. making up the majority of the microbiome (Frese et al., 2015). After weaning (typically when the piglet is 21-28 days old), the populations of *Bacteroidaceae* and *Enterobacteriaceae spp.* decrease in abundance, whilst the *Lactobacillaceae*, *Ruminococcaceae*, *Veillonellaceae* and *Prevotellacea* populations tend to increase in abundance. Populations of *Lachnospiraceae* tend to remain at relatively similar levels of abundance pre and post weaning (Frese et al., 2015).

One of the more drastic changes seen in gut microbiota composition is in the *Prevotella* populations, as there is typically a relatively low population at birth, but it increases very rapidly and dramatically at the time of weaning when a diet that is richer in plant polysaccharides is introduced (Frese et al., 2015). This is characterised by the association of the *Prevotella* genus with consumption of plant polysaccharides which are found in the plant-based diet post-weaning. The increase in *Prevotella* species likely takes over the void vacated by decreases in *Bacteroidetes spp*. which have abundant populations in nursing pigs and are able to hydrolyse milk glycans. Presence of milk glycans in the diet of pigs appears to have a major effect on their gut microbiome, where the microbiota community under this type of diet is labelled a 'milk-oriented microbiome' which is enzymatically and metabolically oriented to the consumption of milk oligosaccharides (Zivkovic et al., 2013). Upon weaning, and hence the introduction of a cereal-based diet, there is a change in orientation of the microbiome and an increased abundance of *Lactobacillaceae* populations which consume plant derived monosaccharides and polysaccharides and produce lactic acid as a by-product of glucose metabolism (Frese et al., 2015*).*

2.2.4. Effect of Microbiota on Swine Production Traits:

Bergmaschi et al. (2020) found several amplicon sequence variants (ASVs) which were significantly correlated with a difference in feed efficiency and fatness traits in three different breeds of pigs – Duroc, Landrace and Large White. At genus level, the sequences produced could be assigned to *Ruminococcus, Clostridium, Eubacterium, Lactobacillus, Bacteroides* and *Prevotella*, from most abundant to least, though most of the sequences (N = 79) could not be assigned to any genera.

The genera *Anaerovibrio*, *Clostridium*, *Faecalibacterium*, *Eubacterium*, and *Ruminococcus* are known to have negative correlations with traits for backfat and feed efficiency in both Landrace and Large White breeds at 73 days of age (Bergmaschi et al., 2020), and *Lactobacillus* was found to have positive correlations with feed efficiency and average daily feed intake in Duroc and Large White breeds at 158 days of age.

A similar study by Chen et al. (2021) sequenced and recovered MAGs from 500 gut microbiome samples from pigs spanning various ages, sexes, breeds, geographical locations, differing levels of domestication (wild boars and Duroc pigs) and from different locations of the gut (lumen samples from jejunum, ileum and caecum as well as faecal samples). The resulting catalogue (referred to as PIGC90) collated 1,358 high-quality MAGs, 4,981 medium-quality MAGs, 2,309 unknown species-level genome bins SGBs and 364 known SGBs. Ten bacterial species were found in the lists of all faecal, ileal and caecal samples: *Lactobacillus amylovorus*, *Ruminococcaceae bacterium*, *Escherichia coli*, *Prevotella copri*, *Bacteroides fragilis*, *Streptococcus suis*, *Phascolarctobacterium succinatutens* and *Salmonella enterica*. Compared to Duroc pigs, it was found that wild boars (in the particular sample) had a greater level of diversity of gut microbiota at genus level; this difference was not observed at the species-level though this is potentially due to poor annotation of the metagenome at species level (Chen et al., 2021). Wild boar had more abundant populations of *Bacteroidetes* and *Bifidobacterium* bacterial species. *Prevotella* and *Lactobacillus* populations, which are associated with fat accumulation and lean meat %, and *Streptococcus* species were all more enriched in the gut microbiota of Duroc pigs. It was suggested that a high propagation of

bacterial species in the gut of Duroc pigs may be due to the high energy and high protein diet they are fed in commercial production systems.

In terms of functional capacity, microbial populations of the small intestine are more involved in digestion of smaller molecule nutrients, while the caecal microbiome has the special ability to degrade molecules such as xylan, pectin and cellulose (Yang et al., 2016). Genera such as *Clostridium* and *SMB53* are more abundant in the small intestine of the pig gut, while *Prevotella*, *Treponema*, *Ruminococcus* and *Faecalibacterium* have a higher abundance in the caecum. It has also been proposed that the ratio of *Bacteroidetes* populations is associated with the level of fatness in pigs (Yang et al., 2016) as leaner breeds of pigs such as Landrace have a greater abundance of Bacteroidetes as well as a greater level of microbial diversity and number of faecal methanogens than more obese breeds such as Erhualian. The ratio of *Bacteroidetes* and *Prevotella* populations, specifically a high abundance of populations, may inhibit fat mass development and inhibit inflammation in low-fatness pigs. Pathogens such as E. *coli*, inflammatory processes, microbial metabolism and nutrient sensing are influential in the high level of fatness in pigs (Yang et al., 2016). The idea that the composition of populations in the gut microbiota are correlated with feed efficiency and fatness traits is relevant in understanding how the intestinal microbial community influences host production traits.

2.2.5. Antimicrobials and Pig Gut Microbiota:

Genes for antimicrobial resistance (AMR) were detected in 327 of the 1,150 MAGs in the study by Holman et al. (2022), including 59 MAGs for tetracycline resistance genes that are commonly associated with pigs, due to tetracycline antibiotics being commonly used on pig farms to treat diseases and, in some countries, also to promote growth. Tetracycline resistance genes have also been known to be spread through farms via pig manure, with abundance of AMR-genes in soil/manure linked to use of antibiotics (Bassitta et al., 2022).
Studies have shown that in-feed antibiotics for piglets can cause a divergence in the gut microbiome and reduce the quantity and diversity of the microbiota (Looft et al., 2012; Kim et al., 2012). It has also been demonstrated that an increase in E. *coli* populations in the ileum occurs post-antibiotic exposure.

Looft et al. (2012) reported that after 14 days of ASP250 antibiotic treatment, piglets had an increased abundance of *Proteobacteria* compared to the pigs who were not given the antibiotic treatment. This shift in abundance of microbial phylotypes was due to an increase in populations of E. *coli*. Antibiotic treatment course also resulted in a decreased abundance of Bacteroidetes microorganisms and an increase of Ruminoccocus populations. Ruminococcus species are commonly found in the hindgut of pigs and function in the degradation of cellulose, so an increase in populations following antibiotic administration may result in a decreased feed conversion ratio (FCR) and improved feed efficiency. The short-term course of low dose in-feed antibiotics also resulted in an increase of abundance and diversity of genes (in the gut microbiota) for antibiotic resistance, including genes for resistance to antibiotics which were not administered (Looft et al., 2012; Hu et al., 2020). This indicates that the use of antibiotics should be used only when necessary to avoid an increase in AMR genes which would make it more and more difficult to treat microbial pathogens in the future.

A key finding of studies into AMR genes and pig gut microbiota is that the results are dependent on the original composition of the microbial community itself and that the swine gut may act as a channel for transference of AMR genes into the environment (strong associations for this have been found in urban sewerage, the human gut and soils) (Looft et al., 2012; Zhao et al., 2018). Because of this, monitoring and control of microbes with AMR genes in reservoirs such as animal digestive tracts should not be under-estimated, especially with a global increase of anthropogenic activity.

2.3. Overview of Farm Management and Practises influencing the Gut Microbiota:

2.3.1. Impact of Cross-Fostering on the Piglet Microbiome:

Cross-fostering is a management practice used on farm to equalise the number of piglets between litters and ensure that every piglet has ownership of a functional teat. This is an important husbandry practice as it relates to colostrum management. Cross fostering is

carried out in the first 24 hours post-farrowing to facilitate maximum absorption of colostrum immunoglobulins before a loss of gut permeability occurs at 24 – 36h of age (Olivero et al., 2019). Sow milk is known to not only affect the neonate offspring microbiota composition, but also their health and disease regulation of the gut. Previous studies indicate that the nursing mother of piglets has a significant effect on the rate of diabetes and expression of anti-inflammatory cytokines in offspring through changes to the gut microbiome (Daft et al., 2015). Currently, there is little know about the effect that cross-fostering has on the intestinal microbiota and immune gene expression of neonates (Maradiaga et al., 2018). It is already known that both breed and nursing mother can differentially influence the microbiota of piglets early in their lives, but it isn't known if it affects the gastrointestinal tract (GIT) microbiota specifically or their immune status (Mu et al., 2019; Mao et al., 2023). There are reports that the microbiota contained in the placenta may impact the early neonatal microbial composition (von Mutius, 2017) and another indicated a transfer of maternal microbiota based off the presence of microbial species in the umbilical cord blood (Willyard, 2018).

Previous cross-fostering experiments have found piglets of differing breeds had identical caecal (Xian et al., 2014) and faecal (Bian et al., 2016) microbiota composition alterations when nursed by a foster sow (Mu et al., 2019). A study by Mu et al. (2019) used an inter-specific cross-fostering system of Yorkshire (a lean-breed) and Meishan (a Chinese obese breed) sows, whereby each sow would nurse a litter comprising 50% their own offspring and 50% non-biological offspring of another breed. Jejunal and colonic microbiota of the piglets they were nursing were analysed at 14 days (pre-weaning) and 49 days (post-weaning) of age. Their findings were that piglets nursed by Meishan sows, regardless of breed, had a decreased abundance of *Streptococcus suis* and an increased abundance of *Cloacibacillus spp.* in the colon, as well as increased interleukin 10 (IL-10) and Foxp-3 positive cells in the colonic mucosa compared to piglets nursed by Yorkshire sows (Mu et al., 2019). The breed effects observed were that Meishan piglets had lower abundances of *Prevotella* genera microbes and a decreased expression of tumour necrosis factor alpha (TNF-a) genes, though an increase in IgA levels. S. *suis* is a potentially pathogenic bacterium, depending on serotype, which colonises the upper respiratory tract in piglets (Feng et al., 2014). *Cloacibacillus spp*. are mucin-degrading bacteria which use mannose, fructose and sialic acid for growth (Looft et al., 2013) so an increase in this microbe is potentially important for regulation of the piglet gut

environment as the substrates are found in sow milk. Interleukin 10 upregulation results in suppressed intestinal inflammation and pathogen invasion as well as increasing Foxp3-positive cells – hence linked with improved anti-inflammatory function in piglet epithelium (Couper et al., 2008, and Sanjabi et al., 2009). There are also other breed-related microbiota differences that have effects on the neonate microbiota which haven't been investigated, such as that Meishan sows had a higher abundance of *Firmicutes* and a lower abundance of *Bacteroidetes* genera microbes than Yorkshire sows (Mu et al., 2019).

A similar inter-specific cross-fostering study by Mao et al. (2023), looked at Duroc and Yorkshire breeds and used 4 groups of piglets whereby two groups were piglets nursed by their birth mothers and the other two were cross-fostered. The main finding was that the cross-fostered piglets had improved growth performance for an extended period post-weaning. The piglets of different breeds showed no significant difference in average daily gain (ADG), which is consistent with a study by Alam et al. (2021), but the cross-fostered piglets appeared to have significantly higher ADG and bodyweight on day 70. The piglets nursed by sows of another breed were found to have an increased alpha diversity of their gut microbiota on both 28 and 70 days old, in comparison with the piglets nursed by their birth mothers (Mao et al., 2023). The changes in beta-diversity analysis over the experimental period showed that the piglet gut microbiota was influenced mainly by the nursing mother pre-weaning, but post-weaning the influence of the nursing mother decreases, and the influence of piglet breed increased. There are typically significant shifts in beta-diversity of gut microbiota around weaning due to weaning stress, diet change and also endocrine system disorder (Koenig et al., 2011; Wang et al., 2019; Yang et al., 2014), so some difference in beta-diversity was expected. The Duroc piglets nursed by Yorkshire sows had a significantly greater abundance of *Prevotella* microbes than the 3 other experimental groups, which is a genus of bacteria associated with fibre digestion and tends to proliferate in the pig gut microbiota after introduction of solid feed. A *Prevotella*-dominated gut microbiota is known to produce greater amounts of short-chain fatty acids (SCFAs) (Chen et al., 2017) which benefits both health and growth performance of the host animal. This is consistent with the growth performance of the Yorkshire-reared Duroc piglets in the study by Mao et al. (2023).

The finding that cross-fostering improved piglet growth performance is also consistent with other studies such as Mu et al. (2019), where there was an impact of nursing mother found in the piglet caecal microbiota (decreased abundance of *Bacteroidetes*), and the fostered piglets had an increased growth rate compared to non-fostered piglets. Cross-fostering within a breed was found to have no significant impact on the composition of piglet gut microbiota, but mRNA expression of toll-like receptors (TLRs) and inflammatory cytokines changed with different locations of the GIT (ileum, jejunum, caecum and colon) (Maradiaga et al., 2019). Toll-like receptors are a type of pattern recognition receptor (PRR) which is expressed on epithelial and immune cells. They recognise bacterial structures, and promote inflammation, which works with binding proteins to activate inflammatory cytokines that fight infections (Balachandran et al., 2015, and Kitazawa et al., 2008). Increased expression of TLRs occurs in the ileum and associated lymph tissues of piglets at 21 days old. Establishing a microbiome in early life is crucial to help stimulate development of the piglets' immune system, especially in terms of increased expression of TLR, TNF-a, IL-10 and Fox3p-positive cell genes.

Conversely, another study reported that piglets which were nursed by their birth mothers scored higher on a bacterial diversity index compared to those that received colostrum from their own dam followed by cross-fostering for the rearing period, or those that were cross-fostered pre-colostrum ingestion and reared by a non-related sow (Maradiaga et al. (2019). Hence the treatment group of gut microbiota of the piglets that remined with their dam had more diversity than those of the other two treatment groups.

Most of the existing literature suggests there is an increased microbial diversity and beneficial impact to growth and health performance for piglets cross-fostered with sows of other breeds (compared to piglets nursed by their birth mothers), but that there is little difference when cross-fostering piglets to sows of the same breed compared to piglets nursed by their birth mothers.

2.3.2. Impact of Differential Housing on Microbiota:

Several studies have investigated the effect of environmental enrichment on the behaviour, welfare and the hypothalamic-pituitary-gut-adrenal (HPGA) axis of pigs (Beattie et al., 1995;

Godyń et al., 2019, and Mkwanazi et al., 2019). Few studies have explored how enrichment resources may impact the gut microbiota of pigs, and their offspring - even though psychosocial stress has been indicated to influence gastrointestinal tract (GIT) pathophysiology and disease susceptibility (Molina-Torres et al., 2019). A study by Wen et al. (2021) investigated the effects of conventional vs enriched housing on pig immune status and gut microbiota in pigs ranging from 0-61 days old. The conventional housing (CH) had 100% slatted flooring, a small 100 cm by 45 cm rubber mat and two metal chains for enrichment in each pen. The enriched housing (EH) gave pigs double the space relative to minimum legal requirements, with a 60:40 ratio between solid and slatted flooring and with a combination of straw, wood shavings, jute bags and broom branches as enrichment substrates (which were all replenished at regular intervals). As well as the known behaviour and welfare impacts that EH has on pigs, those housed in an enriched environment had evidence of a positively influenced immune system development and gut microbiota establishment in early life (Wen et al., 2021). It was proposed that the EH may contribute to increased productivity of pigs and potentially reduce the requirement for antimicrobial use due to increases in interleukin 1 (IL-1) and tumour necrosis factor alpha (TNF-a) genes compared to pigs housed in conventional conditions. This improved productivity of EH pigs was based on the observation of more frequent positive and less frequent aggressive behaviours, higher immune status due to increased haemoglobin levels and a greater abundance of T cells, as well as a significantly more beneficial composition of microbiota (Wen et al., 2021). This is consistent with previous studies such as Van Dixhoorn et al. (2016) which suggested environmental enrichment decreases disease susceptibility, and Giuliotti et al. (2019) which discusses the adverse effects on HPGA axis and immunity in pigs housed in a barren environment.

In terms of gut microbiota, faecal and luminal samples were sequenced using 16S rRNA sequencing and there were differences observed as early as 12 days old between EH and CH pigs; with increased relative abundances of *Prevotella*, *Christensenellacea*, *Ruminococcus*, *Ruminiclostrdium* and *Phascolarctobacterium* and a decreased abundance of *Enterococcus* in EH pigs compared to CH pigs (Wen et al., 2021). The genera with an increased abundance in EH pigs are beneficial due to their association with short-chain fatty acid (SCFA) production and degradation of plant carbohydrates – though this difference in the amount of carbohydrate degrading bacteria may be due to the increased intake of straw in EH pigs. A

higher presence of *Lactobacillus* was found in CH pigs compared to those in the EH treatment, which is a genus of bacteria associated with degradation of lactose (a main component of sow milk and the provided transition diet) (Gänzle et al., 2012). However, at the end of the trial period at day 61 – post-weaning, the CH pigs were found to have a greater variation of ileal and colonic microbiota than the EH pigs.

A similar study by Kubasova et al. (2017) compared the effects of conventional and enriched farrowing pens, which housed sows and piglets during farrowing and lactation. The microbiota of piglets at 1 and 4 days of age were analysed. The conventional housing treatment (N = 18 sows) had a slatted concrete floor with 2.4m^2 of space per sow whilst the enriched housing (N = 21 sows) had a floor covered with a deep layer of hay and had 3.4m^2 of space per sow available. All of the sows in both housing environments were fed a standard gestation and lactation diet. The gut microbiota of 1-day old piglets contained predominantly *Escherichia* and *Clostridium* genera; while at 4 days old, the *Bacteroides* and *Fusobacterium* genera were dominant (Kubasova et al., 2017). This observation is relatively consistent with a study by Mach et al. (2015) which suggests this is a characteristic development of the gut microbiota in pigs. *Prevotella* is typically the most abundant genera of microbes in adult pigs so the reason as to why *Bacteroides* was the most dominant in genera in 4-day old pigs is unknown, though as previously mentioned it is thought to be an important part of piglet gut microbial development (Mach et al., 2015). Differences between the sow microbiota in the two farrowing environments were considered moderate, but there was a significant difference in the diversity of the taxa between sows in an enriched environment vs. a conventional environment. All genera identified in the conventionally housed sow gastrointestinal tracts were from the *Firmicutes* phyla whereas the sows in enriched farrowing pens had genera from the *Bacteroidetes* and *Fibrobacteres* phyla – both of which are characterised by the ability to degrade non-soluble polysaccharides. It is expected these phyla were selected for because of the straw ingested from the enriched environment containing these carbohydrates, which is a consistent conclusion as in the study by Wen et al. (2021).

Other studies investigating the impact of housing environments on gut microbiota explored the disinfection procedures of farrowing pens rather than the level of enrichment. Results from a study by Law et al. (2021) found that the vaginal, milk, skin and gut microbiota of sows

were all minimally affected by differential disinfection of the farrowing environment, but the piglet gut microbiota was significantly impacted by the sanitation procedure. The study had two groups of 3 sows each, introduced to two farrowing environments. The first was cleaned with broad-spectrum disinfectant (D) and the second was cleaned with hot-water power-washing only (not-disinfected, NDe). Each sow had 9 piglets, (N = 27 piglets per farrowing environment). All of the piglets were only exposed to the environments for a period of 3 days. The piglets from the NDe sows had a significantly greater abundance of *Lactobacillus* in the gut and *Enhydrobacter* in their nasal passages compared to the D treatment piglets (Law et al., 2021). At birth the NDe piglets also had increased abundances of *Prevotella* and decreased abundances of *Campylobacter*. There are many benefits of these differences, such as increased SCFA production and carbohydrate degradation, increased lactic acid production, enhanced immunity, inhibition to *Salmonella*, and increased immunoglobulin levels. Decreased abundance of pathogenic genus such as *Enhydrobacter* and *Campylobacter* is consistent with other studies which also showed an increased abundance of *Lactobacillus* and had increased microbial exposure from differential sanitation (Mulder et al., 2009).

Differential disinfection methods between treatments meant that the piglets from disinfected farrowing crates were exposed to a decreased number of bacterial taxa, so their microbiota where characterised differently in early life compared to the piglets from non-disinfected farrowing crates (Law et al., 2021). It was found that increased microbial exposure at birth due to no disinfection was associated with decreased piglet growth performance that persisted post-weaning. Therefore, there is a trade-off that exists between the optimal microbiota development (from increased microbial exposure), and the optimal health and growth performance (from decreased microbial exposure) in pork production systems (Law et al., 2021). Though, because gut microbial tissues are very energetically expensive to develop, and the non-disinfected piglets were producing tissues for a wider variety of microbiota, there is a large likelihood these piglets were not just allocating energy for growth, but also for development of immune responses and upregulating the host mucosal immunity (Scrimshaw et al., 1997).

2.3.3. Impact of Probiotics on Microbiota:

Antimicrobial Resistance (AMR) is the result of natural, genetic processes that have rendered bacteria, viruses, fungi and parasites unresponsive to antimicrobial medicines. As a result, infections become more difficult to treat. In-feed antibiotic usage in pork production systems is commonly used to treat or prevent infectious diseases. In some countries, antibiotics are also used to promote growth (Yun et al., 2021), although this is not permitted in New Zealand. However, the misuse and overuse of antimicrobial medications has contributed to a loss of antibiotic effectiveness, and selection for antibiotic resistance in pathogenic bacteria. The implications of AMR for human and animal health are significant and are driving the exploration for alternative treatments.

One of the largest additives of interest to pork production are probiotics because of their ability to improve immune responses, help maintain intestinal health and improve nutritional efficiency (Pereira et al., 2022). Additionally, as their usage does not pose a food safety risk of pork compared to low-dose in-feed antibiotics, there is an ability to incorporate these into pig diets at beneficial levels. Pereira et al. (2022) reported the benefits probiotics have on physiological, immunological and clinical aspects in different stages of pork production – specifically their influence on gestation, parturition and lactation. Improved immunity during the pigs' growing phase, and better quality meat in the finishing phase of production were reported. The fact that probiotics can be used in any stage of pork production (Yang et al., 2015) is advantageous, but there is not yet any clear consensus about which microbial strains to select, and which inclusion rates or treatment protocols will yield the best results. It has been proposed that to be considered an 'effective probiotic', the microbe must have characteristics such as the ability to resist gastric acid, bile salts and pancreatic enzymes, and also to stick to and colonise the intestinal mucosa (Dubreuil, 2017). Probiotics are beneficial through two main mechanisms, the first being colonisation of intestinal microbial communities via dendritic cells, and the second being adhesion of bacteria to the gut surfaces of the host, which enhances pathogen elimination.

Probiotics can also produce beneficial digestive enzymes that contribute to the pigs' performance through improving digestibility, feed efficiency and increasing weight gain

(Pereira et al., 2022). Commonly used probiotics in pork production are the bacteria *Lactobacillus*, *Pediococcus*, *Enterococcus* and *Weisella*, with *Bacillus* and the yeast *Saccharomyces* also being of growing interest (Pereira et al., 2022; Vasquez et al., 2022). Dietary supplementation of *Lactobacilli* specifically has been shown to not only improve pig performance but decrease the abundance of E. *coli* in intestinal microbiota post-weaning (Yang et al., 2020). Use of probiotics in sows is mainly with the aim of improving animal welfare and reproductive performance through establishment of a healthier gut microbiota. Reproductive performance is observed improve via increased number of piglets, increased growth rates due to higher piglet weight at weaning (Pereira et al., 2022). Probiotics have also been associated with increased feed intake during gestation and lactation, meaning the sow has greater energy supplies for these phases of large energy expenditure. The nursery phase of piglets is typically where probiotic use is the most prevalent, due to evidence of benefits in developing the gut microbiota. Probiotics are likely of most benefit to younger animals (up to post-weaning) which have not yet developed a stable microbiota. For newly weaned piglets, there has been improvement shown in intestinal mucosa, increased digestive enzyme production, improved performance and immunity as well as protective effects against the main pathogens of swine, as reported by Pereira et al. (2022). In grower-finisher stage pigs the influence of dietary probiotics is less evident than in piglets, but there is some evidence of better quality (colour and tenderness) meat, reduced production of faecal gases, and increased feed conversion efficiency (Barba-Vidal et al., 2019).

Vasquez et al. (2022), reported that probiotics can also increase SCFA (short-chain fatty acid) production, specifically the production of butyrate, acetate and propionate in pigs, and can increase lactate levels due to an abundance of lactic acid bacteria such as *Lactobacillus*. This is because the probiotic supplementation enriches the abundance of bacteria from the *Firmicutes* phylum – a phylum which consists of bacterial genera that produce SCFAs such as *Ruminococcus*, *Lactobacillus* and *Lachnospiraceae* . These increases in SCFA production are associated with positive effects on swine performance such as increased growth rate, improved intestinal morphology and cognitive performance, stimulation of host immunity, and protection from pathogens. Microbial metabolites such as BCFAs, phenolic and indolic compounds, ammonia and polyamines, are also influenced by probiotic supplementation (Vasquez et al., 2022). Any changes in fermentation of metabolites always corresponds to

changes in gut microbiota composition, so pig gut microbial composition modulation is likely the main mechanism via which probiotics alter metabolite levels. It is important to note that the extent to which metabolites derived from the gut microbiome are affected by probiotics is inconsistent between studies. Some studies have found that there are no changes to levels of indole or phenol after supplementation of probiotics (Nowak et al., 2017), and others have found no changes in SCFA levels after probiotic treatment (Zhang et al., 2018). It has also been observed that changes in microbiome composition and metabolites may not correspond to an improvement in feed efficiency, growth performance or the other performance indices mentioned above (Valeriano et al., 2017).

The reasons for these inconsistencies are unclear. Many elements could be influential – for example, differing probiotic strains/species, whether it was a single or multiple species probiotic, different dosages, age of pigs, duration of treatment, husbandry practices and environmental factors etc. There is currently no gold standard for the formulation of a probiotic supplement (Pereira et al., 2022 and Vasquez et al., 2022).

2.3.4. Impact of Diet on Microbiota:

Diet is one of the largest determinants of the pig gut microbial composition and it is known that when the diet is altered there are marked changes in microbial ecology and fermentative activity. This is most prevalent in the period of weaning piglets, when the diet is altered from sows' milk to solid feed (Rist et al., 2013). During this period especially, the disrupted and unstable ecosystem allows pathogens to colonise, leading to diarrhoea and other diseases. Because of this, there is increasing interest in improving intestinal health at weaning via dietary additives which beneficially affect the microbiota composition and activity (Rist et al., 2013).

Rist et al. (2013) reported inconsistent results but it appears that an excessive amount of protein reaching the lower gastrointestinal tract (GIT) reduces the incidence of PWD (piglet wasting disease) and inhibits the proliferation of pathogenic bacteria, although the reduced dietary protein also reduces the amount of detrimental fermentation-produced products such as ammonia. The study also proposed that the inclusion of fermentable carbohydrates

may be promising in reducing detrimental protein-fermentation products and proliferation of pathogenic bacteria. The results from Rist et al. (2013) are consistent with those from a study by Haenen et al. (2013) where a diet higher in fermentable (resistant) starch beneficially modulates the microbial composition, SCFA concentrations and gene expression in the GITs of pigs, in comparison to a digestible starch diet.

Zhang et al. (2016) conducted a study on the effects of different dietary fibres on gut microbiota of suckling piglets. There were 12 litters of healthy neonatal piglets included in the study, with 10-11 piglets per litter, and the litters were randomly divided between 4 diets. The effects of a control diet based on wheat bran, were compared to a pure cellulose diet and an alfalfa diet. The piglets all had full access to creep-feeders with their respective test diet being offered between day 7 and day 22 of life, whilst also having full access to milk from their sows. No significant differences in average daily gain or average daily feed intake were found between any of the test diets and the control diet, but there were some significant differences in microbiota composition (Zhang et al., 2016). Findings indicated that the alfalfa diet produced the most beneficial effects on the composition of microbiota, including increased *Clostridium* and *Sporobacter* species in the caecum, increased *Coprococcus* spp. in the distal colon and lowest abundance of *Streptococcus suis* (a pathogenic bacteria) of all the diets. These findings are also consistent with those of other studies (Haenen et al., 2013). *Coprococcus* genera species are characterised by their butyrate-producing abilities, so an increased abundance of the bacteria is beneficial, as butyrate is a metabolite which differentiates T regulatory cells and has anti-inflammatory functions (Lee and Hase., 2014; Mu et al., 2015). The wheat bran diet showed a decrease in *Lactobacillus* spp which are lactic acid-producing bacteria found to be beneficial in the gut microbiota (Orlando et al., 2012), meaning a decrease in these bacteria is likely non-beneficial for the animal. The pure cellulose diet resulted in an increased abundance of *Prevotella* spp which are bacteria important for cellulose degradation, so although suckling piglets are not yet able to digest cellulose, dietary supplementation may induce stimulation of cellulose-degrading bacteria.

Results from Zhang et al. (2016) indicate that a moderate increase of dietary fibre via inclusions of cellulose, alfalfa and wheat bran will affect microbial communities in the piglet gut, and alfalfa supplementation may have particularly beneficial effects. Alfalfa diets are rich

in insoluble fibre (celluloses) and soluble fibres (pectins and fructans) and differs from wheat bran (rich in cellulose) and pure cellulose diets, in that these are made of fully insoluble fibres which have low fermentability in the intestines of pigs (Zhang et al., 2016). These conclusions are consistent with those from a study by Liu et al. (2018) which investigated the effects of diet on gut microbiota diversity in weaned piglets using a control diet, an alfalfa diet and a concentrated fibre diet. The alfalfa diet produced a microbial composition with much higher diversity than the other diets, and also resulted in a marked positive effect on growth performance, SCFA metabolism and a decreased diarrhoea incidence (Liu et al., 2018). The alfalfa diet resulted in increased abundances of *Lactococcus*, *Enterococcus*, *Bacillus*, *Faecalibacter* and *Paenibacillus* species, as well as decreased abundances of *Mycoplasma* and *Helicobacter* species. These indicate that differences in microbiota composition due to the inclusion of alfalfa in the weaner piglets' diet were all beneficial, as the genera of increased abundance are all responsible for regulating the immune system, forming antimicrobial compounds, inhibiting pathogen growth, and repairing gut cells. Conversely, the genera of decreased abundance are pathogenic and induce chronic gastritis, ulcers and inflammation resulting in diarrhoea (Heimesaat et al., 2014).

2.4. Swine Lactation Overview:

2.4.1. Anatomical and Hormonal Changes to the Mammary Gland:

Mammogenesis, or the development of the mammary gland, is a continual process from birth and so there are several parameters which must be met before the adult mammary gland is capable of full function. The most important structural features of the mammary gland are developed before the piglet is born, except for the secretory lobuloalveolar tissue (Grahofer and Plush, 2023, Hurley, 2019).

In the pre-pubertal phase of mammary development, the mammary gland parenchyma grows slowly and consistently until 90 days of age (Sørensen et al., 2002). After puberty, there is continued growth of the mammary gland and elongation and branching of the ductal system within the fat pad, as a response to changing hormone levels which is associated with reproductive cycles – specifically cycles of increased oestrogen (Hurley, 2019). Cyclic gilts will

have more parenchymal mammary tissue than non-cyclic gilts, and due to the increased oestrogen the weight of the mammary gland almost quadruples. The majority of gilts to be used for reproduction are bred soon after puberty so the knowledge of post-pubertal mammogenesis is slim, also the gilts only undergo a limited number of oestrous cycles.

Growth of the mammary gland is limited in the first two thirds of gestation (Hurley, 2019), which has a duration of 114 – 117 days in domestic swine. The 'breakpoint' for growth rate is estimated to be at day 74 of pregnancy where from day 75 to day 105 there is a significant increase in lobuloalveolar development, the amount of parenchymal tissue DNA increases four-fold and total parenchymal DNA increases nine-fold (Weldon et al., 1991; Figure 2.1). The mammary parenchyma gets larger but also denser in epithelial structures, extra-parenchymal tissue also increases by 170% during this period of gestation (Weldon et al., 1991). The growth rate of the individual mammary glands varies according to the location of the glands, the middle glands (3, 4 and 5) grow the largest, followed by the anterior glands (1 and 2) and finally the posterior mammary glands (6, 7 and 8) (Ji et al., 2006).

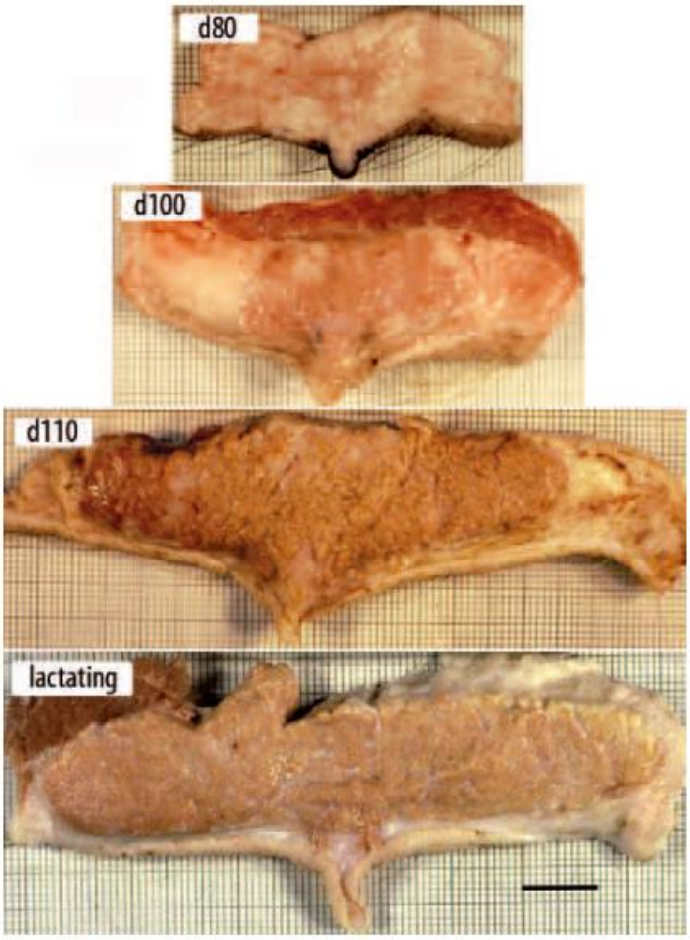

Figure 2.1 - Transverse sections of mammary glands from pregnant gilts during days 80, 100 and 110 of gestation and day 3 of lactation (Source: Farmer and Hurley, 2015).

Development of the mammary gland during pregnancy occurs in response to hormonal stimulation, the hormones considered particularly important in sows are oestrogen, progesterone, relaxin and prolactin. Concentrations of progesterone fluctuate throughout gestation but remain high until just before parturition occurs (DeHoff et al., 1986). Serum progesterone concentrations of the sow rapidly decrease between days 110-112 of pregnancy and then drop further in the final days before farrowing, whilst the fetal progesterone levels decline between days 60-110 of pregnancy and then increase rapidly by day 112. The main oestrogens available in sows are oestrone, oestradiol, estrone sulphate and oestradiol sulphate, the majority of which is produced via oestrogen conversion in the fetus from progesterone (Eldridge-White et al., 1989). Total plasma oestrogen concentrations remain relatively low in early pregnancy until day 75-80, and then steadily increase to the maximal

plasma oestrogen concentration by day 112; concentrations then decline soon after farrowing.

The concentration of relaxin in blood remains low until about day 80 of pregnancy, then increases four-fold by day 110 and increases again by five-fold as a surge 20 hours pre-partum (Eldridge-White et al., 1989). Relaxin, secreted by the corpora lutea, requires pre-exposure to oestrogen to have full effect. It is suggested by Hurley et al. (1991) that an absence of relaxin between day 100-110 of pregnancy causes the mammary tissue to undergo active regression, despite continued exposure to oestrogen from the conceptus.

Circulating concentrations of prolactin are elevated during early pregnancy but remain low until just before parturition (around 2 days prior) where there is a large surge of prolactin (DeHoff et al., 1986). Hypo-prolactinaemia in the first two trimesters of pregnancy does not appear to influence the development of parenchymal mammary gland tissue, instead the relatively low levels of prolactin present in this period are more than sufficient to stimulate the limited amount of tissue development which occurs in this period (Farmer and Hurley, 2015). In contrast, hypo-prolactinaemia in the period of day 90-110 has been found to decrease the amount of mammary parenchymal tissue, total DNA and RNA, and the percentage of protein present (Hurley, 2019). Efforts to induce hyper-prolactinaemia include administration of silymarin (a milk thistle extract), a dopamine antagonist or exogenous porcine prolactin but trials of all have been met with mixed results in regard to mammary tissue development and milk production (Farmer et al., 2014; Vanklompenberg et al., 2013; King et al., 1996*).* Growth hormone influences mammary development in swine but this does not tend to receive as much attention as the effects of prolactin, though administration from day 105 of pregnancy until day 24 of lactation has no effect on milk production or weaning weight of litter (Eldridge-White et al., 1989).

Concentrations of mammogenic hormones change rapidly in the peripartum period where growth of mammary tissues coincides with lactogenesis. There is a prolactin surge beginning 2 days pre-partum and lasting until several days post-partum (Dusza and Krzymowska, 1981), when the circulating prolactin levels decline though concentrations still remain higher post-partum than for majority of pregnancy. Inhibition of the prolactin surge has been found to

have effects on lactogenesis, milk secretion and further development of the gland itself (Hurley, 2019), possible causes for this inhibition include administration of bromocriptine to pregnant sows. Maximal relaxin concentrations are reached a few hours pre-partum, then rapidly decline 48 hours post-partum and are undetectable by 72-120 hours post-partum, this surge coincides with the pre-partum surge of prolactin, the high concentrations of oestrogen and the declining progesterone concentrations (Porter et al., 1992). Administration of relaxin to pregnant gilts in late pregnancy is found to decrease lactation performance and lower survival to weaning, while a relaxin deficiency in this period doesn't appear to have any impact on lactational performance, though it does impact mammary development in late pregnancy (Porter et al., 1992; Kertiles and Anderson, 1979).

There is a substantial amount of mammary tissue growth during lactation where total gland DNA increases by 82% between farrowing and day 21 of lactation. The extent of this development during lactation depends on the degree of milk removal, also referred to as suckling intensity, which is determined by factors such as the frequency and completeness of milk removal, the number and size of suckling offspring, and the hormones released in response to mammary stimulation (Hurley, 2001). The size of mammary glands at parturition is determined partially the by size of the ventral surface in the sow, it is also expected that different developmental patterns occur in the individual glands based on the size of piglets and locations of the glands. Glands 1, 6 and 7 contain the least mammary DNA at parturition and grow rapidly until day 5 of lactation, glands 2 and 3 have an intermediate level of mammary DNA and do not grow much until day 5 of lactation, and finally glands 4 and 5 which contain the most mammary DNA, may produce excess milk than required by the piglets and experience a regression by day 5 of lactation (Kim et al., 1995).

2.4.2. Availability and Composition of Colostrum:

Early, adequate intake of colostrum is a significant determinant of piglet survival in the early (3 – 4 days) suckling period, where the majority (70 – 80%) of losses occur (KilBride et al., 2011). Piglets are born with very little body fat, so depend solely on colostrum as an energy source in the early neonatal stage. Colostrum also has other important attributes for piglet development such as providing a source of passive immunity, nutrients and growth factors,

and supporting thermoregulation (Farmer et al., 2006). Neonatal piglets use colostrum efficiently due to their large capacity for fat deposition and their ability to absorb immunoglobulins during the first 24 hours of life.

Colostrum yield varies greatly between individual sows, with reports ranging from 1.91 to 5.31 kg, and averaging around 3.57 kg. Comparatively, milk production on day 4 of lactation is estimated to range between 4.60-9.64 kg/day and averaging around 8 kg/day. Thus, the average daily milk yield of sows is around double that of the total colostrum yield (Thiel et al., 2002). The factors which influence colostrum production have not been thoroughly studied, though it is at least known that production is influenced by management factors and both sow and piglet characteristics. As litter size is the main factor influencing milk production, this was thought to have an influence on colostrum production also. However, it has been found that there is no impact of litter size on sow colostrum production. As litter size increases beyond having 9-12 piglets per litter, the total colostrum available per piglet decreases by 22-24 grams (Le Dividich et al., 2004). It is also important to note that with increasing litter size there is also a higher likelihood of lower birthweight or lower vitality and low viability piglets being born, which will consume less colostrum than the recommended intake of 240-328 grams/piglet/24 hours (Milligan et al., 2002, Le Dividich et al., 2004). Other factors thought to influence colostrum production include the genotype, age, parity and bodyweight of sows, nursing behaviour, and litter weight (Farmer et al., 2006).

Colostrum is a viscous solution rich in protein, immunoglobulins, sodium and chloride, but low in lactose and potassium, whereas mature milk is a more fluid solution with the reverse constituents (Farmer et al., 2006). The composition of porcine colostrum is relatively well-known but only recently have bioactive compounds been described. These compounds either protect the piglets from infection or modulate their metabolism.

Constituents of colostrum include proteins, non-protein nitrogenous compounds, carbohydrates, lipids (including fat-soluble vitamins), water-soluble vitamins, minerals, ionic constituents, trace compounds and various cell types (Xu, 2003). The composition at time of farrowing is 24-30% dry matter, 15-19% protein, 5-7% fat, 2-3% lactose and 0.63% ash (Farmer et al., 2006). The majority of compositional changes to colostrum occur within the

first 24 hours post-partum but changes continue to occur over the following 4 days (until day 5 of lactation), these changes in composition over the first day post-parturition are shown below in Table 2.1.

Table 2.1 – Average reported concentrations of the major components in sow colostrum:

Colostrum	Time from Parturition (hours):					
	0[1]	3-4	6	12	18	24
Total solid %	26.7	28.1	23.8	20.1	18.4	20.1
Total Protein %	16.6	16.7	13.8	9.6	9.4	7.7
Fat %	6.4	6.1	5.9	5.9	6.4	8.0
Lactose %	2.8	2.7	3.0	3.6	4.1	3.9
Ash %	0.68		0.63	0.64		0.67
Energy kJ/g	6.7		6.0			5.7

Values in above table have been adapted from Farmer and Hurley, 2015 for The Gestating and Lactating Sow, pp195

[1] 0 hours is defined as the time immediately before parturition, during parturition or when the first piglet is born.

Immunoglobulins (IgG, IgA and IgM) make up 80% of the proteins present in colostrum, but the levels of these decrease by half after the first 12 hours of lactation. It is also reported that the whey content (which includes immunoglobulins, beta-lactoglobulin and alpha-lactalbumin) decreases from 14.8% at parturition to only 4.4% 72 hours post-partum, and caseins are present but only in very small amounts (1.5% immediately after farrowing) (Csapó et al., 1996). There are three main amino acids present in colostrum; glutamic acid, leucine and proline which represent 18.1, 9.9 and 9.1% of the total amount of amino acids, respectively. In terms of free amino acid content, colostrum contains 3-8x more valine, methionine, isoleucine, tyrosine and phenylalanine than mature milk (Csapó et al., 1996). Creatine is also present and is so in greater concentrations in swine colostrum than that of other species and it is thought to have an amino acid sparing effect (Kennaugh et al., 1997).

Lactose is the main carbohydrate present in colostrum with a concentration of 3%. The fat content of colostrum is highest 48-72 hours into lactation, at 12.9%, which is more than twice the amount present between 0-12 hours into lactation. Dominant fatty acids in colostrum are oleic acid, palmitic acid, linoleic acid and stearic acid, which together make up 85-90% of the

total fatty acids present. A notable feature of sow milk is that it contains no butyric, capric, caproic, caprylic or lauric acids for the first 72 hours post-partum and no nonadecanoic acid at any stage (Csapó et al., 1996). Carnitine is responsible for transferring fatty acids across the mitochondrial membrane and is very important in neonatal piglets and so is present in high concentrations in colostrum (370 nmol/mL) (Kerner et al., 1984). Concentrations of vitamins (A, D, E and C, but not K) are typically larger in colostrum than mature milk and there are no significant changes to their concentration during the first 72 hours – though there is a slight decrease in vitamin C from 68.4 to 57.9 mg/kg, which approaches the level of significance (Csapó et al., 1996).

The concentrations of different minerals in porcine colostrum have also been widely reported by Csapó et al. (1996). Ash is lower in first-drawn colostrum than mature milk (0.66 vs 0.82%) and potassium, sodium, zinc and copper are all present at greater levels in colostrum while concentrations of calcium, phosphorus, iron and magnesium are greater in mature milk.

Along with the above nutrients, porcine colostrum also contains bioactive compounds, including hormones and a glycoprotein with bactericidal activity known as lactoferrin (Yang et al., 2000). Progesterone, oestradiol, oestrone, somatotrophin and prolactin are all present in colostrum in high concentrations at parturition and then decline (Devillers et al., 2004). Insulin, neurotensin and bombesin have been detected in swine colostrum, and due to their high concentrations and known roles in gastrointestinal physiology, they are suggested to have important biological functions in neonatal piglets (Weström et al., 1987). Insulin-like growth factors (IGF-I and IGF-II) also have a role in porcine gastrointestinal development, and both are present in significant quantities in colostrum. Concentrations of IGF-I are lower than that of IGF-II at farrowing but not later in lactation. Other growth factors such as epidermal growth factor (EGF) and transforming growth factor-B (TGF-B) have also been identified in porcine colostrum. Milk-borne leptin may influence neonatal growth and physiology, the concentration of this is much greater in colostrum than in the systemic circulation of sows.

There are also various types of cells identified in porcine colostrum as reported by Wuryastuti et al. (1993). Neutrophils and lymphocytes are the predominant cells with macrophages,

eosinophils and epithelial cells are also present but in smaller numbers, various cellular metabolites and nucleotides have also been identified (Atwood and Hartmann., 1995).

The composition of colostral secretions changes rapidly over time. Compositional analysis has shown the transition of colostrum to mature milk occurs within 1-2 days post-partum and that concentrations of IgG and Na^+ decrease while K^+ stabilises over this period (Wilcox et al., 1982). Lowering the Na^+:K^+ ratio is consistent with the paracellular pathway of secretory epithelial cells closing. Increasing active secretion of lactose increases osmotic pressure, hence drawing in a large volume of water and reduces the total solids.

2.4.3. Establishment of Lactation and the Milk Ejection Reflex:

The first of two stages of lactogenesis is secretory differentiation, which occurs 8-10 days pre-partum. This stage is where mammary epithelial cells differentiate in order to have the capacity to synthesise the unique constituents of milk (Pang and Hartmann, 2007). Secretory differentiation is based on changes to mammary cell structure and metabolism, and the increasing concentration of lactose in urine (Hartmann et al., 1984). Secretory activation is the second stage of lactogenesis, where copious secretion of milk occurs at approximately 1.5 days post-partum (Hurley, 2019; Thiel et al., 2014). Before parturition, tight junctions between mammary epithelial cells become leaky, allowing exchange between extracellular spaces and the alveoli lumen. At the onset of milk secretion, the secretory granules are transferred to the alveoli lumen which become larger. Colostral and lipid droplets are present in porcine mammary tissue at around day 105 of pregnancy with marked distension of the alveoli lumen by day 112 and further differentiation again by day 1 of lactation (Farmer et al., 2006). Cellular events which occur in lactogenesis include, expansion of the endoplasmic reticulum (where milk synthesis occurs) between day 105 and 112 of pregnancy, establishment of cell polarity (day 1 of lactation), presence of dilated cisternae on endoplasmic reticulum, and secretory vesicles and microvilli on day 4 of lactation. Microvilli are important as secretory products migrate towards the apical ends of cell and are secreted via exocytosis at microvilli-level, the mammary glands become engorged in late pregnancy as alveoli expand from secretions and fat globules which accumulate at apical ends of cells (Kensinger et al., 1986).

Lactogenesis is controlled by various 'reproductive' hormones such as oestrogen, progesterone, prolactin, and oxytocin; and 'metabolic' hormones such as growth hormone, corticoids, insulin, and thyroxin (Schams, 1976). Prolactin is known to be an essential hormone in the control of lactogenesis, specifically for the maintenance of lactation in swine (Schams, 1976). The release of milk into the ducts of the udder and the teat canal is known to be mediated by a neurohormonal reflex, called the milk ejection reflex involving oxytocin. Oxytocin is released from the posterior pituitary gland after tactile stimulation by the piglets of the udder, then is carried by the circulatory system to the mammary gland (Grosvenor and Mena, 1974) which then causes contraction of the alveolar myoepithelium.

The milk ejection reflex in response to suckling stimulation has been widely studied, and a distinct pattern of nursing, suckling and milk ejection has been observed (Ellendorff et al., 1982). The mean duration of the suckling period was found to be 6.3 minutes, and each milk ejection event occurred within a mean time of 2.4 minutes from the initial pre-ejection massaging of teats by piglets (Ellendorff et al., 1982). Milk ejection is coincidental with a silent suckling (milk withdrawal) period of 7-38 seconds whilst milk is being consumed. In the sow, initiation of the nursing period is indicated by phases of slow and then faster grunting (vocalisation) noises emitted by the sow. The grunting begins around 25-30 seconds before intramammary pressure rises (Fraser, 1977) and acts as a vocal signal to piglets to encourage them to begin the pre-ejection massage stimulation of the udder. Reflex milk ejection appears to only occur if the sow is relaxed. If sows are disturbed or stressed they often refused to nurse and even if they did not refuse nursing they often failed at milk ejection (Ellendorff et al., 1982).

An increased blood concentration of oxytocin was detected 30 seconds before milk ejection, while the concentration of lysine-vasopressin was not found to increase above basal levels (Ellendorff et al., 1982). During incomplete sucklings it was found that there is no increase in blood oxytocin or vasopressin levels. Complete period of suckling and hence complete milk removal is important for the continuation of milk secretion. The intermittent and spurt-like release of 25 μm of oxytocin is concluded to be the cause of reflex milk ejections. The necessity of oxytocin release in order for milk let-down to occur is suggested to be due to the

fact that pig mammary glands have no cisternae for milk storage. The amount of oxytocin released is not dependent on the length of massaging or the number of piglets massaging the teats. It has also been shown that electrical stimulation of the pig neurohypophysis resulted in an oxytocin release and a subsequent milk ejection (Wakerley and Lincoln, 1973).

2.4.4. Nursing Behaviour, Teat Order and Teat Fidelity:

Survival and growth of piglets during the first few weeks of life is almost entirely dependent on the piglet-sow interactions. Usually all of the piglets in a litter suckle at the same time, and nursing events are frequent and 'cyclical', occurring approximately every 50 minutes towards the end of lactation with wide variation between individuals (Grahofer and Plush, 2023). The pattern of suckling and nursing evolved primarily because the sows' mammary glands have no cistern to store milk between nursing events, so the milk is only available when ejected following the release of oxytocin (De Passillé and Rushen, 1989*)*. During parturition, colostrum can be expressed from the teat easily so piglets can suckle continuously. This 'on-demand' ability to suckle ends around 11 hours post-farrowing, though there is some contention as to the point at which colostrum secretion is transitioned to cyclical milk secretion (Hemsworth et al., 1976).

During the first few hours after birth, suckling bouts are frequent and tend to involve only a few piglets at a time, but as the piglets age the frequency of suckling bouts decrease and the number of piglets present at each bout increase (De Passillé and Rushen, 1989). Despite claims that suckling changes from continuous early after birth to synchronised suckling, there isn't an identified point in time where suckling is continuous, and nor when it is solely cyclical/synchronised (De Passillé and Rushen, 1989*)*. The criteria used to define and determine the start of synchronised suckling is also misleading – it is said that continuous suckling ends whenever the first synchronised suckle occurs except there is no specified definition of what a synchronised suckle is, it is just assumed to be when all the piglets suckle together for the first time (Hemsworth et al., 1976). It is also important to note that just because the first synchronised suckle has occurred, doesn't mean that all following bouts will also be synchronised.

Soon after parturition, a typical nursing structure is established which includes 5 main phases (see Figure 2.2) – initiation of nursing, pre-ejection, milk ejection, post-ejection and finally termination of nursing. The nursing interval gets longer as lactation progresses and eventually ends up at approximately 50 minutes long by the end of lactation (Grahofer and Plush, 2023).

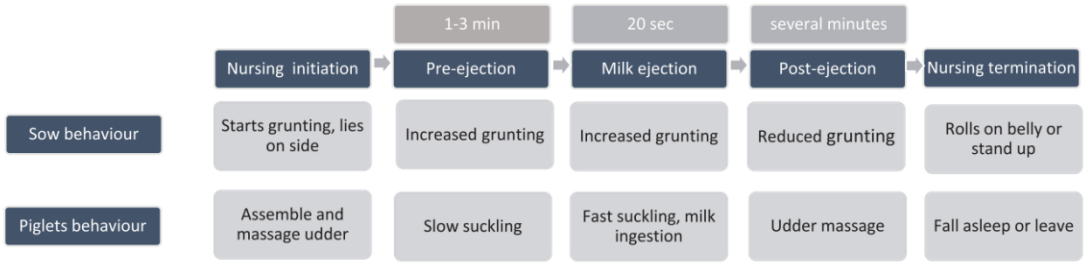

Figure 2.2 - Structured nursing behaviour in pigs, figure (Source: Grahofer and Plush, for Animal Frontiers 2023).

It is widely accepted that piglets tend to suckle from the same teat throughout lactation, though little had been detailed about how this teat fidelity comes about until more recent years. The consistency of teat choice becomes increasingly progressive over the first 7-10 days of lactation, but teat preference is thought to occur after only two days (McBride, 1963). Competition within a litter for different teats is important in swine production, and due to the difference in piglet body sizes within a litter, it can produce due glands with differential milk production (De Passillé et al., 1988). This comes about as the stronger, larger piglets are better able to massage their teat segment more frequently and/or for longer. The advantage conferred through such behaviour is increased blood flow and nutrient supply to stimulated teats, resulting in increased milk yield. This is described as the 'restaurant hypothesis' (Algers and Jensen, 1985) whereby piglets order the size of their future meals through udder stimulation.

Piglets are considered unusual compared to neonates of other species as they engage in agonistic behaviours during suckling, at a very early age, with fighting having been recorded frequently only a few hours after birth. Scheel et al. (1977) suggested that success or failure in these early fights over teats will correspond to varied growth rates within the litter. There are four proposed ways in which piglets who lose these teat order fights may experience decreased weight gain/body weight; infrequent suckling, difficulty establishing 'ownership' of

a teat (piglets who do not establish a teat fidelity tend to grow at a slower rate), inability to suckle as much after birth so fewer immunoglobulins (colostrum) are ingested, and finally they may not have access to the more productive anterior teats (De Passillé and Rushen, 1989).

Competition between suckling piglets may be divided into direct and indirect competition. Indirect competition may involve competing for milk via extra stimulation of the teats while direct competition includes actions that mean some piglets are excluded from the udder. The results of both kinds of competition are apparent as varied growth rates (Thomson and Fraser, 1986).

It has been suggested by Jeppesen (1982) that piglets are influenced by sounds from the mother in the first 24 hours after birth which attract the piglets to the front teats. The piglets then space themselves along the udder and learn to recognise where they suckle. Each piglets' saliva may contain an odourous substance which helps them to recognise the teats once they have suckled for the first time (Jeppesen, 1982). Another study has suggested that this odourous saliva and the recognition of neighbours is involved in the formation of teat-order (McBride, 1963).

Other studies (McBride, 1965; Gill and Thomson, 1956) have proposed there is a preference for suckling the anterior teats because these have a greater milk yield, of around 15.3%, than the posterior teats. The piglets suckling either of the front teats is found to have superior weight gain and typically have a higher rank in the dominance hierarchy of the litter (Scheel et al., 1977). In modern sows, the anterior and middle mammary glands tend to be more developed than the posterior mammary glands (Kim et al., 2000), and it appears that piglets who feed from the anterior 5 teats gain weight faster than those who nurse the remaining posterior glands. The first 5 glands also have larger wet and dry weights, and a greater DNA and protein content comparative to the remaining glands, with the piglets who nurse these heavier glands gaining weight faster. There is little difference between the anterior and middle mammary glands, but a significant difference between the anterior/middle glands and the posterior mammary glands. This functional superiority of the anterior and middle

mammary glands has a positive correlation with the increase in bodyweight of nursing piglets (Kim et al., 2000).

Studies of correlations between teat order and piglet weights have produced relatively controversial results. Some have concluded that in the fights to determine teat-order the larger individuals have more success, and so gain ownership over the front teats. Others have proposed that males, due to larger birthweight, may be more likely to occupy anterior teats than females (Scheel et al., 1977), whilst others have observed the opposite and concluded the growth of an individual depends on the number and size of neighbours (Fraser and Thompson, 1979). Suckling the most anterior pair of teats means the piglet will only have 3 neighbours maximum while a piglet suckling the next most anterior or any further back than these (except the furthest most posterior pair) will have up to 5 neighbours. Rosillion-Warnier and Parquay (1984) did not observe any agonistic behaviour during the formation of teat order in their study, regardless of the litter size and also concluded that sex, birthweight (except for very low birthweights) and birth-order had no effect on the formation of teat-order. This is consistent with other studies such as Fraser and Thompson (1979) and is contradictory of findings from studies such as (Hartstock and Graves, 1976; Scheel et al., 1977). Despite larger litter sizes having no impact on agonistic activity, they still cause a strain on the teat order development, littermate competition, and also on the capability of the sows to adequately feed their litters (Kobek-Kjeldager et al., 2020).

2.5. Summary:

A comprehensive gene catalogue of uncultivated microbiota is useful for studies of the gut microbiota itself, but the corresponding microbial entities of MAGs need to be confirmed via cultivation, isolation and analysis of the microbes (*Chen et al, 2021*). Though from metagenomic studies to date, we can see that there is a significant impact on the gut microbiota of having an abrupt and distinct change of diet at weaning, at both a taxonomic and metagenomic level. Because of how advantageous pigs are to human medical research, it would be invaluable to be able eventually produce a comprehensive gene catalogue of the gut microbiota in pigs, though this would require a large amount of time and resources due

to the fact that the pig gut microbial populations are, for the most part, poorly characterised, especially to a strain level. Future studies should focus on expanding the amount of known and characterised bacteria and other microorganisms in the microbiome. This would allow an increased level of manipulation of the microbiome and if a greater understanding of the influence of the microbiota have on production traits and general health, especially the microorganisms which influence fat and protein deposition.

This literature review has also discussed some of the larger areas of farm-management and practises within the pork industry which have evidence of influence on the gut microbiota and subsequently the overall productive performance of pigs – those specifically being differential nutrition, use of probiotics, level of housing enrichment, cross-fostering, and disinfection of pens. Results and conclusions are typically inconsistent from previous studies on the above farming management practises. It is evident that these factors play some role in the succession and composition of microbiota in pigs, but to what extent the microbiota is influenced by these practices is still unknown. Because of this, the area requires further conclusive research to determine initially what the consistent influence of each practise is (if there is one) on the microbiota, and then how to best manipulate to pig gut microbiota via farm management to result in the most optimal productive performance.

Intake of colostrum is important as piglets require the energy source and passive immunity transfer, especially as they are only able to absorb the immunoglobulins for a short period of time after birth, and are born without fat so the nutrients provided by colostrum are necessary to permit thermo-regulation. Colostrum yields differ between sows but the overall yield of colostrum is about half of the sows' day production of milk during lactation. The composition of colostrum is relatively well documented, and at different stages of lactation - right after farrowing, just before transition to mature milk etcetera, which is important as the composition of colostral secretion change rapidly as lactation continues.

The anterior and middle mammary glands produce the most milk during lactation and hence the piglets who nurse these glands will grow faster, the anterior and middle mammary glands also contain more mammary DNA and protein content than the posterior glands. This is important as piglets show a preference for these anterior and middle teats during the first

few hours after birth, some studied concluded this is due to a larger teat, others have said it is due to piglets moving toward the sow's grunting noises to suckle and others that this is because those suckling anterior teats have less neighbours than the middle or posterior teats. There is plenty of contradiction between studies on various areas of swine lactation, specifically the behavioural aspects of lactation and no real conclusions which have been agreed upon.

Chapter 3. Materials and methods:

The collection of samples for this study was approved by the Massey University Animal Ethics committee (application number 22/37). The samples were collected in February 2023 from sows and piglets at a commercial farrow-to-finish indoor pig farm. The farrowing accommodation consisted of conventional farrowing pens with crates and fully slatted flooring. The pens featured a forward creep with a heated solid concrete floor and overhead infrared heat lamps. The creep area was maintained at 33°C whilst the temperature in the farrowing rooms was maintained at 22°C.

Samples were collected from N= 21 sows (N = 17 milk samples and N = 20 udder skin swabs) and N = 63 piglets (N = 63 piglet faecal samples) within 72 hours of parturition. The farm had a farrowing cycle of approximately 20 sows per week, with most (~70%) farrowing on Thursday and Friday. The farm was visited each Saturday for three successive weeks to ensure that a sufficient number of piglets and sows were sampled within the first 72 hours of their farrowing event. Farm records were used to exclude sows which farrowed outside of this window.

Udder skin samples were collected using a sterile swab (brand: Copan, 150mm long, rayon tipped and individually packaged) that was pre-moistened in milli-Q water and rubbed along the ventral udder surface for at least 20 seconds to ensure good transfer of bacteria from skin onto the swab. Milk samples were collected by waiting for a suckling event to occur where milk let down was initiated naturally. Once milk let down occurred, a minimum of 1.5ml of milk was captured into a sterile Eppendorf tube, which was then closed and stored on ice. Piglet faecal samples were obtained using the same brand of sterile swab pre-moistened in sterile milli-Q water. Piglets were lifted and held by a handler, whilst another handler inserted the tip of the swab into the rectum of the piglet. The swab was rotated in a gentle clockwise direction and remained in the rectal passage for 10 seconds to ensure good bacterial transfer. On removal the piglet was assessed for any discomfort then sprayed with a stock-safe marker paint to avoid sampling the same piglet multiple times. Three piglets from each sow were sampled. Sows and piglets were excluded from sampling if they or their piglets had received any antimicrobial treatments (Chodhury et al., 2019).

All samples were kept on ice within an insulated container for transport back to Massey University (approximately 1 hour travel time) at which point they were frozen at -80°C until DNA extraction was performed. The Extraction protocol for DNA was conducted using the Presto Stool DNA Extraction Kit, as per manufacturer's instructions, with one minor change: in place of stool samples, the swab tip was placed into the bead beating tube. In the case of milk samples, a spun down sample was used instead, to reduce the presence of proteins which impact extraction processes.

DNA extraction yields were determined using standard Qubit protocols with 2µl of DNA extraction and 198µl of qubit reagent. DNA yields are presented in the appendix (Stacked bar-plots in section 8.4), but in general DNA quantities ranged from 0.5ng/µl to 570ng/µl, indicating a variance in the extraction efficiencies. Extracted DNA samples underwent 16S metabarcoding sequencing at the Massey University Genome Service in February 2024. An amplification round was conducted using protocol outlined in Kozich et al. (2013) on an Illumina MiSeq 2X 250 base paired end run using version 2 chemistry. From a total of 105 samples taken, 83 samples were of a high enough quality following extraction and sequencing for further analysis of the microbial composition, meaning 83 samples passed the required checks of Q30, read quality, DNA concentration and purity ratios. Of the 83 samples available for analysis there were 17 milk samples, 17 skin swab samples and 49 piglet faecal samples. The combination of milk, skin swab and piglet faecal samples per litter are presented in table 3.1. Replicate samples were taken but not processed, and only pen groups with either sow samples and piglet samples, or only sow samples were processed.

All sequencing runs contained a water blank sample to control for contamination throughout the extraction and sequencing process. Water used for the sample collection was also used to detect contamination.

In preparation for metabarcoding analyses, a copy of the greengenes 16S database was downloaded in Kraken2 (Wood et al., 2019), on 20 February 2024. See Appendix 8.1 for the code and methodology followed in Python software, and Appendix 8.2 and 8.3 for code and methodology followed in R.Studios to produce stacked bar-plots and alpha and beta diversity

indices. A Macbook Pro using version 11.1 of MacOS Big Sur was used to run version 3.6.15 of Python through Anaconda.

Chapter 4. Results:

A varying number of reads were sequenced from different samples, even from within the same pen. For example, the milk sample of sow 676 only had 25,000 reads whilst the piglets suckling from this sow had between 125,000 – 160,000 reads, shown below in Table 3.1.

Table 3.1 - Number of reads from the samples sequenced:

Sow ID	MILK	SOW SKIN	PIGLET 1	PIGLET 2	PIGLET 3
519	82505	100370	93516	71036	102034
581	85403	106565	140253	125565	164917
582	66310	112585	77422	138783	-
586	66254	107999	130110	117105	148395
591	67413	102348	71271	148593	148395
592	52735	71101	116766	130586	-
617	104333	114591	106587	-	-
676	24972	117232	125408	159924	139561
679	-	76178	93708	100472	-
742	81762	108222	121642	99801	135008
744	-	59253	154483	144591	128326
745	32161	106786	122410	126451	-
752	-	117698	102656	123047	51812
803	79289	70673	155161	-	-
843	-	-	161026	-	-
892	10168	113550	103617	117944	123184
893	25881	-	140833	-	-
898	92788	-	89053	127804	-
PEN 2*	93335	143697	110811	126180	104606
PEN 5*	70816	-	115159	84809	97105
PEN 8*	78966	150013	121785	122639	-
AVERAGE	65593.6	104638.88			

*Sows in pens 2, 5 and 8 did not have ear tags and thus were identified by their pen number within the farrowing room.

The original kraken reports for each sample illustrate that there were no E. *coli* bacteria present in any of the samples (milk, sow skin and piglet faecal samples). Despite this, there are still a few pathogenic bacterial families present in a majority of the samples, such as Streptococcus, Staphylococcus and Campylobacter species – especially S. *aureus*. Other

pathogenic species which showed slightly less of a presence were Salmonella and Mycobacteria, and a few samples also showed a presence of Listeria species.

Mycobacteria was more present in the sow skin and milk samples. Only a few piglet faecal samples showed any presence of this bacteria, and when it was present, there was only a small number of reads of it.

4.1. Relative (%) Abundance Plots:

From Figure 4.1, below, we can see that the most prominent phyla in milk samples are *Firmicutes* and *Proteobacteria*, followed by *Actinobacteria* and *Bacteroidetes*. Samples from sows 581, 591 and 898 contained the least abundance of the *Firmicutes* phylum, milk samples from sow 581, 591 and Pen 2 contained the greatest abundance of the *Proteobacteria* phylum. The milk sample from sow 617 has the greatest abundance of bacteria from the *Bacteroidetes* phylum, followed by sow 519, 592 and Pen 8.

Figures 3 – 8 present stacked bar-plots of read proportions of different samples such as sow skin, sow milk, and piglet faecal samples. The different coloured bars are a proportion of the total number of reads for that sample, meaning the size of the bar reflects the percentage of reads present that are associated with that phylum of bacteria or archaea.

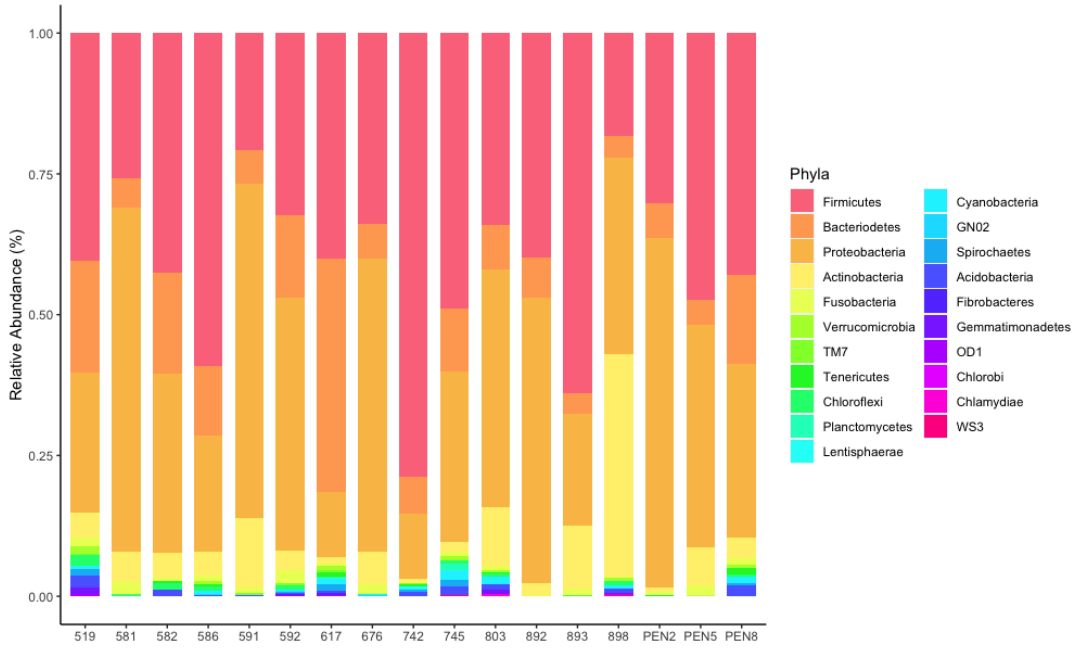

Figure 4.1 - Relative abundance plot of milk sample reads, showing abundances of different bacterial phyla present in the sow milk samples

The milk from two sows (582 and 803), contained reads identified to be from the *Chlamydiae* phylum. For sow 803, 0.25% (219 reads) of the milk sample reads were identified as this phylum, whereas for sow 582 only one read from the sample was identified to be *Chlamydiae*.

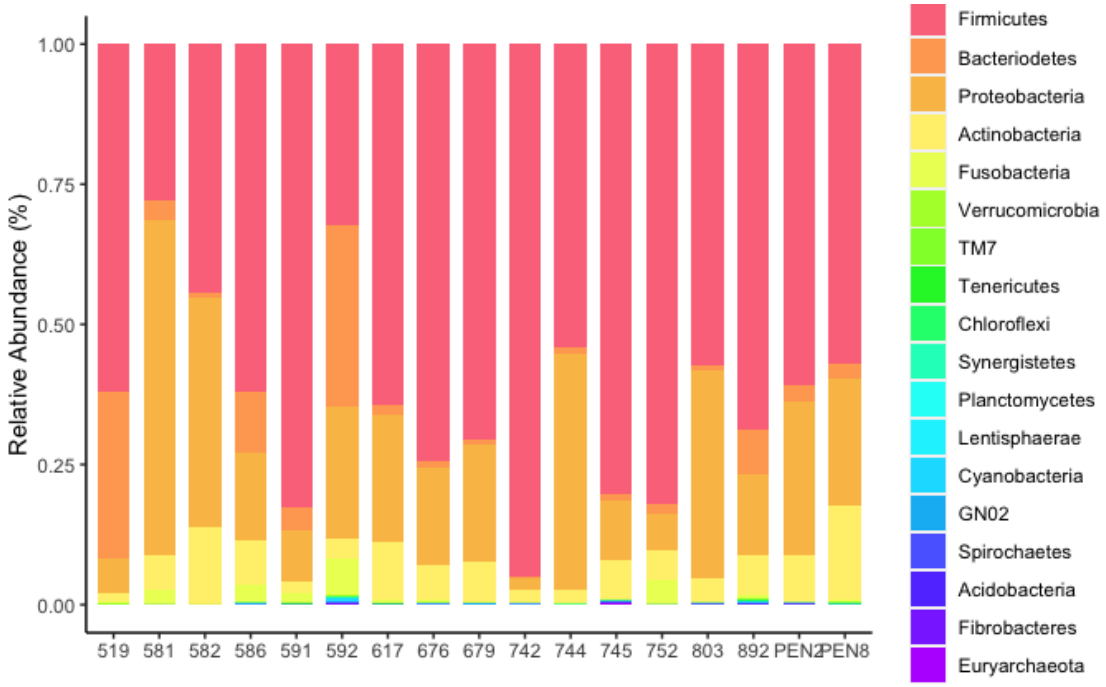

Figure 3.2 - Relative abundance (%) plot of sow skin sample reads, showing abundances of different bacterial and archaeal phyla present in sow skin samples.

In Figure 4.2 we can see that similarly to the milk samples (Figure 4.1) most sow skin samples had *Firmicutes* and *Proteobacteria* as the most abundant phyla, followed by *Bacteroidetes* and *Actinobacteria*. We can see that the skin sample from sow 581 contains the greatest abundance of bacteria from the *Proteobacteria* phylum, while the sample from sow 742 appears to contain the lowest abundance of bacteria from this phylum. In terms of the *Firmicutes* phylum, the skin sample from sow 742 contains the greatest abundance (followed by sow 591), whilst the skin sample from sow 581 contains the smallest abundance of bacteria from the *Firmicutes* phylum (followed by sow 592).

There was also a presence of *Euryarchaeota* (Archaea) in 11 of the 17 samples, with one sample, sow 745, having 0.33% of the total reads identified as *Euryarchaeota*. The skin samples from sow 519 and sow 592 were identified to contain the greatest abundances of bacteria from the *Bacteroidetes* phylum, followed by samples from sows 586 and 892.

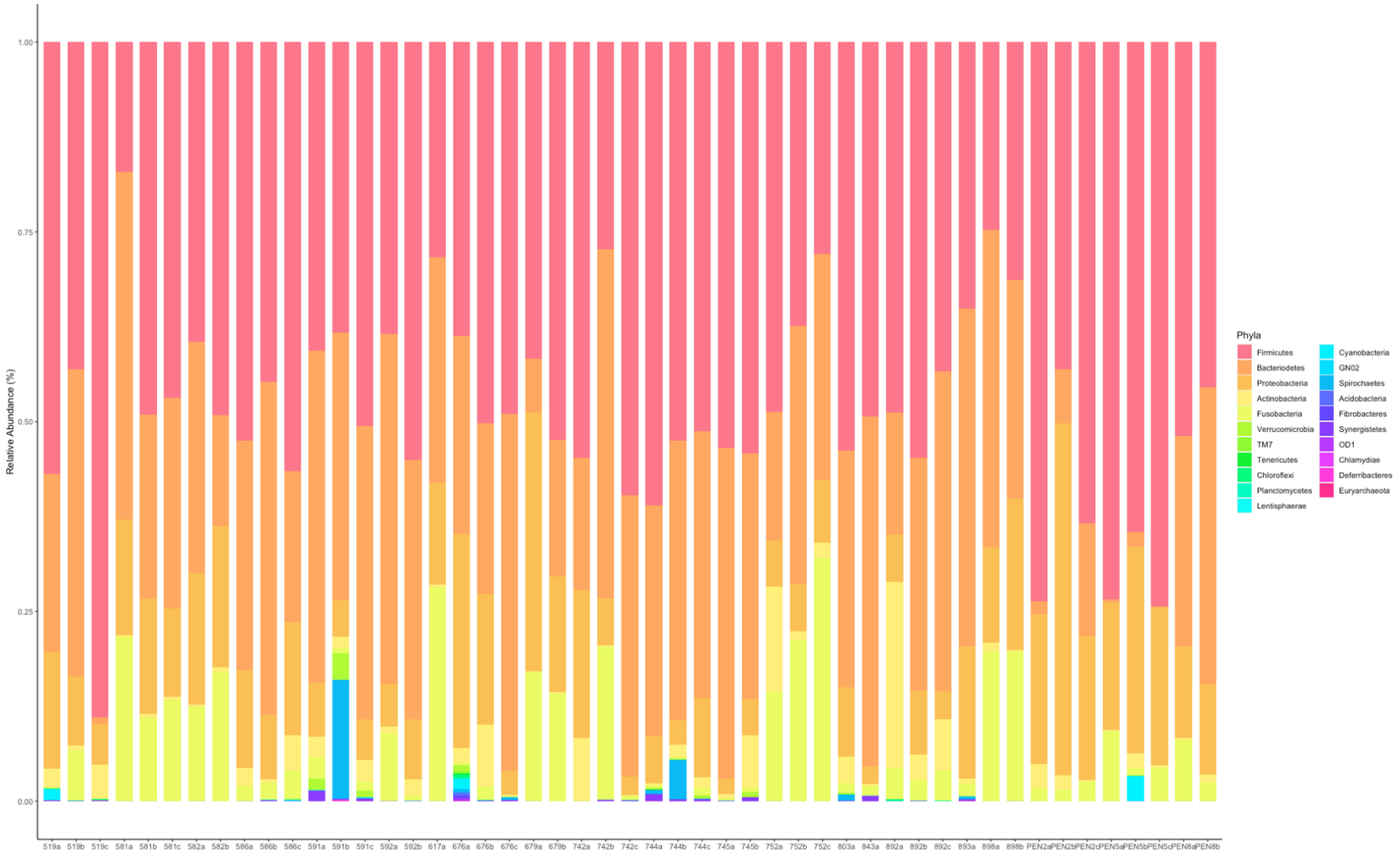

Figure 4.3 - Relative abundance (%) plot of sample reads from piglet faeces[1], showing abundances of bacterial and archaeal phyla present in piglet faecal samples.

[1] the faeces of each individual piglet in each numbered pen (hence a, b, and cs in plot labels)

In the above figure (Figure 4.3), we can see that there is some variation between the groups of piglets in each pen, though there is obviously more variation within some groups than others. For example, the 3 piglets from Sow 519 appear to have varying abundances of bacteria from the *Firmicutes* and *Bacteroidetes* phyla. Specifically, the sample from piglet 519c shows a much lower abundance of bacteria from these phyla than the samples from its litter mates in the samples from piglets 519a and 519b.
Piglets 591b and 744b show a significantly greater % abundance of bacteria from the *Spirochaetes* phylum than all the other piglets. Similarly, piglets 519a, 676a and Pen 5b show a greater abundance of bacteria from the *Cyanobacteria* phylum, and piglets 519a and 744a show a greater abundance of bacteria from the *Synergistetes* phylum.

The *Spirochaetes* phylum include genera of bacteria such as *Treponema*, *Leptospira* and *Borrelia*. Interestingly, the genus classifications of *Spirochaetes* found in piglet 591b and 744b included *Treponema*, *Spirochaeta* and *Sphaerochaeta*. The *Treponema* genus of bacteria is known to include strains which can cause syphilis, though none of these strains were detected in either of the two piglets, instead the strains detected are ones known to cause periodontal lesions.

Piglets with reads belonging to the *Leptospirae* classification (all had between 1-11 reads) - 581a, 581b, 582a, 586, 617a, 752a, 752b, 898b, Pen8a. None of the Pen2 piglets (a, b or c) had any bacterial reads from the *Spirochaetes* phylum detected in their samples, but at least one of the piglets in every other pen had some abundance of *Spirochaetes* detected.

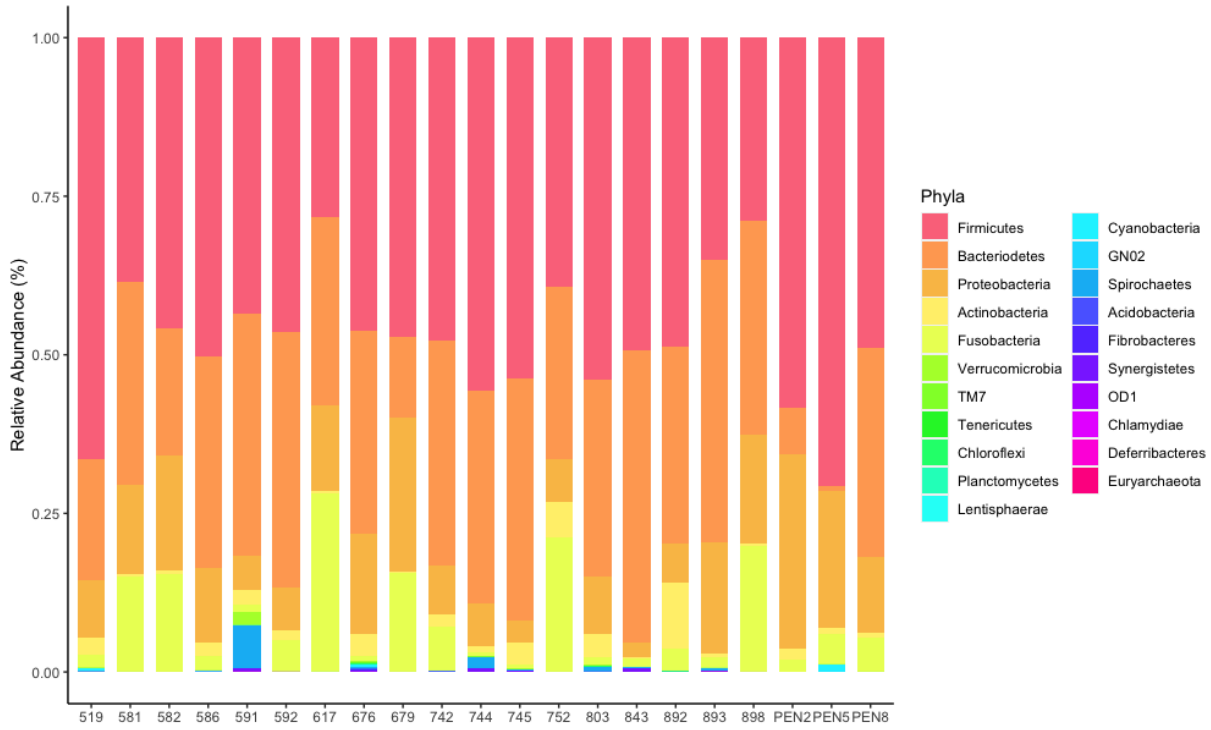

Figure 4.4 - Relative abundance (%) plot of the average sample reads from piglets in each pen (at phylum level), showing the average abundances of bacterial and archaeal phyla present in the piglet faecal samples.

In Figure 4.4 (above) compared to Figure 4.3, since the phylogenetic compositions of all of the piglets from the same pens have been averaged, we lose some of the diversity and the significant differences between piglets within a pen get lost. Despite this, we can still see that piglets from pen 591 and 744 still on average have a greater abundance of *Spirochaetes*, and that piglets in pen 519 and PEN2 have, on average, a greater abundance of *Firmicutes* compared to piglets from other pens. The piglets in PEN5 have the lowest abundance of reads from the *Bacteroidetes* phylum, followed next by the piglets in PEN2 – the rest of the piglets in all other pens appear to have relatively similar abundances of *Bacteroidetes* bacteria present.

Piglets in pen 617 have the greatest abundance of Fusobacterial reads, followed by piglets in pen 752 and 898. Not all of the piglets seem to have abundances of *Actinobacteria*, those that do appear to have relatively similar abundances of reads for that phylum, though piglets in pen 892 do have the greatest abundance of *Actinobacteria*.

Similarly to the milk samples (Figure 4.1), there is a presence of *Euryarchaeota* (Archaea) in the piglet samples, with 16 of the 21 pens (shown in Figure 4.4, the average reads of each pen) having a presence of *Euryarchaeota*. The piglet from the pen 893 (piglet 893a) had the largest average number of reads for this phyla of Archaea with 53 reads, while the rest of the 15 pens with Archaea detected had an average of between 25-0.5 reads.

It appears that the category of samples with the most phylogenetic variation are the milk samples, with the all of the milk samples having consistently larger abundances of the lesser common phyla such as *Verrucomicrobia*, *Tenericutes*, *Chloroflexi*, *Cyanobacteria*, *Spirochaetes*, *Acidobacteria*.

Milk and sow skin samples have relatively consistent abundances of *Actinobacteria*, except for the milk sample from 898 which has quite a large abundance of *Actinobacteria* detected. None of the piglet faecal samples were very abundant in *Actinobacteria*, nor were the abundances very consistent across all of the piglets.

The piglet faecal samples appear to typically have a larger abundance of *Fusobacteria* compared to their respective milk and sow skin samples. There is also a trend of sow skin samples containing larger abundances of *Firmicutes* phyla, and the piglet faecal samples containing larger abundances of *Bacteroidetes* compared to their respective milk and sow samples from the same pen numbers.

The abundances of *Firmicutes* and *Bacteroidetes* in all of the piglet faecal samples are more consistent than the abundances of the two phyla across the milk or sow skin samples. There is far more variation in abundances of *Firmicutes* and *Bacteroidetes* within the milk and sow skin samples – the sow and milk samples tend to have either very large or small abundances of the phyla.

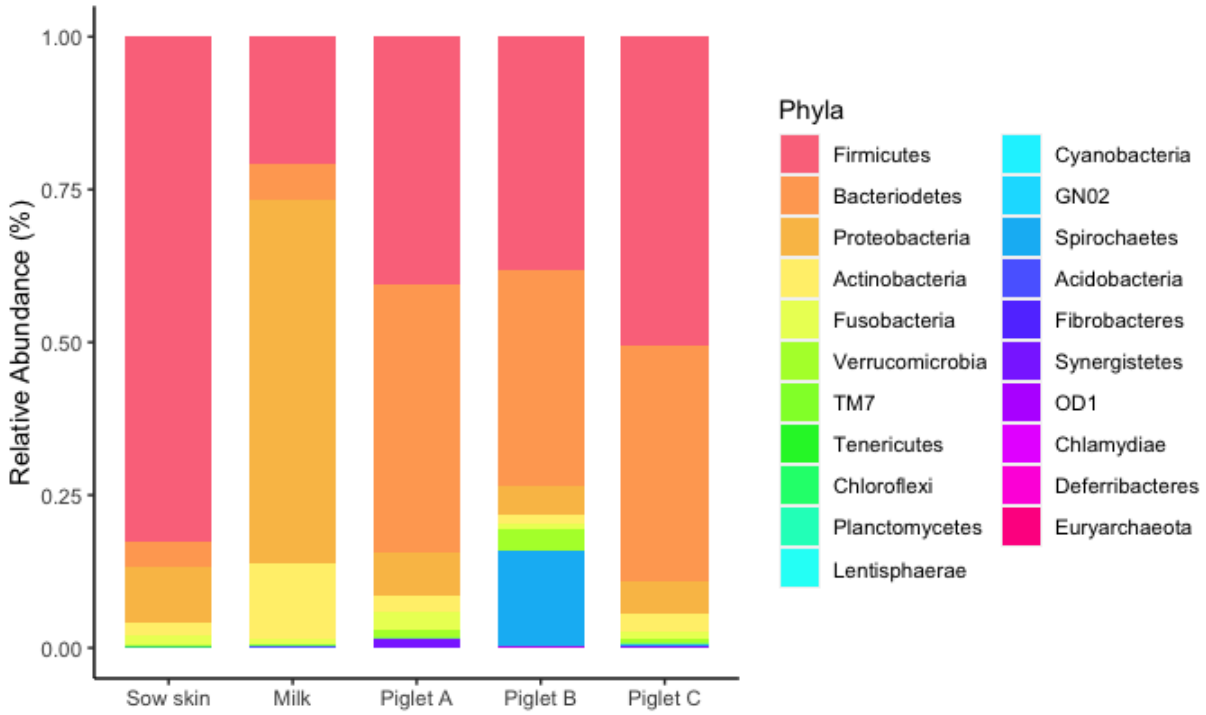

Figure 4.5 – Relative (%) abundance plot of phyla, showing all sample reads from Pen 591

One of the pens with a more significant difference between the samples is pen 591, as shown above in Figure 4.5. Figure 4.1 shows milk sample from pen 591 having a very large abundance of *Proteobacteria*, followed by smaller abundances of *Firmicutes*, *Actinobacteria*, and then *Bacteroidetes* being the smallest of the main 4 phyla. Whilst in Figure 4.1 we can see the pen 591 sow skin sample has a very large abundance of *Firmicutes* (the second largest of all sow skin samples), and then very small abundances of *Proteobacteria*, *Bacteroidetes* and then *Actinobacteria* – the sow skin sample also showed a similar abundance of *Fusobacteria* to the abundance of *Actinobacteria*. In Figure 4.4, showing the average sample reads from the piglets in each pen, we can see that the piglets from pen 591 have similar sized abundances of *Firmicutes* and *Bacteroidetes*, smaller abundances of *Proteobacteria* and *Actinobact eria*, but also have a large abundance of bacteria from the *Spirochaetes* phyla – due as the sample from piglet 591b detected a particularly large abundance of this *Treponema* family bacteria.

This stark difference in abundances of *Spirochaetes* bacteria between piglet 519b and the other two piglets from this sow (591a and c) leads to wondering about cross-fostering and whether the piglet originally was with another sow and then moved to be with piglet 591. Because of how young the piglets were when samples were taken it seems unlikely that cross-

fostering would have made such a large difference in abundance of *Spirochaetes* bacteria present in the microbiome after less than 24 hours with the foster-sow. Although, the rest of the abundances of each phyla are relatively similar to the phylogenetic compositions of piglet 591a and 591c, all 3 piglets have similar abundances of *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia*. They also have similar abundances of *Firmicutes* bacteria, though piglet 591b has a smaller abundance than the other two piglets from this pen.

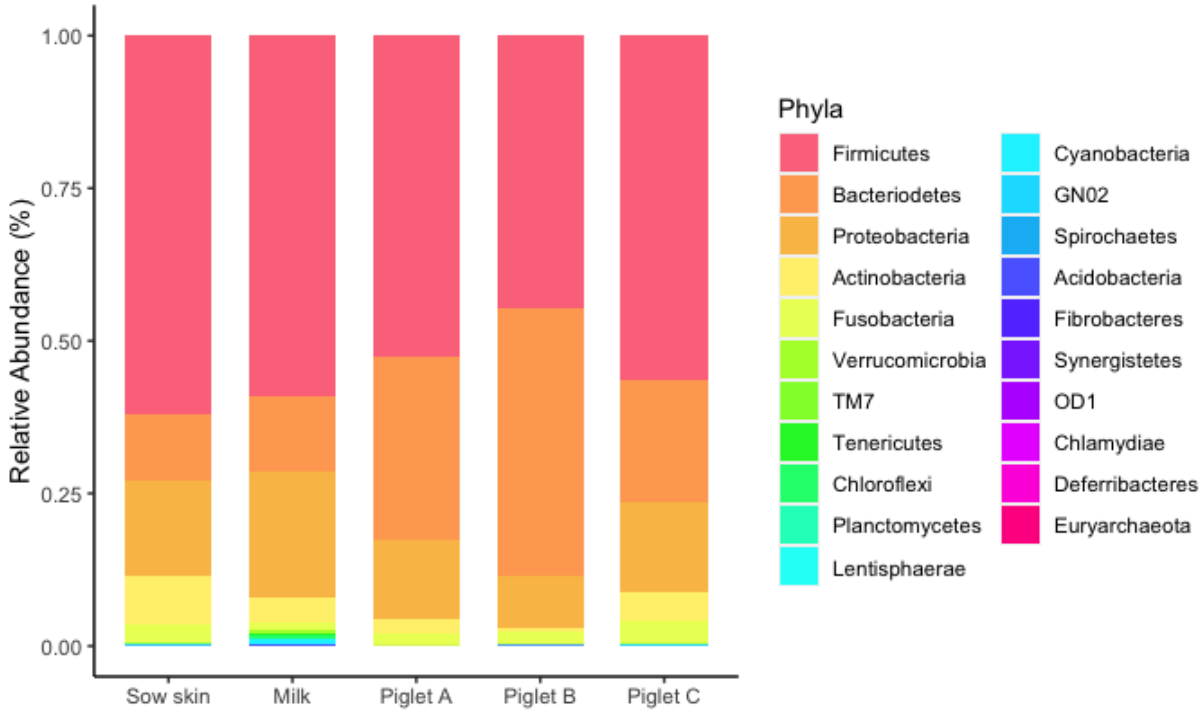

Figure 4.6 - Relative (%) abundance plot of phyla, showing all sample reads from Pen 586

The samples from pen 586 (Figure 4.6) show the least differences between samples in the relative abundance of microbial populations compared to the difference between samples in other pens. The samples have relatively consistent abundances of *Firmicutes*, *Bacteriodetes*, and *Proteobacteria*; while the abundances of *Actinobacteria* are much more varied. Piglet B has a slightly greater amount of *Bacteriodetes* in comparison to the other piglets, but this difference is smaller than the differences between samples within each other pen group. Piglet B also appears to have the smallest abundance of *Actinobacteria*, with the sow skin sample having the greatest abundance. The milk sample has greater abundances of the lesser common phyla compared to the other categories of samples, following the trend previously

mentioned. The samples of this pen also follow the afore-mentioned trend of the piglet faecal samples contained greater and more consistent abundances of *Bacteroidetes*, although the samples contradict the trend that piglet faecal samples contained greater amounts of *Firmicutes* – although since the abundances of *Firmicutes* across all 5 samples are relatively consistent, this contradiction is negligible. There is also the possibility for competing organisms which go undetected in other samples, and that presence of this decreases abundances of *Bacteroidetes* and *Firmicutes* but does not show up on the stacked bar-plots. Effectively, other competing organisms can cause decreased abundances of different bacterial phyla in the gut microbiota, resulting in relative abundances that go against the trend.

4.2. Krona Plots (Snapshots of Bacterial Compositions):

The below Krona plot snapshots show an example of the uses Krona plots have as a tool for comparing microbial compositions. Figures 9 – 11 present Krona plot snapshots which shows the percentage of reads present in a group of samples, being sow skin, sow milk or piglet faecal samples, and the taxonomic levels related to these reads beginning with the super-kingdom down to the family level and the associated abundances of identified spectra. The snapshots are taken at with the phylum level of bacteria at the innermost of the plot, and with the species at the outermost of the plot. The interactive nature of the html version of these Krona plots allows us to file through the layers of the compositions, looking first at the phylogenetic level closest to the centre of the plot, and becoming more and more specific in classification as the layers get closer to the edge of the plot, where we can see abundances of specific strains of bacteria.

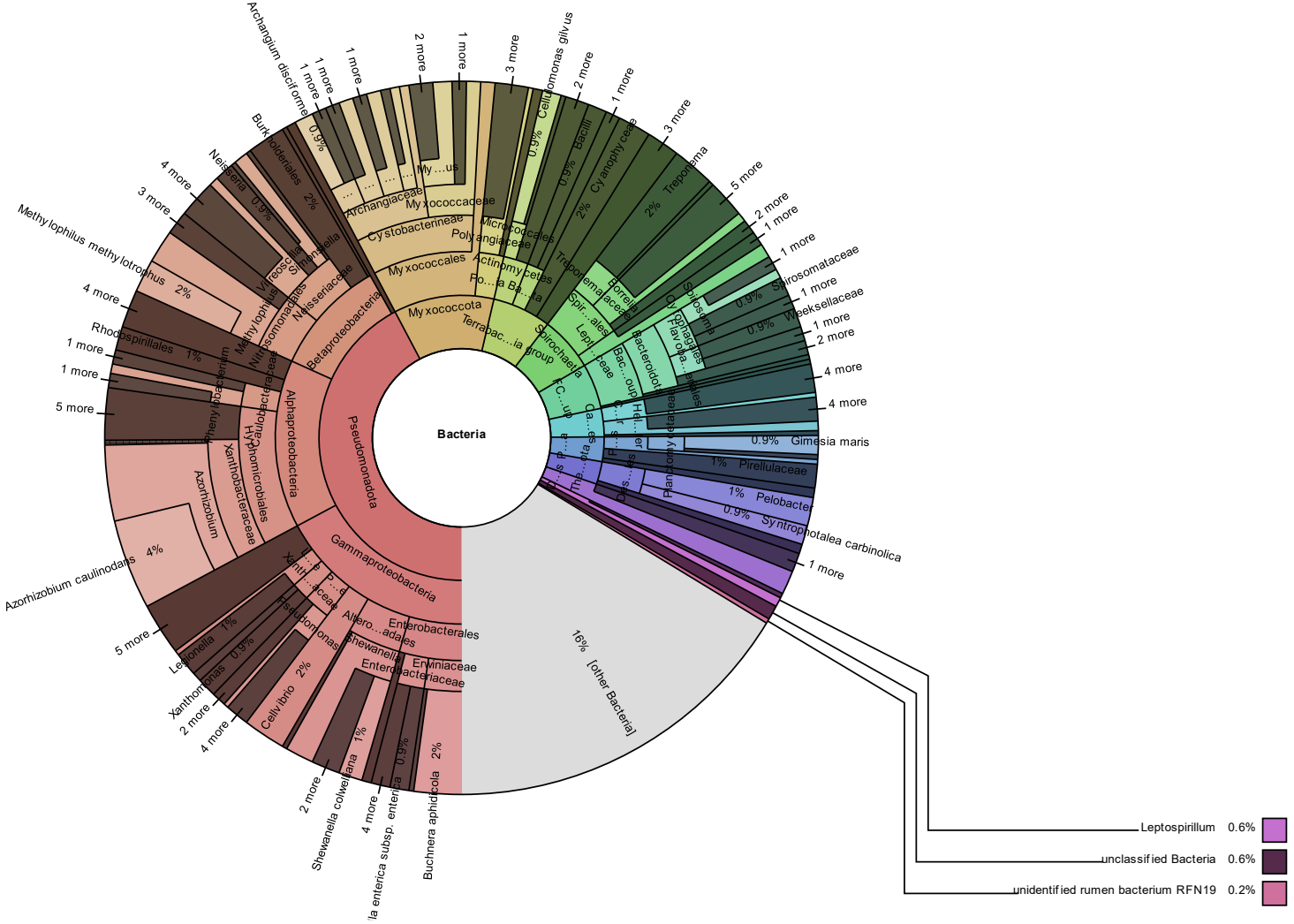

Figure 4.7 - Krona Plot of Average Milk Sample Bacterial Compositions

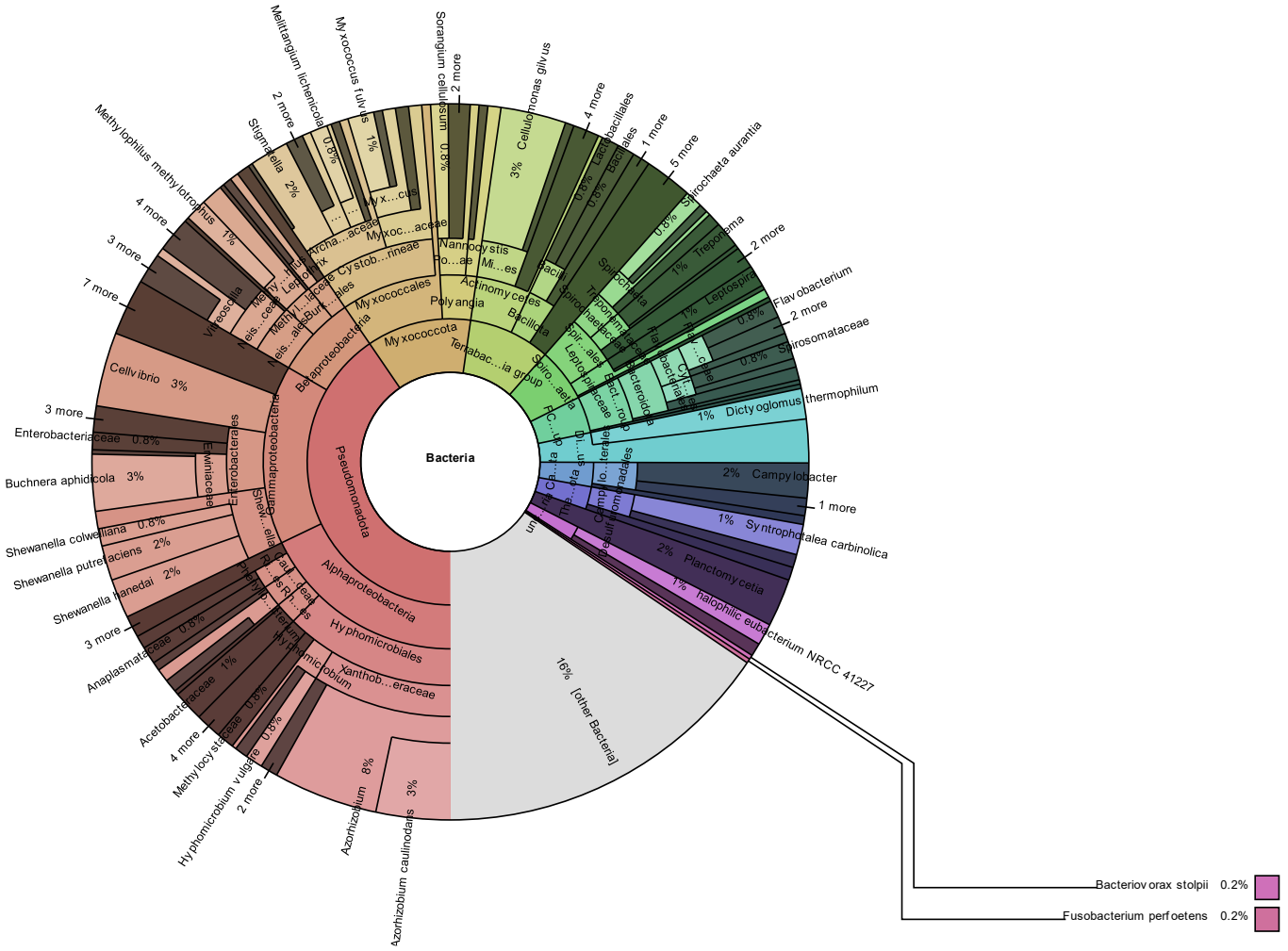

Figure 4.8 - Krona Plot of Average Sow Skin Sample Bacterial Compositions

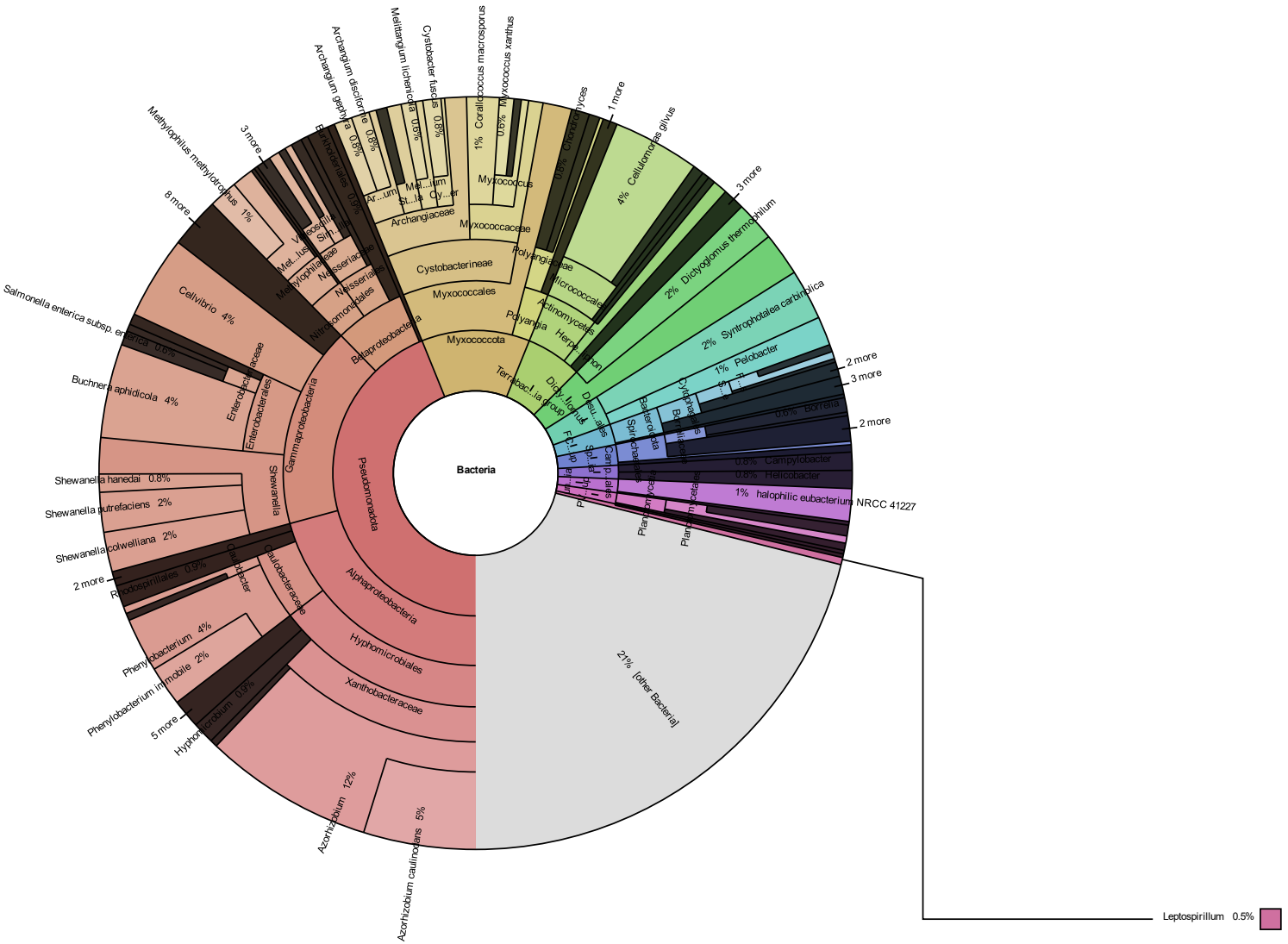

Figure 4.9 - Krona Plot of Average Piglet Faecal Sample Bacterial Composition

4.3. Diversity Indices:

The samples from pen 582 all show beta-diversity indexes of above 0.8, except for the beta-diversity index between the samples of Piglet 1 and Piglet 2 which has a value of 0.427. This indicates that the samples in pen 582 contain mostly different species of bacteria from one another.

In Pen 892, the beta-diversity indexes between the milk sample and Piglet 1, as well as the index between the milk sample and Piglet 3, both have a value of 1. The beta-diversity index for the milk sample and Piglet 2 has a value of 0.987 which indicates that 98.7% of the species present in each sample are different. The indexes with a value of 1 indicate that 100% of the species present in Piglet 1 and 3 differ from those present in the milk sample.

The lowest beta-diversity index value is 0.131, between the samples of Piglet 1 and Piglet 3 from PEN5. The next lowest is 0.149 between samples of Piglet 1 and 2 from pen 586, then 0.281 between samples of Piglet 2 and 3 from pen 892. As lower values indicate a higher amount of similarity, these samples contain the greatest numbers of the same species as each other. This level of dissimilarity can be seen above (Figure 4.9) in the areas of condensed red/orange squares along the red diagonal line from the top left to bottom right corner of the plot – there are these condensed areas align with the parts of the plot that correspond to groups 586, 892 and PEN5.

Bray-Curtis Dissimilarity Index (beta-diversity index):

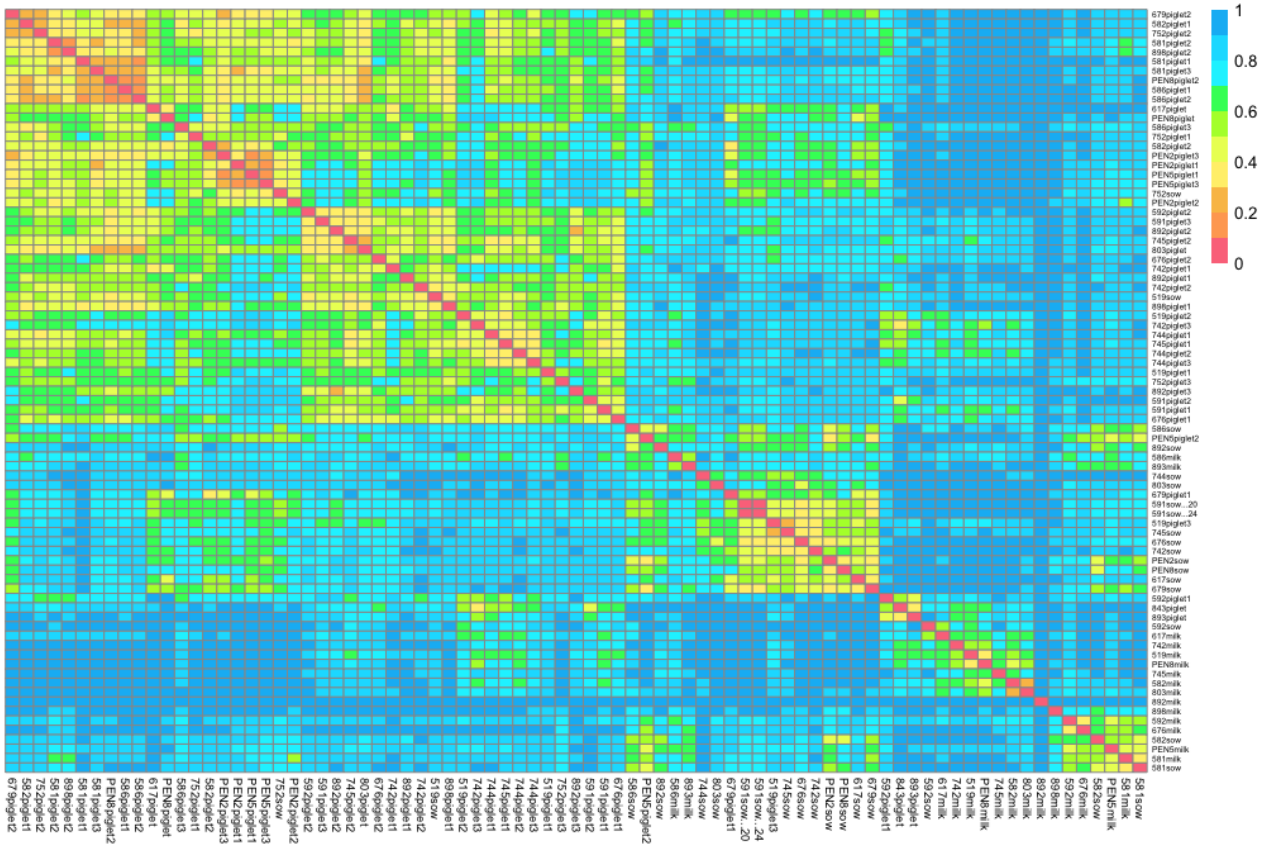

Figure 4.10 - Heatmap of Beta-diversity Indices

Alpha-diversity Index (Shannon index):

Shannon's index quantifies the uncertainty in prediction a species identity for an individual, taken at random from the dataset. For example, if a community has a low diversity, we can be fairly certain of an organism we may choose at random à the higher the index, the more diverse the species in the particular environment are; so the samples in this research with a higher Shannon's index will have a greater diversity of species compared to those with lower Shannon's index values.

Below in Table 3.2 we can see, piglet 1 from PEN 2 has the lowest alpha-diversity index with a value of 1.34, the next lowest values being 1.40 and 1.44 from PEN 5 piglet 1 and the PEN 8 milk sample respectively. On the other end of the spectrum, the sample with the highest alpha-diversity index is the Pen 2 sow sample with a value of 2.76. The next two highest alpha-diversity indexes are 2.72 and 2.70 from 676 sow and 676 piglet 2, respectively.

Table 3.2 – Alpha-diversity Indices:

Sow grouping	MILK	SOW SKIN	PIGLET 1	PIGLET 2	PIGLET 3
519	1.8289	2.1351	2.5629	2.3343	2.2195
581	2.2195	2.1298	1.6275	2.0863	1.8362
582	1.7858	2.5215	1.6852	1.7493	-
586	2.4820	2.5257	1.9840	1.6751	2.1199
591	2.3288	2.3320	2.4703	2.5146	2.4879
592	2.6640	-	1.7919	2.3499	-
617	2.2412	2.1779	1.9856	-	-
676	1.9160	2.7205	2.4205	2.7016	-
679	-	1.6678	1.5090	1.7976	-
742	1.6755	1.9338	2.4271	1.9982	2.2710
744	-	2.0622	2.5384	2.3536	2.6433
745	1.566	2.5184	2.2906	2.4216	-
752	-	2.0872	2.1519	2.0272	1.6994
803	2.0205	2.4245	2.4753	-	-
843	-	-	2.0698	-	-
892	1.6350	2.6532	2.1440	2.4113	1.7060
893	2.0767	-	2.1181	-	-
898	2.3532	-	1.9331	2.0025	-
PEN 2	-	2.7557	1.3399	1.9649	1.5622
PEN 5	2.5704	-	1.4029	2.0847	1.4958
PEN 8	1.4388	2.7133	1.5498	1.8358	-

Chapter 5. Discussion:

5.1. Spirochaetes:

At the level of reads of the Spirochaetes genus identified through this research, we would expect the piglets to have been exposed to *Leptospirae* bacteria but it is unlikely to cause disease. The sows within this study are routinely vaccinated against *Leptospirae* bacteria so although the piglets will not have been directly vaccinated themselves, they will have ingested antibodies through suckling colostrum and milk from the vaccinated sows.

There is also the possibility that these reads may be due to urine run-off from the sows onto the udder skin where the piglets are suckling, hence ingesting the bacteria – though none of the sow udder skin samples contained any reads for *Leptospirae* bacteria so this is an unlikely cause of ingestion. Due to the Animal Status Declaration (ASD) forms required by the Leptospirosis control programme to be completed whenever pigs are moved between commercial properties in New Zealand. Because of this, commercial pig farms are mostly free of the disease, or at least more-so than any other livestock industry in New Zealand (Worksafe NZ, 2016).

In the period of competition for teat access and establishment of teat order/preference, piglets have been known to defend preferred teats using their needle teeth (Fraser and Thompson, 1991). During these 'fights' the piglets are known to inflict injuries to the faces of their siblings, these facial lacerations may result in facial skin necrosis which forms crusted ulcerations and may extend further towards the mouth and eyes of the piglets. The lesions can later produce routes for further infection. Piglets which suckle on the more anterior or posterior teats are thought to receive fewer lesions than piglets that suckle middle teats. The bacterial strains of Spirochaetes found most prevalent in the piglet faecal samples were Treponema strains that are known to cause periodontal lesions – *T. socranskii* and *T. amylovora* (Karlsson et al., 2014). Periodontal lesions, and the bacteria that cause them, are passed between individuals predominantly through saliva.

Cross-fostering piglets, which is used to manage survivability of piglets born into larger litters or low-birth weight piglets, this involves moving piglets into unfamiliar litters (Chou et al.,

2022) which is associated with aggressive behaviour between the unfamiliar piglets fighting for a place in the teat order (Horrell, 1982). There is relatively contradictory evidence around facial lesions and cross-fostered piglets, where some articles have reported that the fostered piglets tend to have more lesions than the sow's biological piglets (Roberts and Martineau, 2001; Straw et al., 1998; Horrell and Bennett, 1981) while other articles have reported cross-fostered litters have lower facial lesion scores than non-cross-fostered litters (Hansson and Lundeheim, 2012).

5.2. Phylogenetic variation within categories of samples – specifically sow milk samples:

The microbiota of milk has yet to be systematically investigated in sows, but a study by Chen et al. (2018) aimed to categorise the variations in bacterial diversity and composition over the course of the lactational period using 16S rRNA sequencing. It was reported that the bacterial composition of colostrum changed significantly but was relatively stable in the transitional and mature milk. *Firmicutes* and *Proteobacteria* are consistently the dominant phyla in sow milk, with the next most abundant phyla being *Actinobacteria*, *Bacteroidetes* and *Fusobacteria* (Chen et al., 2018). The findings from Chen et al. (2018) are consistent with the results from this research which found the same order of most abundant phyla for sow milk samples. Although, only 12 phyla were identified across the samples from Chen at al. (2018) (n=160) compared to the 21 phyla identified across the milk samples in this investigation (n=17).

The average alpha-diversity (Shannon diversity index) of sow milk microbiota from this research is 2.05 (ranging between diversity values of 1.34 to 2.76) which is inconsistent with the approximate average Shannon diversity index value of sow milk microbiota found by Chen et al. (2018) and Chen et al. (2022) with diversity values of 5 (ranging between diversities of 4.5 and 6), and 5 (ranging between values of 3 and 6), respectively. This may be due to the fact that both studies had a greater number of sow milk samples (N = 160 and N = 139, respectively) than this research (N = 16), and also that the samples from the other studies

were taken over time increments (7 times over 3 weeks, and 9 times over 4 weeks respectively).

In this research, a common trend across the stacked bar-plots for the samples (Figures 4.1, 4.2 & 4.3) is that the sow milk sample plot indicates a greater variation in phyla, with lesser common phyla present at relatively large proportions (between 100 and 1500 reads) in comparison to the abundances of these phyla in the sow skin and piglet faecal sample plots.

Interestingly, Chen et al. (2018) found that the alpha-diversity of milk microbiota appeared to decrease between samples on day 0 to the samples on day 1 and 3.
The beta-diversity indices from Chen et al. (2018) indicate that the microbiota of sow milk samples differed significantly at all different time periods sampled with the exception of similarities between the microbiota on days 1 and 3, and days 7, 10 and 14.

Chen et al. (2022) found that the relative contribution of milk and sow udder/skin microbiota to the formation of the piglet gut microbiota was lower than other sources of maternal microbial transmission. Though this is inconsistent with previous reports on the microbiota of breast-fed infants – this may be due to the fact that previous studies only sequenced samples of milk and areolar skin and ignored other maternal and environmental pathways for transmission (Chen et al., 2018; Law et al., 2017). Instead of sow milk bacteria majorly contributing to the colonisation of the piglet gut microbiota, Chen et al. (2022) indicated that initially the greatest maternal source of bacteria for the piglets is the sow vaginal microbiota up until day 21 where the greatest source of bacteria becomes the sow faecal matter. In terms of environmental sources of bacteria, the slatted flooring of farrowing pens is suggested to be an important source of bacteria for colonisation of the piglet gut microbiota (Chen et al, 2022), but that the bacterial environment on the slatted flooring differs from that of sow faecal matter and so it is important instead because of other activities such as feeding and disinfection practises.

5.3. Presence of Pathogenic Bacterial Strains:

In this study, none of the samples taken were found to have any reads from the *Escherichia* genus indicating no presence of *E*. *coli* bacteria in any of the piglets, sow milk or sow skin sampled from the farm. This is interesting as *E. coli* is a prevalent bacterial pathogen in young piglets (Kubosova et al., 2017; Holman et al., 2022), the fact that there are no reads for this in any samples collected for this study indicates that either the environment is clean, or at least clean to the point where the bacteria is not picked up on sow skin or in faecal matter; or the bacteria had not yet had enough time between birth and samples taken (24 hours) to colonise the gut of the piglets and be excreted in faecal matter; or are in low enough numbers to not be detected using these methods.

Kubosova et al. (2017) reported that the gut microbiota samples of 1-day old piglets contained predominantly *Escherichia* and *Clostridium* genus bacteria, which is inconsistent with the 1-day old piglets sampled for this research which were found to have no reads for *Escherichia* bacteria. In terms of the *Clostridium* genus bacteria, there was a presence of reads in all piglet faecal samples taken with levels of abundance ranging from 11 to 6967 reads and an average amount of *Clostridium* reads of 2265. In most piglet samples, the *Clostridia* class was one of the most abundant which is consistent with the study by Kubasova et al. (2017).

A trend found from Holman et al. (2022) was that the MAGs (metagenome-assembled genomes) indicating *Escherichia* presence decreased when piglets under-went weaning, whilst the abundance of *Clostridium* MAGs were found to increase post-weaning.

Lack of reads for *Escherichia* bacteria in this data also indicates that the commercial farm used for sampling in this research has upkept pen disinfection and management practises such as slatted flooring to separate faecal matter from the lying area, resulting in the skin of sows being left visibly clean. As *Clostridial* bacteria are a much hardier bacteria than *Escherichia* bacteria, there is still a presence of *Clostridium* found on the sow skin and in the piglet faecal matter. It is likely that if any *Escherichia* bacteria is present in the sows of the farm, it would be present in their faecal matter but this was not sampled as a part of this research. If *Escherichia* bacteria were present in the sow faecal matter in the pens, it is also likely that at

the time of sampling the piglets had not yet exhibited exploratory behaviours or ventured far from the sow into the forward creeps of the pen, so piglets would have little contact with the dunging area at this time. If samples were taken from the piglets beyond 72 hours or one week after birth. *Escherichia* bacteria may have been present in the piglet faecal matter (assuming its presence in sow faecal matter) as the bacteria would have had a longer period to colonise the piglet gut .

In terms of *Clostridium* bacteria, in this study all of the sow skin samples and all but one of the piglet faecal samples contained the strain *C. perfringens*, a bacteria known to have 5/five classified toxinotypes (A, B, C, D and E). The most prevalent toxinotype of *C. perfringens* in swine is type B, responsible for necrotising enteritis in piglets, followed by type E which is responsible for enteritis (Uzal et al., 2010). The only piglet faecal sample not containing reads for *C. perfringens* is the sample from pen group 843, there is only one recorded sample from this pen so we cannot be sure whether the sow or other piglets in the pen had contracted this strain bacteria or not – although other *Clostridium* bacterial strains were present in piglet faecal sample 843, just not *C. perfringens*. The only other samples without reads for *C. perfringens* was the pen group 582 milk sample, though this sample did show reads for other strains of *Clostridium* bacteria, and the other samples from pen group 582 (piglets and sow skin) did show presence of C*. perfringens*.

Pen group 892 milk sample was not able to provide a deeper read for *Clostridium* bacteria than at the genus level so we cannot be sure of presence though *C. perfringens* bacteria is present in other samples from the same pen group. Similarly the PEN2 milk sample has reads at family level (*Clostridiacaeae*) only, though other samples from the PEN2 group contain reads for *C. perfringens*.

Other pathogenic bacteria of interest with presence in this study were *Salmonella* serovars, *Staphylococcus* specifically *S. aureus* as well as an unkonwn species of the *Listeria* genus, and some species of *Campylobacter*. *Salmonella* serovars are present across various samples of sow milk, sow skin and piglet faeces with abundances of between 1-22 reads, specifically serovars of *S. enterica* which are increasingly known to cause clinical symptoms of salmonella in pigs and is one of the most common isolated serotypes in diseased pigs (Naberhaus et al.,

2019). Piglets of weaning age are the most commonly susceptible to infection from salmonella bacteria (Won et al., 2022), so it is likely too early in their lives at the time the samples for this study were taken for enough bacteria to colonise the gut and cause clinical symptoms of salmonellosis. *Staphylococcus* bacteria is present in almost every sample taken (sow milk, sow skin and piglet faeces) for this research, with a majority of the piglet faecal samples having a presence of *S. aureus*, a bacteria known to cause mastitis (Holmes et al., 2011). As the read levels do not go further than species level, we cannot tell if the strain of S. aureus bacteria present is the methicillin-resistant strain (MRSA). Methicillin-resistant strain strains are also known to be zoonotic, bacteria can be transferred to humans in contact with infected pigs and can then result in postpartum mastitis in human females (Holmes et al., 2011). There are also a few samples with reads for *S. sciuri* which is an emerging pathogenic bacteria known to cause exudative epidermitis (facial dermatitis) in piglets, a communicable skin disease also caused by the staphylococci bacteria *S. hyicus* (Chen et al., 2007). Acute forms of exudative epidermitis typically affect suckling piglets but more chronic forms are seen in weaning-aged piglets.

A couple of swabs from piglet faeces showed presence of *Listeria* bacteria but only in an abundance of 1-2 reads. Because of the lack of reads at genus level, there were no identified species of *Listeria* present in samples but the most common species of *Listeria* is *L. monocytogenes* which is a major pathogenic foodborne bacteria that can infect humans through contaminated pork (Kanuganti et al., 2002). When *Listeria* bacteria colonises the intestines of pigs, it may also contaminate the carcase and surrounding environments in the abattoir. Even when present at low levels in meat at processing plants, the bacteria multiplies during refrigeration. Listeria bacteria is more commonly identified in ground pork (mince) than deep muscle which suggests that contamination occurs in meat processing stage (Uyttendaele et al., 1999; Kanuganti et al., 2002).

Campylobacter is another pathogenic bacteria with presence in the samples from this study, but typically only identified to a family or genus level, a few samples identified *Campylobacter* to a species level but this was less common. *C. fetus*, *C. rectus, C. ureolyticus,* and *C. subantarcticus* were species identified in a couple of piglet faecal samples, all of these bacteria cause issues in the gastrointestinal tract of pigs but are not the most prevalent

species of *Campylobacter* bacteria (*C. coli* and *C. jejuni*). Pigs are typically infected with *Campylobacter* bacteria early in life but are known as symptomless carriers, resulting in contaminated meat post-slaughter (Steinhauserova et al., 2001). Another pathogenic bacteria from the same order as *Campylobacter* was also identified in some piglet faecal samples; *Helicobacter pylori* which is responsible for erosion and ulcers in the stomach of pigs, although this species was only present in a few samples with an abundance of between 1-14 reads.

5.4. Presence of Probiotic Bacterial Strains:

Pediococcus is a genus of probiotic bacteria from the family *Lactobacillaceae*, and is known for its use in treatment of constipation and diarrhoea as well as its use to enhance immune responses. *Pediococcus* was found to have a relative abundance of 1-2 reads in 10 of the 49 different piglet samples, with each of these 10 samples being from a different pen group. The metagenomic analysis in this research was unable to identify a more specific/lower classification of the *Pediococcus* bacteria present in the samples but the species most known for probiotic characteristics and its ability to colonise the gut of small animals is *P. acidilactici*.

Lactococcus is a genus of probiotic bacteria, also from the family *Lactobacillaceae*, known for producing lactic acid as the major or sole product of glucose fermentation. In terms of probiotic effects, the species of *Lactococcus*, namely *L. lactis*, has many strains some of which are known for their probiotic functions and others known for being highly adaptable environmental pathogens which cause a range of diseases across humans and animals (Wu et al., 2023). *Lactococcus* was found to have a relative abundance ranging between 1-10 reads in 30 of the 49 piglet samples analysed by this research, these samples were spread across 17 pen groups with 9 of the pen groups having multiple samples with presence of *Lactococcus* bacteria. Similarly, with the *Pediococcus* reads, the metagenomic analysis did not identify reads at a more specific level than the genus so we do not know which specific strains of *Lactococcus* were present in the samples.

Another genus of the family *Lactobacillaceae*, *Lactobacillus* is known to have similar probiotic effects (Hermann-Bank et al., 2015). These bacteria are known to colonise the intestines of

new-born piglets quickly after birth and hence become a stable member of the gut microbiota (Kenworthy and Crabb, 1963). Piglets with low abundances of these bacteria, specifically the species *L. acidophilus* will likely have gastrointestinal issues as these bacteria are diminished in diarrhoetic piglets. *Lactobacillus* bacteria is present in every sample of piglet faeces with reads specific enough to determine species of the bacteria present, except for the sample from PEN8 piglet 2 which only showed a read depth to genus level for *Lactobacillus* bacteria. The most common species of Lactobacillus bacteria present in piglet faecal samples are *L. mucosae* and *L. reuteri*, followed by *L. zeae*, *L. iners* and *L. delbrueckii*. *Lactobacillus mucosae* is a probiotic bacteria which adheres to the intestinal epithelium and induces greater mucus production in-turn reinforcing the intestinal barrier (Khaneghah et al., 2020; Misra et al., 2019), making these bacteria especially beneficial in neonatal piglets as they are exposed to increasing amounts of pathogenic bacteria. *Lactobacillus reuteri* is a bacterium capable of producing antimicrobials which inhibits colonisation of pathogenic microbes in the gut microbiota of the host, as well as reducing the production of pro-inflammatory cytokines and promoting development of regulatory T cells (Mu et al., 2018).

Lactobacillus and *Pediococcus* genera bacteria are known to be commonly used as probiotics in pork production systems (Pereira et al., 2022) and *Lactobacillus* bacteria specifically has been shown to improve both the overall performance of the piglet but also decrease abundance of *E. coli* bacteria colonising the gut microbiota post-weaning (Yang et al., 2020). Additive probiotic use is most prevalent when piglets are in the nursery-stage (pre-weaning), so around the age of the piglets in this study, as there is evidence of benefit whilst the gut microbiota is first developing and not yet stable (Barba-Vidal et al., 2019). This is ideal seeing a presence of these bacteria already in the samples from the piglets of this study, meaning the piglets already have a good basis of probiotic bacteria without providing any as an additive/externally in-feed.

5.5. Bacteria with low levels of reads but common across samples:

In the sow milk samples, a relatively low abundance of *Acidobacteria* reads was observed, but it remained relatively common across all samples. The ‘low’ abundance of reads was still

greater than the abundance of *Acidobacteria* reads from sow skin samples and piglet faecal samples. *Acidobacteria* makes up an average of 0.53% of total reads from sow milk, vs an average of 0.01% of total reads in either sow skin or piglet faecal samples. *Acidobacteria* is a phyla of bacteria which typically inhabit terrestrial and aquatic habitats, and are especially abundant in acidic soils (Dedysh and Damasté, 2018). Interestingly, there are no other studies that have characterised sow milk and identified *Acidobacteria* as a phyla present in the microbial composition, but a study on cows' milk by Liu et al. (2015) identified *Acidobacteria* reads at an abundance of 0.04% of the total reads (0.009% and 0.034% of reads in each of the two populations sampled) which is far less than the average of 0.53% of total reads present in the milk samples of this study.

Cyanobacteria is another phylum identified to have a low level of reads in sow milk but relatively similar read levels across all sow milk samples, and a greater average abundance compared to that of sow skin and piglet faecal samples – an average of 0.40%, 0.08% and 0.07% respectively. *Cyanobacteria* is a phyla of bacteria known for their role in photosynthesis and fixation of nitrogen, but also for causing large and occasionally toxic bacterial blooms in bodies of water (Whittons and Potts, 2012). In terms of livestock, a species of *Cyanobacteria*, *Arthrospira platensis* (commonly known as spirulina) is given as a feed supplement as there have been studies indicating improvements to productivity and the ability to both combat effects of malnutrition as well as metabolic diseases associated with over-eating (Lugarà et al., 2022; Holman and Malau-Aduli, 2013). There is very little known about the *Cyanobacteria* which colonise the mammalian gut, but due to their relation to chloroplasts, *Cyanobacteria* colonisation in the gut microbiota is likely to do with the level of plant-based compounds present in sow diets (Piccolo et al., 2017). As sow faecal matter was not sampled the abundance of *Cyanobacteria* present in the gut microbiota of sows in this study cannot be known. It can be postulated that it would be present due to the abundances of the bacterial phyla in the sow's milk. There have also been studies which contradict the benefits of *Cyanobacteria* (spirulina) in livestock in terms of the toxin-producing *Cyanobacteria* species such as blue-green algae toxicosis (McGorum et al., 2015).

The *Chloroflexi* phylum includes bacteria which carry out the reductive dehalogenation of chlorinated organic compounds to produce energy (Bandopadhyay and Shade, 2024). Several

species of Chloroflexi are photosynthetic and most members of the *Chloroflexi* phylum are known as sludge bacteria but the role that *Chloroflexi* bacteria play in mammal milk microbiota are currently unknown (Williamson et al., 2022). Chloroflexi bacteria have been identified in the sow milk samples of this study, again at relatively low levels but still greater abundances than those of the sow skin and piglet faecal samples – average read abundances of 0.40%, 0.03% and 0.01% respectively (sow milk, sow skin and piglet faeces). Williamson et al. (2022) have stated that both *Cyanobacteria* and *Chloroflexi* have been found present in samples of mammal milk but at low levels which is consistent with the milk samples from this study where almost all samples contain reads for these phyla, though there has not been very much research on this prior to the study by Williamson et al. (2022).

5.6. Bacteria with high levels of reads and common across samples:

Interestingly, when the abundance of *Firmicutes* bacteria in a sample from this research was greater, the abundance of *Bacteroidetes* bacteria was lower with the same in reverse. *Firmicutes* is consistently the most prevalent phyla in sow skin samples, *Proteobacteria* and *Firmicutes* are the most prevalent in sow milk and *Firmicutes* and *Bacteroidetes* are the most prevalent in piglet faeces. The dominance of *Firmicutes* and *Bacteroidetes* phyla in new-born piglet gut microbiota is consistent with a study by Wang et al. (2019).

Bacteria in the *Firmicutes* and *Bacteroidetes* phyla are capable of metabolising starch, fructans and lactose, with some enzymes from these pathways only present in either of the bacterial phyla – such as an extracellular glucan-branching enzyme which coverts starch to pullulanases (Wang et al., 2019). This enzyme is thought to be crucial in the main pathway of starch degradation in the swine GIT where a-1,4-glucanotransferase catalyses glucan chain transfer to form glucosidic linkages and is an enzyme found only in *Firmicutes*. The enzyme is thought to improve accessibility to insoluble starch (Rumbak et al., 1991) and is commonly found in the gut microbiota of humans, chickens, pigs and cattle (Lee et al., 2014). Wang et al. (2019) found a high abundance of starch-utilising enzymes present in faecal samples which indicates that wheat starch may be a major source of carbohydrates for microbiota in the colon of swine. The distribution of extracellular and periplasmic enzymes used for starch

degradation through the GIT shows a 'high level of metabolic cooperativity' which is similar to that of human starch-degrading communities (Wang et al., 2019; Flint et al., 2012).

Bacteroidetes and *Lactobacilli* bacteria are known for degrading the low molecular-weight fructans found in wheat. *Bacteroidetes* bacteria metabolise fructans via intracellular, extracellular or periplasmic endo-fructanase enzymes, at least one of which type is present in every fructan-degrading Bacteroidete. *B. thetaiotaomicron* is a species known for fructan utilisation but has not been identified in any piglet faecal samples in this study, whilst *B. caccae, B. ovatus, B. fragilis* and *B. uniformis* are species known to ferment and utilise insulin (a b2-1 fructan) with an efficiency similar to the use of glucose (Sonnenburg et al., 2010) where at least one of these bacteria has been identified in each of the piglet faecal samples of this study. The metagenomic study by Wang et al. (2019) was the first to identify presence of extracellular fructanases in intestinal *Lactobacilli* bacteria – these fructanases have been previously identified only in oral streptococci and sourdough so presence of these enzymes in intestinal Lactobacilli is thought to reflect a specific nutritional requirements of pigs.

Lactose accounts for around 27% of sow milk solids (Klobasa et al., 1987) which makes it a major carbohydrate in the diet of suckling piglets, and so the ability to hydrolyse lactose is important. Lactose hydrolysis is catalysed by enzymes in Firmicutes and Lactobacilli, intracellular GH2 b-galactosidase and GH42 b-galactosidase respectively and occurs in the stomach where *Firmicutes* populations colonise and there is plenty of dietary lactose readily available (Wang et al., 2019). *Lactobacillus delbrueckii* is a species of *Firmicutes* bacteria that is specialised in lactose conversion, but is found to decrease in abundance post-weaning (Wang et al., 2019) and is no longer detected in piglet intestines at all after 2-3 weeks post-weaning (Yang et al., 2015).

A number of studies have looked at anaerobic digestion through microbial populations and reported that populations of *Firmicutes* and *Bacteroidetes* bacteria are typically abundant in terms of anaerobic digestive processes (Chen et al., 2016; Kampmann et al., 2012; Liu et al., 2009; McGarvey et al., 2007; and Sundberg et al., 2013). Chen et al. (2016) found a strong correlation between the abundance of *Firmicutes* and *Bacteroidetes* population and the

methanogenic digestive process performance of the animal where anaerobic digestion is important for reduction of greenhouse gas emission.

Another important bacteria grouping to piglet development are those in the *Prevotella* genus, where a supposed drastic change occurs between birth and weaning in terms of abundance of populations – typically the abundance of these populations at birth are very small, this rapidly increases as time continues towards weaning (Frese et al., 2015; Liu et al., 2019; Amat et al., 2020). This is thought to be due to the increased plant matter ingested by piglets as they age and their diet changes post-weaning, as *Prevotella* populations degrade plant polysaccharides into short-chain fatty acids (Liu et al., 2019). The increase in *Prevotella* takes over from the abundant *Bacteroides* populations at birth, since the piglets' diet is changing, they no longer need as many bacterial populations to hydrolyse milk glycans and the piglet gut is no longer known as a 'milk-oriented microbiome' (Frese et al., 2015).

In this study, there are quite varied abundances of *Prevotella* genus populations found in the piglet faecal samples, ranging from 1 read to 33873 reads – so varying from a relatively insignificant amount of reads to rather significant sized populations of *Prevotella*. Not all samples could be read to a species level for *Prevotella*, but when it was possible to identify a species the most common was *P. copri* which is a bacteria known to be highly abundant in piglet gut microbiota post-weaning (Amat et al., 2020), so it is interesting *P. copri* is also present in samples of newborn piglets. Amat et al. (2020) stated that the relative abundance of *Prevotella* populations in suckling piglets is 1% where in this study the average relative abundance of *Prevotella* populations in piglet faecal samples is 3% though due to such a large range of read abundances, this differs between piglets – Piglet 1 from pen 592 identified 33973 reads for the *Prevotella* genus which made up 27.5% of the total reads for that piglet while Piglet 1 from pen 582 identified 1 read which makes up less than 0.01% of the total reads. The smaller sized populations of *Prevotella* identified in this study (piglets with between 1 and 100 reads) are much more consistent with previous literature whilst the piglets with upwards of 1000 reads assigned to the *Prevotella* genus are inconsistent with previous studies.

5.7. Limitations and future recommendations:

Although this study has identified similar bacteria from milk, sow skin and piglet faeces there are limitations to the study scope. As a study of this sort, using metagenomic sequencing, has not be done before, it is likely more samples were needed to better characterise the microbiota and to better characterise the microbial composition of the areas sampled (sow skin, sow milk and piglet faeces). Something else this study could have done differently was ensuring the cross-fostered piglets were known, as well as this knowing which was the biological sow and the cross-fostered sow for each piglet that was moved as in this study this was not known and may have affected some results.

In terms of future research, taking swabs of more variables than just sow milk and sow skin to see the effect on piglet gut microbiota would be ideal. Similarly to how Chen et al. (2023) took many samples of sow faeces, sow vaginal swabs, water trough samples, airborne samples and swabs of the slatted flooring, as well as the sow udder skin and sow milk samples. Another type of sample that may be beneficial to look at may be the sow diet. The difference between Chen et al. (2023) and this study, as well as the increased number and types of samples, is that their study used 16S rRNA sequencing and not metagenomic sequencing as used in this study.

A second recommendation to look at in the future could be to mark which piglets are born first (birth order) and also look at this compared with the teat order of piglets if it is possible to determine – in order to see if this may affect how the piglets' gut microbiota is colonised. Frequency of testing is a third recommendation for any future research where this study only sequenced bacteria from samples taken around 24 hours post-parturition, it would be beneficial to follow up with taking and sequencing samples in the days and weeks after this time (at time-points of 2 days, 3 days, 5 days, 7 days, 10 days, 14 days, 21 days, 28 days etc), another potentially beneficial time of sampling would be directly after birth to get an idea of the least-colonised version of a piglet gut microbiota after only maternal transfer of bacteria, before it is rapidly colonised from a variety of external/environmental variables.

Chapter 6. Conclusion:

This study has been a preliminary analysis of the microbiota of sow skin and sow milk to determine potential sources for colonisation for piglet gut microbiota (sampled indirectly via piglet faecal matter). The metagenomic analyses involved have revealed many similarities and differences between pen groups but also within categories of sample types (sow skin, sow milk and piglet faeces). Overall the piglet samples have been dominated by populations of *Firmicutes* and *Bacteroidetes*, whilst sow milk and skin samples were dominated by populations of *Firmicutes* and *Proteobacteria*. The sow milk samples displayed a greater presence of less common phyla such as *Cyanobacteria*, *Chloroflexi* and *Acidobacteria*, compared to other samples, but these phyla are only present at relatively low levels of reads. A high abundance of *Spirochaetes* was identified in some piglet samples compared to all other samples, it was determined to be a large population of *Treponema* bacteria. These bacteria are an indication that facial necrosis-causing bacteria are present in these piglets and potentially also present in the sow saliva, but with the current data it cannot be tested as these were not sampled as part of this research.

In terms of pathogenic bacterial populations, there was no reads for *E. coli* bacteria present in any samples taken for this research. This may be very beneficial as E. coli is typically a species which rapidly colonises the guts of new-born piglets. *Clostridium*, another bacterial phylum known to rapidly colonise piglet guts, was found to be widely abundant in the piglet faecal samples so it is likely that the disinfection protocols of the piggery were strong enough to get rid of any *E. coli* but as *Clostridium* is hardier it may remain in the environment. It is likely that if sufficient *E.coli* is present in the environment, it would've been picked up in samples of the slatted flooring, and that at this age piglets have not yet ventured away from the sow to pick up the bacteria.

Pediococcus and *Lactococcus* are common probiotic bacterial families found across piglet samples which include species known to increase overall production and have a protective function against *E. coli* bacteria post-weaning. These bacteria are typically fed as additives pre-weaning due to their benefits on a developing gut microbiota, so the fact these piglets already have a good basis of probiotic bacterial populations is ideal for their future productive performance.

This research study has been successful in the characterisation of bacterial populations present in the sow udder skin, sow milk and piglet faecal matter that was sampled. It has been successful in achieving what was set out to do – performing the initial steps to fully characterise microbiomes present in the New Zealand commercial pork industry, and further to determining the main pathways of transmission of bacteria for neonate piglets'.

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Chapter 8. Appendix:

8.1. Code used in Kraken and Krona:

(Kraken2 is either in environment soph2 or soph3, braken is in soph4)

cd Documents/bioinfo

conda install kraken2

kraken2 --paired --classified-out cseqs#.fq PEN8SOW_R1.fastq.trimmed.gz PEN8SOW_R2.fastq.trimmed.gz --db 16s --use-names --report PEN8SOW

python combine_kreports.py -r 519SOW 581SOW 582SOW 586SOW 592SOW 592SOW_2 617SOW 679SOW 742SOW 744SOW 745SOW 752SOW 803SOW PEN2SOW PEN8SOW -o combinedsowreport

conda install krona

ktUpdateTaxonomy.sh

ktImportTaxonomy -n Bacteria -o krona_reports/sowkrona.html krona_reports/*SOW

beta_diverity.py -i {list of filename.bracken} –type bracken

alpha_diversity.py -f {filename.bracken – individual only} -a Sh

alpha or -a meaning the type of alpha-diversity, Sh (Shannon Index) used above, other options include BP, Si, Isi, and F

8.2. RStudio's code used to produce stacked bar-plots:

```
library(ggplot2)
library(reshape2)
X519phyla = X519_phyla
X519phyla_melt = melt(X519phyla, id = c("PEN519"))
X519phyla_melt$PEN519 <-
factor(X519phyla_melt$PEN519,levels=unique(X519phyla_melt$PEN519))
```

```
ggplot(X519phyla_melt, aes(fill=variable, y=value, x=PEN519)) +
        geom_bar(position="fill", stat="identity", width=0.7) +
labs(x= "", y = "Relative Abundance (%)", fill= "Phyla") +  scale_fill_manual(values = CPCOL) +
theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(), panel.background = element_blank()) +
        theme(axis.line = element_line())
```

Use the colour picker tool to determine a colour palette manually:

```
CPCOL = c("#FC778B", "#FFA861", "#FAC055", "#FFEF7A", "#EAFF63", "#AFFF36", "#8FFF33", "#13F231", "#05FA7B", "#00FFC4", "#0DFFF7", "#00F2FF", "#05DEFF", "#08BAF5", "#5C6CFF", "#6445FF", "#8B3DFF", "#B936FF", "#E83BFF", "#FF00DD", "#FF0890")
```

8.3. RStudio's code for heatmaps:

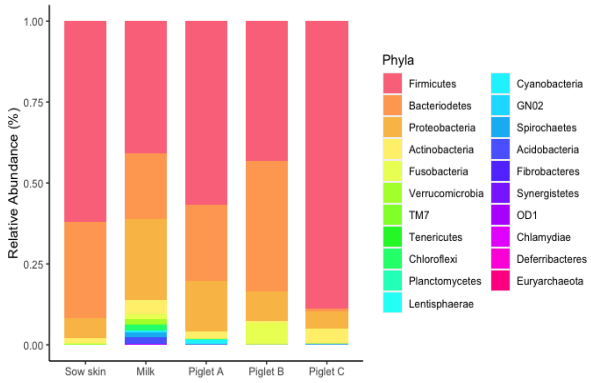
```
library(pheatmap)
library(viridis)
library(readxl)
library(RColorBrewer)
betadiversity2 <- as.matrix(betadiversities)
rownames(betadiversity2) <- colnames(betadiversity2)
pheatmap(betadiversity2, display_numbers = F, fontsize_col = 7, fontsize_row = 5, color=COLS, symmetrical=TRUE, treeheight_row = 0, treeheight_col = 0)
```

Use the colour picker tool to determine a colour palette manually:

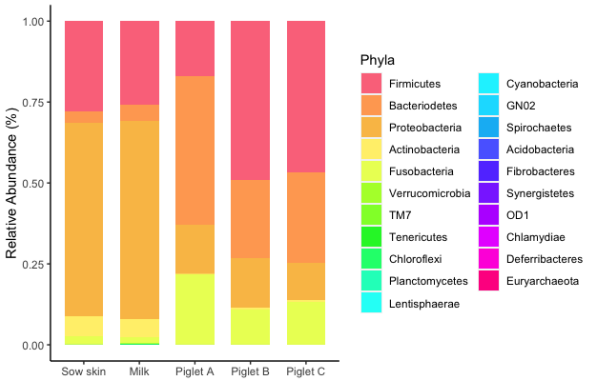
```
COLS = c("#FC778B", "#FFA861", "#FAC055", "#FFEF7A", "#EAFF63", "#AFFF36", "#33FF66", "#00F2FF", "#05DEFF", "#08BAF5")
```

8.4. Stacked bar-plots of all individual pen-groups

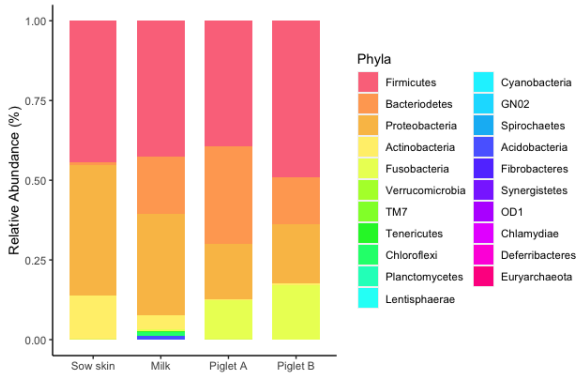
Pen 519



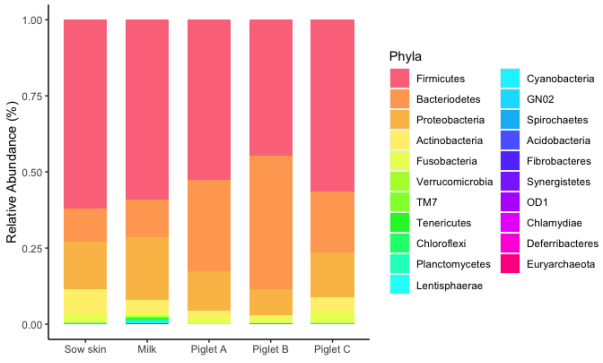
Pen 581



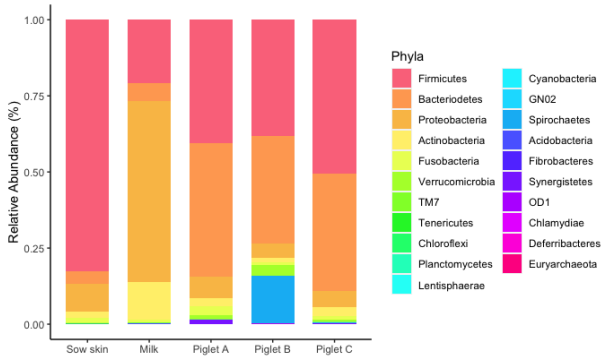
Pen 582



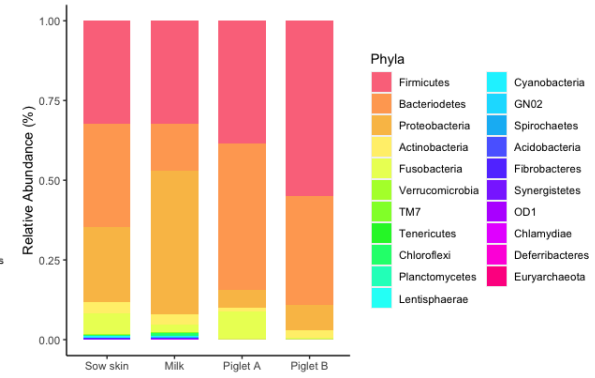
Pen 586



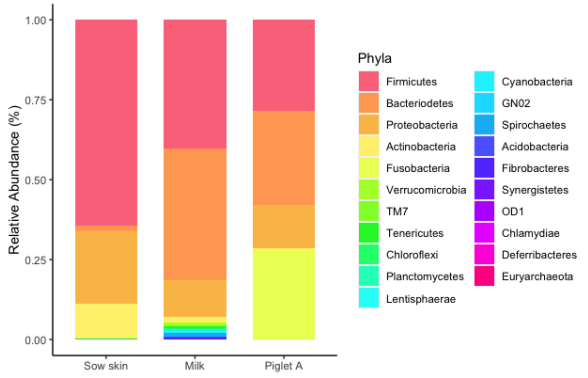
Pen 591



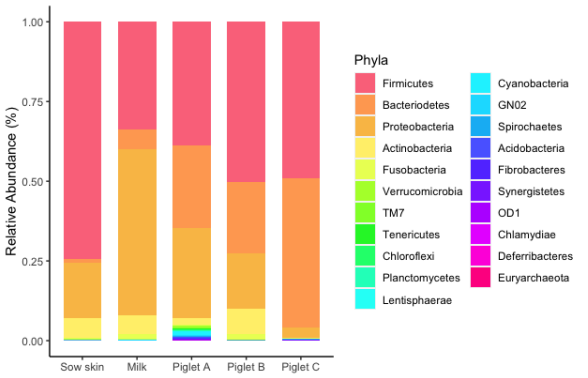
Pen 592



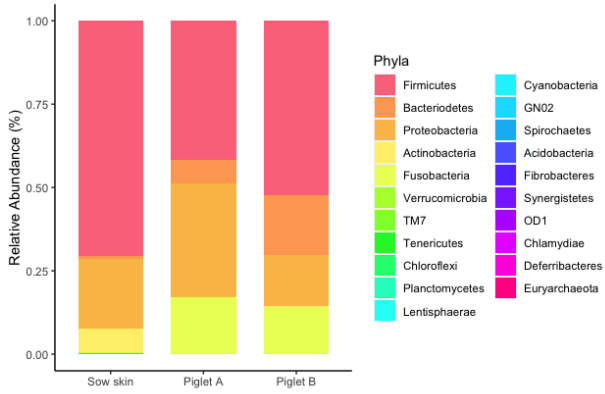
Pen 617



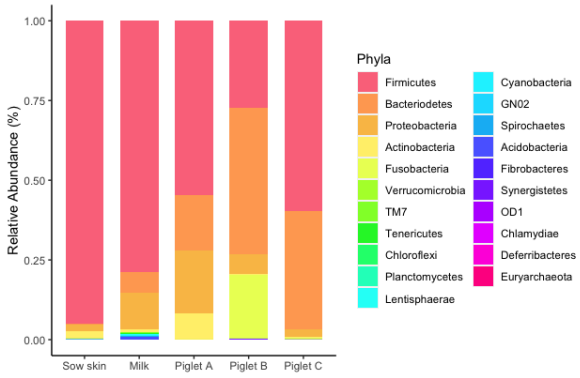
Pen 676



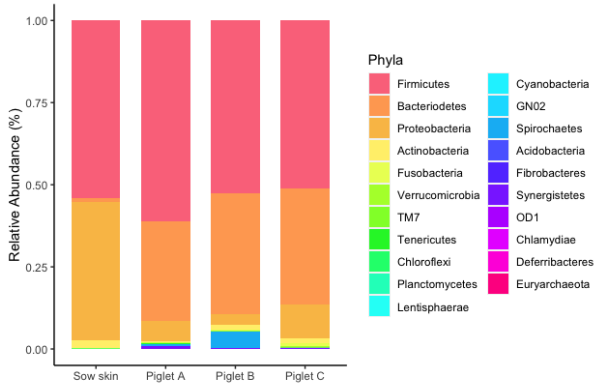
Pen 679



Pen 742



Pen 744



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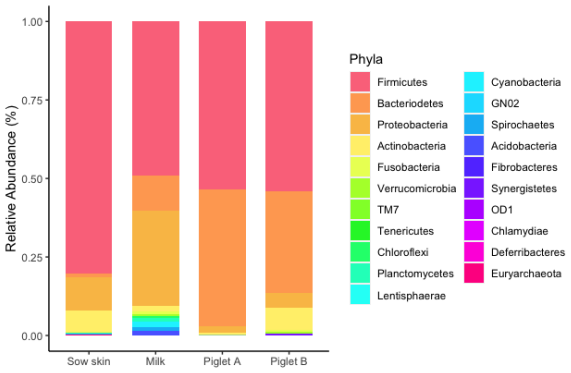

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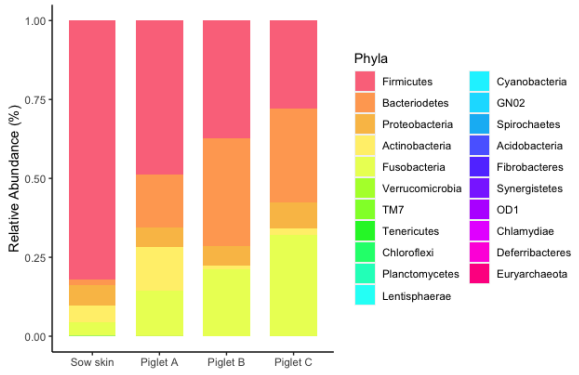

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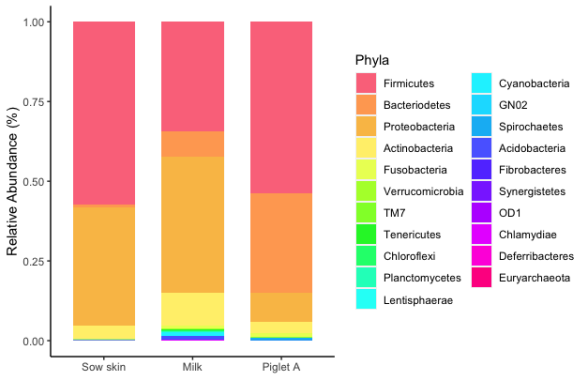

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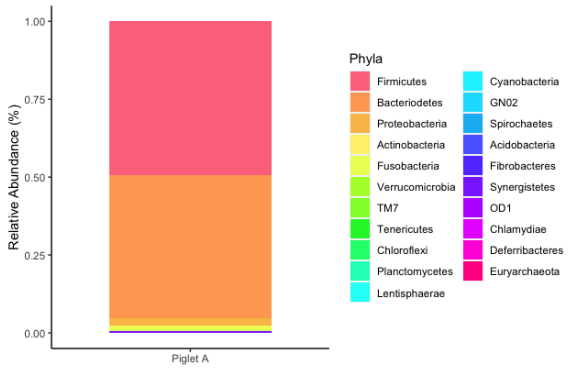

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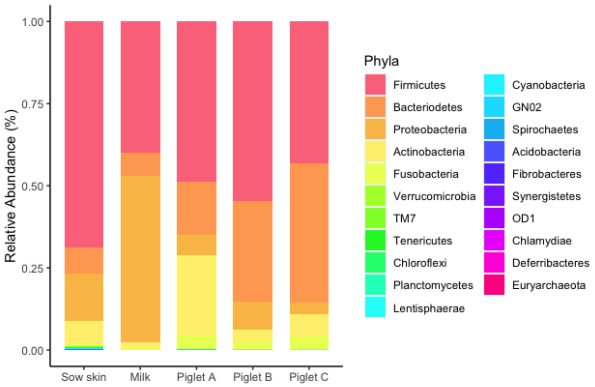

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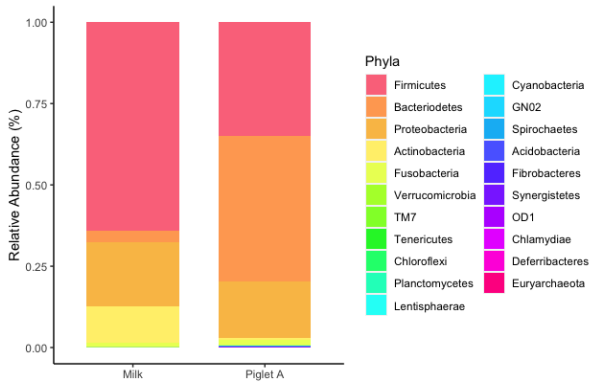

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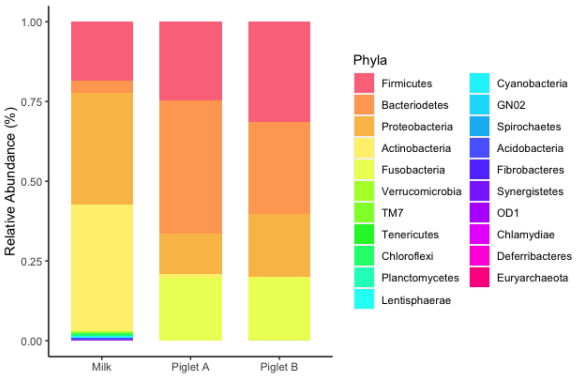
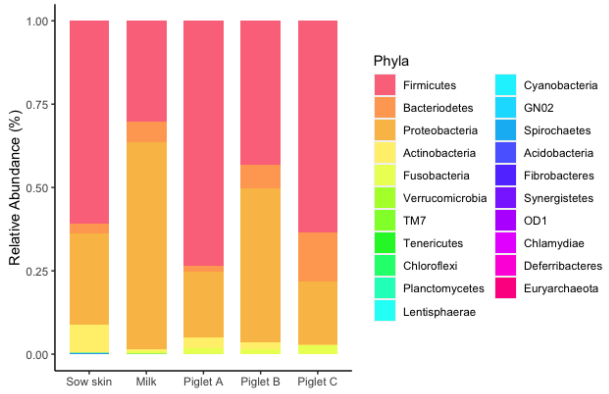
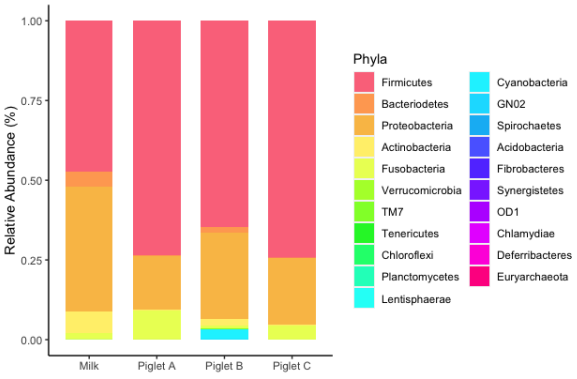


PEN2



PEN5



PEN8