The Fungus Among Us: Exploring the Porcine Mycobiome

by

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Abstract

Microbes in the intestinal tract are essential for host health and development. While the role of bacteria in these process is well studied, the role of fungi (the mycobiota) have received less attention. However, fungi have been shown to alter immune system development and intestinal architecture. The objectives of this thesis were to explore the impact of antibiotic treatment on fungal community structure, to track and profile the pig mycobiome over 1 production cycle with comparisons to commercially raised and feral pigs, and to determine the impact of *Kazachstania slooffiae* on bacterial community structure, metabolite production, intestinal and immune system development.

In study 1, 32 piglets from 4 different litters were randomly assigned to one of 4 treatment groups: placebo (P) amoxicillin (A) amoxicillin + clavulanic acid (AC) or gentamicin + ampicillin (GA). Bacterial and fungal community structure were investigated by sequencing the 16S rRNA gene and the internal transcribed spacer-2 (ITS2) rRNA gene, respectively. Total bacteria and total fungi were quantified by quantitative polymerase chain reaction. This study showed that antibiotics did not alter fungal community composition ($P = 0.834$), however, AC treatment increased the ratio of total fungi to total bacteria ($P = 0.027$). Additionally, the maternal mycobiome drove piglet mycobiome composition, especially with regard to the yeast *K. slooffiae*. We found that piglets were more similar to their maternal sow than to any of the other sows in the study ($P \leq 0.05$).

In study 2, 2 piglets from 12 different litters were fecal sampled at 11 days of age, the day before weaning, 7 days after weaning and 119 days after weaning. Additionally, 8 sows in a commercial facility and feral pigs were sampled. Fungal community structure was evaluated via sequencing of the ITS2 rRNA gene. We found that piglets clustered by sow *K. slooffiae* status at

11 days of age ($P = 0.087$) and 119 days after weaning ($P = 0.046$), but not on the day before ($P = 0.297$) or 7 days after weaning ($P = 0.859$). Piglets were more similar to their maternal sow than a random sow at 11 days of age ($P < 0.001$) but not after ($P \geq 0.05$). Additionally piglets clustered with their litter mate at 11 days of age ($P = 0.006$) and 119 days after weaning ($P = 0.007$), but not on the day before weaning ($P = 0.184$) or 1 week after weaning ($P = 0.087$). Together these results suggests that what piglets are exposed to in the farrowing pen can have long-term impacts on mycobiome composition. Commercial sows were found to be variable in the amount of *K. slooffiae* present. Feral pigs had a more complex mycobiome consisting predominantly of fungi associated with soil.

In study 3 we used a gnotobiotic piglet model to study the impact of *K. slooffiae* on bacterial community structure, metabolite production, and immune system and intestinal development. We found that *K. sloofffiae* altered the bacterial community and increased the amount of total bacteria present in the intestine ($P \leq 0.05$). *K. slooffiae* colonization altered the ileal metabolome including increasing butyrate levels ($P = 0.032$). *K. slooffie* also resulted in a greater villus height to crypt depth ratio in the ileum ($P = 0.028$) suggesting increased absorptive capacity and the immune system was altered both in terms of cytokine production and immune cell phenotype ($P \leq 0.05$).

In summary, this thesis shows that shaping early life fungal exposure may have long-term impacts on mycobiota composition, and that *K. slooffiae* is an active member of the core pig mycobiota that may play a role in pig health.

Preface

This thesis is an original work by Tausha Louise Prisnee.

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TP designed the experiment, conducted the experiment, collected and analyzed the data and wrote the manuscript. JF assisted with data analysis and edited and approved the manuscript. NED assisted with sample collection, data analysis, and edited and approved the manuscript. HL assisted with sample collection, and edited and approved the manuscript. TJ assisted with data analysis, edited and approved the manuscript. BW designed the experiment, supervised data analysis, edited, and approved the manuscript. This study was approved by the Animal Care and Use Committee of the University of Alberta and conducted in accordance with the guidelines of the Canadian Council on Animal Care animal use protocol number AUP00000922.

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Chapter 1: Introduction

1.1 Characterization of fungi and yeast

The gastrointestinal tract is home to a vast array of microorganisms, which include bacteria, fungi, archaea, protozoa, and viruses (1). Together, these microorganisms can be referred to as the intestinal microbiome. Compared to bacteria, fungi have received much less attention, despite being important members of the intestinal microbiome. The collection of fungi in the intestinal tract is referred to as the mycobiota. The kingdom Fungi represents a diverse collection of eukaryotic organisms ranging from single celled yeast to multicellular mushrooms (2). There are several markers that are used to delineate Fungi from microbes belonging to other kingdoms. Historically, Fungi were classified as plants, however, they were later separated due to their lack of chloroplasts and their ability to acquire nutrients from decomposition (3). The next major marker that has been used to identify fungi is the presence of ergosterol in the cell membrane (4). Ergosterol is a molecule that is similar in function to cholesterol in mammals in that it regulates fluidity of the cellular membrane (5). Ergosterol has been used to identify fungal biomass (6); and is frequently used as a target for antifungal drugs due to its presence in all major fungal pathogens (7). However, it should be noted that it's not only fungi that contain ergosterol as it is present in some Protozoa, Archaeplastida, and Amoebozoa (4). Another feature that has been used to define fungi is the structure of the fungal cell wall. The innermost layer of the cell wall is highly conserved among fungi and consists predominantly of β-(1,3) glucan and chitin which form a scaffold around the cell (8). Depending on the organism β-(1,6), β-(1,4), α-(1,3) and α-(1,4) linkages may also be present (9). Attached the layer of glucans and chitin are

glycoproteins (including mannoproteins) and in some fungi, melanin. Glycoproteins have several different cellular functions such as protection, structural integrity, mating, and immune recognition by the host (10, 11). Melanin is used by some fungi such as *Aspergillus* species and may enhance cell wall rigidity, thereby allowing them to remain turgid when dehydrated and to enable the hyphae of pathogenic fungi to penetrate host tissue (8).

Fungi can be broken down into 5 major phyla – Chytridiomycota, Zygomycota, Glomeromycota, Basidomycota, and Ascomycota (12). In the pig gastrointestinal tract (GIT), the dominate phyla are Basidomycota and Ascomycota, with the majority of fungi being yeast belonging to Ascomycota (13). As previously mentioned, yeast are unicellular fungi which can belong to either Basidomycota (e.g. *Cryptococcus neoformans*) or Ascomycota (e.g. *Saccharomyces cerevisiae*) (14). Yeast can be found in soil, plants, animals, insects, and water (15, 16), and has been consumed by humans in the form of bread or fermented beverages for thousands of years (17). Yeasts are facultative anaerobes (18), meaning they can survive both with and without oxygen, allowing them to live in the intestinal environment. Yeasts grow optimally at 25-30 °C, however they can grow over a large temperature range, from 0-47 °C. Yeasts are typically quite acid tolerant and can grow under conditions as low as pH 4.0-4.5, however they do not grow well under alkaline conditions (19), making them well suited to the GIT. Yeasts such as *S. cerevisiae* can reproduce both sexually and asexually. Asexual reproduction occurs via budding, a process in which both haploid and diploid cells produce a genetically identical bud, which will grow until it reaches a certain size, and then produce a bud of its own. The haploid version of *S. cerevisiae* can exist as two mating types, **a** and α, and they mate with the opposite mating partner to form a diploid **a**/α cell. Under stressful conditions, diploid cells can undergo sporulation where each diploid cell will produce 4 haploid spores (20).

Conditions for spore production can include the absence of nitrogen and the presence of a non-fermentable carbon source (21). Some yeasts, such as *Schizosaccharomyces pombe*, are able to reproduce via binary fission, similar to bacteria (22). Yeast of both reproduction types, that is, budding and binary fission, are present in the gut microbiome of humans (23, 24). To the authors knowledge, only budding yeast have been identified in the pig.

1.2 Fungi in the pig

Piglets are born with sterile gastrointestinal tracts (25) and are colonized with microbes shortly after birth (26). Fungi have been shown to colonize the piglet GIT as early as post-natal day (PND) 1 (27). On PND 1 the piglet mycobiome is composed predominantly of *Cladosporiaceae*, *Malasseziaceae*, *Dipodascaceae*, and *Nectriaceae*, although the mycobiome at this age shows a high degree of individual variation (27). *Cladosporiaceae* is a family of fungi that includes molds that are present in the environment (28), and it is likely that piglets are obtaining these fungi from exploring surfaces in the farrowing pen. In humans, *Malassezia*, the sole genera from the family *Malasseziaceae* is commonly isolated from skin (29), and can be passed to the infants GIT via breastfeeding and skin-to-skin contact (30). Pigs also have *Malassezia* on their skin (31), and as such, it is likely that their GITs are colonized from suckling. In humans, fungi are considered to have high intra-individual and inter-individual variability, especially when compared to bacteria (23), and it is likely that fungal colonization in pigs is similar.

By PND 3 fungi have increased in abundance from approximately 10 colony forming units (CFU) /g on PND 1 to 100 CFU/g of feces when cultured on Sabouraud dextrose agar plus cefoperazone, and no major increases are seen after PND 3 until 1 week post weaning (27). Prior

to weaning fungal α-diversity, as measured by Shannon index, decreases, and species evenness is variable (27, 32). However, β-diversity prior to weaning stayed relatively constant, with pre-wean and post-weaning pigs clustering separately (27, 32). During the pre-weaning stage, one study found that the fecal mycobiome was dominated by Mucoromycota and Basidiomycota, and found that the families *Cladosporaceae*, *Mucoraceae*, *Symbiotraphinaceae*, and *Trichosporonaceae* were significantly correlated with pre-wean feces (32).

On the day of weaning, fungal Shannon index was observed to increase sharply, but decrease again by 1 week post-weaning, before rising again by 2 weeks post weaning (27). This is in contrast with bacterial Shannon index which in the same pigs increased over time and was the highest at 2 weeks post weaning (27). When β-diversity was measured by Bray-Curtis dissimilarity, piglets at 1 and 2 weeks post weaning clustered together (27), and additionally cluster with adult pigs (32). This suggests that the dietary change to solid feed instead of a milk based diet drives mycobiota composition during the weaning transition. Adult pigs do have slightly different mycobiotas than pigs undergoing the weaning transition, with adults having greater mean relative abundance of *Dipodascaceae* and *Neocallimastigaceae* and lower mean relative abundance of *Wallemia*, which may be due to the change in diet (32). Post-weaning, there is an increase in the genera *Kazachstania*, *Wallemia*, and *Hyphopichia* (27, 32, 33).

Compared to nursery and weanling pigs, the mature pig mycobiome is less well characterized. Summers et al. (2019) found that non-gravid adult pigs had 5.18 CFU of fungi per gram of feces, which did not differ statistically from postpartum sows who had 5.86 CFU/g of feces (27). Li et al. (2020) found that there are some differences between adult pigs of different breeds in terms of fungal composition. They found that Chenghua pigs were dominated by the genera *Loreleia*, *Russila*, *Candida*, *Nephroma*, and *Metshnikowia*. Yorkshire pigs were

dominated by the genera *Loreleia*, *Russula*, *Candida*, *Metschnikowia*, and *Bullera*. Finally Tibetan pigs were dominated by the genera *Russula*, *Nephroma*, *Candida*, *Loreleia*, and *Metschnikowia* (34). It should be noted that the pigs used in this study were all housed in different environments depending on their breed. Additionally, while the pigs in the Chenghua and Yorkshire groups were fed a corn and soybean based diet, the Tibetan pigs were fed a grass silage based diet. Therefore, it is not possible to conclude that the differences were based on breed, but that the differences may be based on environmental factors such as diet and intensity of rearing practices.

In 35-day-old piglets, Arfkan et al. (2019) found that the dominate genera throughout the entire GIT were *Kazachstania*, *Hyphopichia* and *Wallemia*, however the authors note that in contrast to *Kazachstania*, *Hyphopichia* and *Wallemia* are most likely non-colonizing and are simply being ingested and not establishing in the GIT. This differed compared to bacteria, where several organisms such as *Helicobacteraceae* were decreased in the lower GIT compared to the upper GIT. Fungal α-diversity was highest in the stomach and then in the colon. In the same pigs, bacterial α-diversity increased throughout the GIT. The presence of increased fungal diversity in the stomach likely has to do with fungi being acid tolerant and also likely having decreased competition for resources with bacteria in the stomach. Bacteria showed decreased dispersion in feces whereas fungi did not (13). These findings, which suggest that many of the fungi in the tract are simply passengers and do not truly colonize is similar to what is proposed for humans, where a few core mycobiota are noted with a high prevalence of 'passengers' (35). As outlined by Suhr and Hallen-Adams (2015) there are likely a few reasons for this trend, the first of which is that many fungi, including those commonly found in the human intestine, such as *Penicillium* do not grow at 37 °C. *Wallemia*, which is also commonly found in the pig gut, is

an extreme xerophile and would not be able to grow at the water-activity of the mammalian body (36).

1.2.1 *Kazachstania slooffiae* in pigs

While many bacterial species have been consistently found in pig populations around the world, relatively few fungal species are consistent across studies. *Kazachstania slooffiae* is a species of yeast that is commonly isolated from the pig GIT (13, 27, 32, 37, 38). *K. slooffiae* is a particularly interesting yeast, as it is found in pigs from different geographic locations and under different farm conditions (13, 27, 32, 33, 37-42). It has been found in the United States, Germany, Spain, and China, and in pigs raised under commercial and experimental farm conditions with different diets (13, 27, 32, 33, 37-42). There is no known environmental reservoir for *K. slooffiae*, and its presence in pigs from diverse environments suggests that it is a yeast that is host adapted. As a result of its ubiquitous presence in pig populations, it is also the best characterized pig yeast species. *K. slooffiae* is a budding yeast belonging to the *Kazachstania* (*Arxiozyma*) *telluris* complex, which also contains *K*azachstania *bovina* which is commonly found in cattle, and *K*azachstania *pintolopesii* which is commonly isolated from rodents (43). *K. slooffiae* is considered a commensal organism (38). There may be some benefits of *K. slooffiae* for the pig, and a previous study by Urubschurov et al. (2017) suggested that *K. slooffiae* may be used as an amino acid source for pigs, as it is high in the limiting amino acid lysine and found that the abundance of both *K. slooffiae* and total yeasts correlated positively with total short chain fatty acids (SCFAs) (44). Some interesting interactions with the bacterial community have been noted. *K. slooffiae* abundance has been positively correlated bacteria species such as *Lactobacillus* (13). When grown in the supernatant of *Lactobacillus acidophilus*

isolated from pigs, *K. slooffiae* has been shown to have increased biofilm complexity, suggesting that a positive correlation between *L. acidophilus* and *K. slooffiae* may be present in the pig GIT (38). Additionally, Summers et al. (2021) found that the supernatant *Enterococcus faecalis* isolated from piglets decreased *K. slooffiae* growth, suggesting that a negative correlation may exist between the two in the piglet GIT, and found no difference is *K. slooffiae* growth when grown in the supernatant from *Lactobacillus fermentum* isolated from humans (38). This suggests that bacteria that are not host adapted do not have the same impact on *K. slooffiae* growth. This may be important when considering the use of bacterial probiotics, as if the goal is to increase *K. slooffiae* growth, using organisms which are not adapted to the pig may not result in increased *K. slooffiae* growth. Recently, Hu et al. (2023) showed that *K. slooffiae* is able to promote glycolysis in the intestinal epithelium by lysine desuccinylation, suggesting that *K. slooffiae* is an important commensal in the pig GIT and that supplementation with *K. slooffiae* could be used as an intestinal protectant for pigs with an insufficient intestinal energy supply, such as those with diarrhea (45). While largely considered to be commensal, one study identified *Kazachstania* as a genus that was in higher abundance in pigs with diarrhea compared to healthy pigs (39). However, there may be an alternate explanation to this finding. Since *K. slooffiae* appears to be a core microbe among pigs, that even when the mycobiome is disturbed by diarrhea, it stays in the gut, thereby representing a larger relative abundance of the intestinal mycobiota. The literature that has thus far been mentioned in this review has relied on both culture and next generation sequencing (NGS) based approaches to characterize the mycobiome. However, there are a number of challenges that exist when it comes to characterizing fungi in the intestinal environment.

1.3 The study of fungi

Until recently, the field of microbiology relied on culture-based approaches to characterize organisms, both in the GIT and elsewhere. Previous research has identified that only 27% of fungal species found in the human GIT are identifiable by culture-based methods (46). While this number is low, it is important to note that it is possible to culture some of the most abundant fungal organisms in the human GIT such as *S. cerevisiae*, multiple species of *Candida* and *Malassezia* (23, 47).

1.3.1 Fungal culture

There are multiple types of culture media commonly used in fungal studies. Perhaps the most commonly used media for are Sabouraud dextrose agar and Potato dextrose agar (48). However, other media such as yeast extract peptone dextrose (YPD) are commonly used to grow yeast such as *S. cerevisiae* (49), which is commonly used in the food industry. In general, yeast require several different media components for growth. They require a carbon source such as dextrose, a source of nitrogen and amino acids, and vitamins. This can be achieved by using cell wall hydrolysate which provides a source of nitrogen, amino acids and vitamins, with the addition of dextrose or glucose (50). Many more complex medias have been used to isolate yeast and filamentous fungi from the GIT. Hamad et al. (2017) used a culturomics approach to study the human mycobiome and found that they were able to isolate 73.2% of the identified yeast strains in the GIT from Dixon agar, followed by 53.7% from modified Schadler agar, 19.5% from Sabouraud agar, 14.6% on Banana agar and 12.2% from Potato Dextrose Agar (47). This shows that to date, most studies that have relied solely on fungal culture using media other than Dixon media may be missing a significant proportion of fungal species in the intestine. Dixon

media allows for the growth of *Malassezia* species (51), which are lipophilic fungi (52). This is because Dixon media contains lipids, in the case of the study by Hamad et al., from oleic acid and olive oil (47). Regardless of media used, when isolating fungi from a mixed microbial community containing bacteria, the use of antibiotics is necessary. Antibiotic usage varies between studies, but chloramphenicol is commonly used, as is oxytetracycline (53), and sometimes chloramphenicol is used in combination with gentamicin (54). Hamad et al. (2017) used a combination of imipenem, colistin, and vancomycin to successfully culture yeast strains on Dixon media (47). Regardless of the antibiotics used, they must be broad spectrum enough to eliminate all bacteria that may grow on the media, and tests should be carried out prior to sample collection and plating to ensure that there is no bacterial resistance that could cause inaccurate plate counts.

In culture dependent studies, it is also important to pay attention to the conditions in which the microbes are grown. In the human intestine, one study found that all isolated fungi were able to grow aerobically. Additionally, they found that all isolated fungi were able to grow at 22 °C and 28 °C but not 42 °C, although they did not try 37 °C (47). Another study using human fecal samples found no difference between aerobic or anaerobic culture conditions on fungal counts irrespective of media type when incubated at 37 °C (55). While a temperature of 37 °C may not allow for the growth of all fungi found in the intestine, isolation at 37 °C gives a good idea of which organisms are capable of reproduction at body temperature and may indicate that these organisms are able to colonize the GIT, not just flow through it as is the case with many fungi.

Another component of culture dependent studies is identification once organisms have been cultured. Perhaps the most traditional method of identification is colony morphology. In

pigs, yeast isolated from the GIT has several distinct colony morphologies (40). *S. cerevisiae* colony morphology is altered by glucose availability, with increased glucose resulting in increased convexity of colonies (56). *K. slooffiae* grown on yeast potato dextrose agar displays a concave morphology and when grown on Sabouraud dextrose agar display a flat morphology (38). While identification by colony morphology is a simple and inexpensive method of identification, it also proves difficult when differentiating between yeast in the GIT. This is because many yeasts of the pig GIT have similar colony morphology (40). Therefore, other methods must be used to help identify yeast in the GIT.

One method of identifying fungal taxonomy is through Sanger sequencing. Sanger sequencing allows for the generation of a DNA sequence (57) which can then be used to identify a microbe by comparing the sequence against reference data bases, such as the one maintained by the National Institutes of Health, the Basic Local Alignment Search tool. One drawback to Sanger sequencing is that the microbes must be isolated and prepared for sequencing, which can be quite time consuming when sequencing is required for many isolates.

Another commonly used method is the use of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF-MS has multiple benefits for isolate identification, including speed, accuracy, ease of use and cost effectiveness (58). However, there are some limitations to the use of MALDI-TOF MS, that include an inability to discriminate between closely related species, incorrect identification due to incomplete spectra databases, and a need for considerable biomass for identification (59).

1.3.2 Next generation sequencing

The advent of next generation sequencing (NGS) has helped make studying the microbiome more feasible. Next generation sequencing refers to the practice of sequencing DNA fragments in parallel, thereby allowing for many sequences to be generated at one time (60). One of the main benefits of NGS is the time saved. Prior to the advent of NGS, it took approximately a decade to sequence the human genome using Sanger sequencing, and now the human genome can be sequenced in one day (60). Therefore, many microbiome studies choose to use NGS to better understand the microbiome.

1.3.3 DNA extraction

Prior to performing any NGS assays, DNA must be extracted from fungi. This too poses several challenges. Fungi are often more difficult to extract than bacteria as fungi have thick and complex cell walls which resist lysis (61, 62). As such there is a wide variation in the success of fungal DNA recovery between commercial kits (63). However, there are several factors that contribute to successful fungal DNA extraction. The first factor is mechanical disruption of the cell wall, which involves breaking up the cell wall typically using glass or ceramic beads and a tissue homogenizer (64). Bead beating followed by proteinase K digestion has been shown to increase DNA recovery by 10 to 100 fold, depending on the type of fungi, compared to a commercial DNA extraction kit alone (64). This brings us to another important aspect of fungal extraction – chemical cell wall lysis. A variety of commercial kits are available which contain proprietary lysis buffers. In one study the Fungi/Yeast Genomic DNA Isolation Kit (Norgen BioTek Corp, ON, CAN) outperformed the Qiamp DNA extraction kit (Qiagen, DUS, DUE) or

phenol chloroform isoamyl alcohol extraction after the fungi in all groups had been treated with proteinase K (65). Another study identified that a combination of bead beating and the DNeasy Powersoil Kit (Quigen, DUS, DUE) yielded the highest amount of DNA compared to phenol chloroform and other commercially available kits, although increased DNA fragmentation was observed with this method (66). Commercial kits are frequently updated with new ones becoming available all the time. It is recommended that extraction controls be included in order to make sure that the extraction method is suitable for the type of sample being studied. Extraction bias is frequently present in microbiome studies (67). However, there are some best practices that can be used to help minimize the impact of these biases. Randomizing samples during extraction can help decrease batch effects. Samples should be extracted using the same extraction kit lot where possible, and when not possible the extraction kit lot numbers and dates should be included as confounding variables during data analysis (68).

1.3.4 Fungal marker gene regions and challenges

There are, however, several challenges when it comes to studying the mycobiome compared to the studies that focus on bacteria. For one, there is less consensus on which region of the fungal genome is ideal for sequencing. Primers used in fungal sequencing studies typically cover one of 4 regions: 18S (small subunit (SSU)), Internal transcribed spacer (ITS) 1, ITS2 or 28S (large subunit (LSU)). In between the ITS1 and ITS2 regions is the 5.8S region, which is typically conserved within species with only negligible variation (69). The 18S SSU region is frequently used when looking at classification on phylum level or above, as this region typically does not contain enough hypervariable regions to be able to classify fungi at a species level (70). Although depending on the primer set 75% accuracy at the genus level can be achieved (71). The

28S LSU region allows for species level classification in some yeasts, although this region is sometimes not sufficient to resolve differences in closely related species (72). With these challenges in mind, the ITS1 and ITS2 regions have become popular targets for studies where identification at the species level is desired (69). One challenge of using either of the ITS regions is that there is sometimes considerable intraspecific variation in ITS sequences (69). On average, there is more intraspecific variation in the ITS1 region compared to the ITS2 region (69). However, the results of one study found that in 34% of the fungi studied the ITS2 region was more variable than the ITS1 region (69). Another challenge of using the ITS regions is length variation between organisms (73). One study found that the ITS1 region varied from 9 base pairs to 1181 base pairs and ITS2 ranged from 14 to 730 base pairs (74). However, the ITS2 region is less variable and therefore may result in less taxonomic bias than ITS1 (73, 74).

Once sequenced, reads need to be classified using a database. One challenge of fungal taxonomic classification is that fungal databases are often incomplete or incorrect (75). Another challenge is that there is no agreed upon database which is best used for certain regions, and as a result studies tend to differ in the database used.

With all of these challenges in mind, it may be advantageous to base primer selection on the fungal community being studied. In the case of the pig mycobiome, a study was completed by Arfken et al. (2023) which looked at a fungal mock community based on the piglet mycobiome to try and determine the best target gene (18S, ITS1 or ITS2) and the best database (ITS1 and ITS2 – UNITE and 18S – SILVA) (76). The authors of this study concluded that no one marker-database combination performed better consistently. However, they did find that the ITS markers were slightly better than 18S, although they consistently were unable to identify *Lichtheimia corymbifera*, which is commonly seen in the piglet mycobiome (76). With no

consensus on which region is best, multiple studies have concluded that using multiple primer sets and markers may be advantageous (76, 77).

1.4 Fungal interactions with the host

1.4.1 Recognition and interaction with the innate immune system

Fungal recognition by the host immune system occurs when pattern recognition receptors (PRRs) on immune cells recognize pathogen-associated molecular patterns (PAMPs) associated with the fungal cell components (78). Several toll-like receptors (TLR) on phagocytes and dendritic cells recognize different parts of the fungal cell wall that act as PAMPs (78). Dendritic cell-associated C- type lectin-1 (dectin-1) is expressed on immune cells such as dendritic cells, macrophages, and neutrophils. Dectin-1 is able to recognize β-glucan on fungal cell walls (Figure 1.1) and activates phagocytosis, the production of reactive oxygen species, and cytokines such as interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) (79, 80). However, not all β-Oglucans and β-glucan presentations are able to induce an immune response via Dectin-1, with one study showing that soluble β-glucans did not activate Dectin-1 (79). Instead, they found that β-glucan must be presented to Dectin-1 in an immobilized form, such as on the yeast cell wall (79). Another study found that β-glucan size was an important factor contributing to cytokine production by human dendritic cells, and showed that large β-glucans stimulated dendritic cells to produce significantly more IL-1β, IL-6 and IL-23 than smaller β-glucans (81). This may have implications on the effectiveness of yeast and yeast cell wall products fed as pre and probiotics as preparations containing small or fractionated β-glucans may not produce an immune response,

and it is unclear if this is a positive or negative outcome when looking at the effectiveness of yeast based pre and probiotics.

Another important PAMP on fungal cell walls are mannans. Different mannans are recognized by different TLRs. Phospholipomannan from *Candida albicans* has been shown to be sensed by TLR2 (82). However, O-linked mannans, also from *Candida,* are recognized by TLR4 whereas mannose receptor cluster of differentiation (CD) 206 or CD209 recognizes N-linked mannans (83, 84). There are also other Dectin receptors which bind with mannans. Dectin-2 recognizes high mannose structures such as N-linked mannans and α-mannans, and Dectin-3 recognizes α-mannans (85). Additionally, fungal DNA is able to stimulate TLR9 (85), and the IL-1 receptor (IL-1R) recognizes certain fungi such as *Candida albicans* in both yeast and hyphal form (78). Chitin in the fungal cell wall is also recognized by the innate immune system by fibrinogen C containing domain 1 (FIBCD1), which has been shown to control the level to which fungi are able to colonize the murine GIT and also helps to reduce fungal driven intestinal inflammation (86).

Once PRRs on immune cells have been stimulated by fungi or fungal components, the gene myeloid differentiation primary response 88 (MYD88) is activated (78). This will cause the production of pro-inflammatory cytokines, especially IL-12 by dendritic cells, induction of respiratory burst and degranulation, and T helper 1 (Th1) cell differentiation (78). Another important gene in fungal recognition and immune response is Caspase recruitment domain-containing protein 9 (CARD9). CARD9 activates the transcription factor nuclear factor kappa B (NF-κB) (87), which then causes an inflammatory response (88). CARD9 is essential for host protection against fungal pathogens, as CARD9 deficiency is characterized by increase susceptibility to fungal infections from organisms such as *Candida* (89).

1.4.2 Interaction with the adaptive immune system

In addition to the innate response, the adaptive immune system also responds to fungi. The T helper cell subtypes Th1 and Th17 promote the phagocytic clearance of fungi by releasing the proinflammatory cytokines interferon-γ (IFN- γ) and IL-17A/F (90). Th2 immune responses are often associated with fungal persistence and have detrimental impacts on the host during fungal infection (90, 91). The role of regulatory T cells is to balance the pro and anti-inflammatory responses (90). The activation of naïve T cells to specific fungal antigens depends on their interaction with dendritic cells that present fungal antigen (90). Th17 cells produce the cytokines IL-17A, IL-17F, and IL-22 and are implicated in the inflammatory response to fungi (90), and memory T cells for *C. albicans* are part of the Th17 subset (92, 93). While Th17 responses are largely influenced by *C. albicans* in the human intestine, Th17 cells are cross-reactive to other fungi, which may drive an allergic inflammatory responses in the lung to airborne fungi (94). Fungal colonization has been shown to alter the systemic immune system in early life, suggesting that fungi play a role in early life immune system development (95)

1.4.3 Interaction with host epithelial cells

The intestinal lumen is lined with epithelial cells that will come into contact with the microbes in the GIT (96). While the field of mycobiome research is still relatively new, research into *C. albicans* can give us insight into how fungi interact with the host epithelial cells, as it is a well studied opportunistic pathogen. When *C. albicans* is in its hyphal form, it is able to adhere to host epithelial cells. Adherence occurs when Hyphal Wall Protein 1 (Hwp1) interacts with an

unidentified host cell wall protein causing the covalent attachment of *C. albicans* to the host (97). However, Hwp1 is not the only adhesin expressed on the fungal cell wall, the Agglutinin-Like Sequence (ALS) family of genes are also expressed in fungi in both yeast and hyphal forms.

These genes, along with Hwp1 promote biofilm formation both *in vitro* and *in vivo* (98). Adherence to epithelial cells is followed by either cellular invasion, which is achieved by endocytosis or fungal penetration of the host cell wall. Additionally, *C. albicans* may also become disseminated in the host by degrading tight junction proteins such as E-cadherin via proteolysis (99).

1.5 Interaction of fungi with bacteria

Fungi and bacteria live together in the GIT and as such interact with one another. Perhaps the most well known example of fungal-bacterial interaction is during antibiotic administration. *C. albicans* is a member of the normal human and pig mycobiota (23, 40). However, when antibiotics are given, they decrease the bacterial community in the GIT. In one study, administration of vancomycin to mice caused the decrease in *Bacteriodales*, *Deferribacterales*, *Erysipelotrichales*, and *Clostridales*, which then allowed for *C. albicans* colonization and disseminated candidiasis (100). There is also evidence that changes in the fungal community due to the administration of antifungals can alter the bacterial community. Decreases in *Penicillium brevicompactum* and *Candida tropicalis* in the murine gut following antifungal treatment have been shown to result in the decreased relative abundance of *Bacteroides*, *Allobaculum*, *Clostridium*, *Desulfovibrio*, and *Lactobacillus* and increased relative abundance of *Corprococcus*, *Anaerostipes*, and *Streptococcus*, which suggests that bacterial and fungal communities in the gut are co-dependent (101).

1.5.1 Physical interactions between fungi and bacteria

Physical interactions between fungi and bacteria include the attachment of bacteria to fungal filaments causing decreased filamentation of *C. albicans* (101, 102) and the adherence of *C. albicans* to the cell surface of *Streptococcus gordonii* (103). Additionally, bacteria and fungi can form biofilms together. *C. albicans* and *Staphylococcus aureus* aggregate to form biofilms that help to evade host defense mechanisms and promote antibiotic resistance (104). Biofilms containing both fungi and bacteria are metabolically diverse, and it has been suggested that this diversity leads to increased virulence (105). However, some multi-kingdom biofilm formation may be beneficial to the host. In *Burkholderia cenocepacia* and *C. albicans* aggregated biofilm, *B. cenocepacia* has been shown to interfere with the transition to hyphal form, thus potentially decreasing the pathogenicity of *C. albicans* (106).

1.5.2 Chemical interactions between fungi and bacteria

Chemical exchanges also exist between bacteria and fungi (102). The quorum sensing molecule 3-oxo-C12 homoserine lactone, which is produced by *Pseudomonas aeruginosa* has been shown to inhibit *C. albicans* hyphae formation without reducing the total amount of *C. albicans* (107). *C. albicans* has also been shown to form biofilms with *Streptococcus mutans*, which is commonly found in dental carries (108, 109). Communication with quorum sensing molecules as well as cross feeding has been shown to occur between these two organisms, resulting in increased biomass of biofilms (108). Interactions between fungi and bacteria are common outside of the body as well and have been studied to the greatest extent in fermented food production. Interaction between *Lactobacillus* species and *Saccharomyces cerevisiae* occurs

frequently in food systems and one example is the production of growth factors by *S. cerevisiae* such as CO_2 and amino acids such as valine and leucine, which promote the growth of *Lactobacillus* species (110). Additionally, lactate and acetate from *Lactobacillus* can induce *S. cerevisiae* to decrease ethanol production, creating a lower stress environment for the *Lactobacillus* (110). With these well-studied interactions in mind, we can speculate that other fungi and bacteria interact chemically in the intestinal tract.

1.5.3 Interactions within the intestinal microbiome

Supplementing animals and humans with yeast has been shown to alter bacterial community composition. *Saccharomyces cerevisiae* has been shown to alter the piglet bacterial community in a dose dependent manner (111). However, β-glucan derived from either yeast or oats showed either no or only modest changes in the bacterial community (112, 113). A number of interactions have been found between fungi and bacteria in the piglet intestine, with 21 day old piglets showing 93 different interactions and 35 day old piglets showing 142 interactions, with interactions being identified using a co-occurrence network (32). In the previously mentioned study, the fungi *Aspergillus* and *Hyphochia* showed the most interactions, with some of them being negative such as the interaction between *Aspergillus* and *Ruminococcaceae* UCG-004 (32). *K. slooffiae* is also able to alter bacterial community composition, with *K. slooffiae* administration increasing bacterial richness in a dose dependent manner in pigs given *K. slooffiae* 1-3 times starting 5 days after weaning, with doses 1 day apart (44). *K. slooffiae* also increased bacterial α-diversity compared to a placebo and was correlated with increases in the select bacterial genera, including *Prevotella*, *Dialister*, *Acidaminococcus*, *Christensenellaceae*, *Ruminococcaceae* and S24-7 (44).

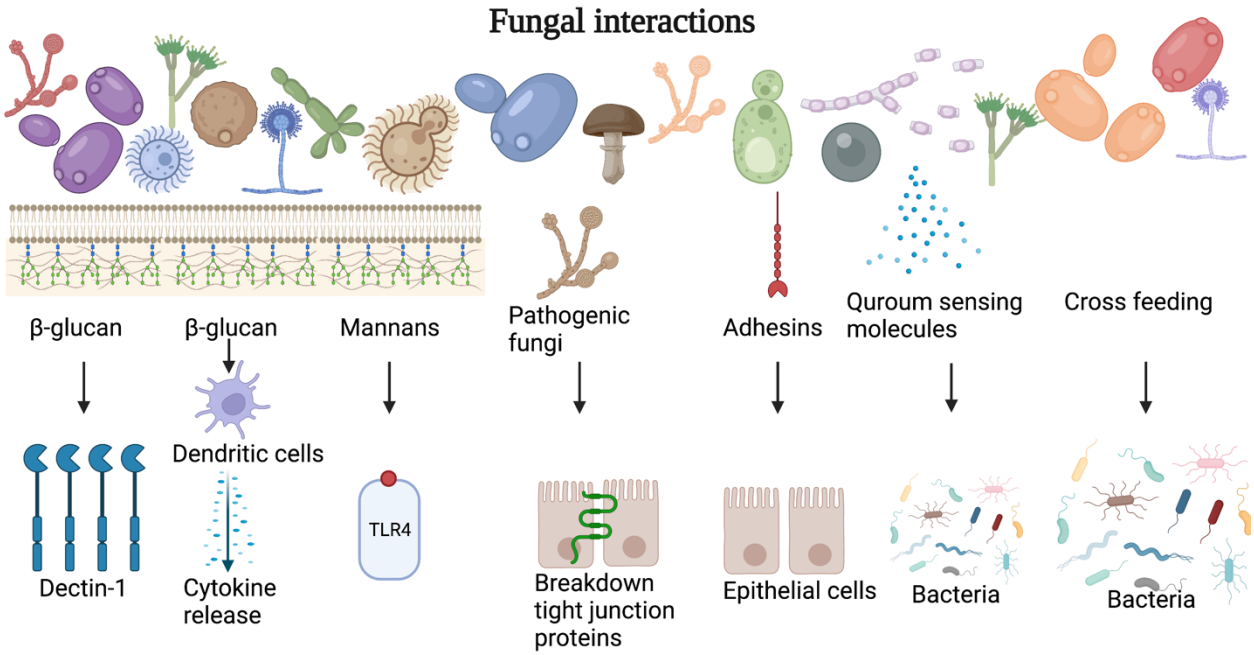

Figure 1.1. Interactions between fungi and the host. Figure was creased with BioRender.com.

1.6 The role of fungi in health and disease

1.6.1 Impact on sows

Yeast and yeast fractional products have long been fed to pigs in order to improve health and production parameters. By far the most common yeast fed to pigs is *S. cerevisiae* followed closely by *Saccharomyces boulardii*. There are a number of health benefits associated with feeding yeast to pigs. Feeding *S. cerevisiae* to sows during late gestation has been shown to improve the quality of colostrum by increasing the concentration of protein, lactose, and non-fat solids, and increase sow plasma concentrations of immunoglobin G (IgG) (114). Galliano et al. (2013) found that *S. cerevisiae* was able to increase IgG concentration in colostrum and milk and

that milk IgA levels were maintained for longer in sows fed *S. cerevisiae* (115). Feeding *S. cerevisiae* to sows has also been shown to shorten farrowing duration, increase feed intake during lactation, and minimize backfat loss during lactation, thereby improving sow health (116). Supplementation of sows can also impact piglets later in life, with one study showing that piglets from sows supplemented with yeast derived β-glucan and casein hydrolysate had decreased incidence of diarrhea at weaning, decreased feed intake after weaning, and improved feed efficiency (117).

1.6.2 Impact on suckling piglets

When suckling piglets are fed yeast there are also a number of proposed benefits. Sun et al. (2022) found that supplementing piglets with *S. boulardii* increased IgG levels, which the authors suggested may have led to the observed increase in post-weaning performance (116). Another study found that supplementing piglets from birth to 28 days of age with *S. cerevisiae* increased piglet body weight and average daily gain, possibly due to changes in the bacterial community (111). Creep feed containing active dry yeast has been shown to increase feed consumption (118), which is important because increased creep feed consumption has been associated with increased post weaning feed intake and increased post weaning performance (119).

1.6.3 Impact on post weaning pigs

The post weaning period is a time of social, environmental, and dietary stress in pigs (120). Following weaning, piglets can show decreased barrier function and nutrient adsorption, depending on factors such as time of weaning and preweaning feed intake (120). In the post

weaning period, diarrhea is common and may be caused by a number of factors including proliferation of enterotoxigenic *Escherichia coli*, microbial dysbiosis, and abrupt changes in diet and environment (121, 122). Supplementation with live yeast has been shown to impact the health and performance of pigs in the post weaning period. Trckova et al. (2014) found that feeding *S. cerevisiae* to pigs from birth through to the post weaning period and then challenging them with enterotoxigenic *E. coli* resulted in decreased diarrhea scores, decreased duration of diarrhea, and decreased shedding of enterotoxigenic *E. coli* (123). This may be because yeast cell wall has been shown to be able to attach to some enteric pathogens such as *Salmonella* and enterotoxigenic *E. coli* to bind the hosts intestinal epithelium, although binding capabilities appear to be strain specific (124). Shen et al. (2009) found that adding dry *S. cerevisiae* to the diets of post weaning pigs altered immune system parameters, with the finding that supplemented pigs had decreased IFN-γ, decreased T helper cells, and increased jejunal villus height and villus height to crypt depth ratios (125). However, this study did not find any differences in growth performance, despite altered intestinal morphology (125).

Yeast does not necessarily have to be live to have an impact on host parameters. Lee et al. (2021) looked at including yeast cell wall in the diets following weaning and found that pigs fed a diet supplemented with yeast cell wall had a lower incidence of diarrhea, as well as an increased villus height to crypt depth ratio in the duodenum and jejunum, decreased IL-1β and TFN-α, and increased ileal gene expression of tight junction proteins (126). Hydrolyzed yeast derived protein from *S. cerevisiae* is another product that can be added to feed. In the post weaning period, supplementation with this product has been shown to increase body weight, average daily gain and average daily feed intake (127). In addition, hydrolyzed yeast derived protein also increased jejunal villus height to crypt depth ratio and increased short chain fatty

acid concentrations in the intestine (127). In a study that compared the use of three different yeast preparations on a number of intestinal and immunological parameters, Jiang et al. (2015) found that for early-weaned (14 days of age) piglets, feeding both live yeast as well as super fine yeast (baker's yeast) improved a number of pig health parameters. Feeding live or super fine yeast enhanced feed conversion, improved intestinal development, increased serum IgA, IL-2 and IL-6 levels, and increased the ratio of CD4+/CD8+ ratio, compared to heat killed yeast or control pigs (128). It is unclear why the heat killed yeast did not elicit the same responses as the live or super fine yeast, since non-viable yeast cell wall products were successful in eliciting physiologic changes in other studies.

1.7 The role of fungi in human health

In pig research much of the research focus is rightfully placed on pig production and growth. However, exploring the impact of fungi on human health, through both human and rodent models, can provide us with insight into how yeast impacts the mammalian body.

1.7.1 The role of fungi in gastrointestinal disease

Inflammatory bowel disease (IBD) involves the chronic inflammation of the gastrointestinal tract that can flare and subside over time. In humans, the intestinal fungal community is skewed during an IBD flare (129). During periods of active disease, the mycobiome has an increased Basidiomycota/Ascomycota ratio, a decreased relative abundance of *S. cerevisiae* and an increase in *C. albicans* (129). While it is not completely understood how *C. albicans* contributes to IBD severity, it has been proposed that IL-17 mediated inflammation may play an important role in IBD pathogenesis (130). Candidalysin is a toxin produced by

pathogenic *C. albicans* in its hyphal form (131). This toxin is able to directly damage epithelial membranes, and activates epithelial immunity (131). Additionally, candidalysin accounts for the induction of Th17 induced IL-17A, a pro-inflammatory cytokine which is a common feature of inflammatory conditions such as IBD (132, 133). Candidalysin may also contribute to other autoimmune diseases such as type 1 diabetes and rheumatoid arthritis (134). On the other hand *S. boulardii* has been shown to inhibit IBD by causing the accumulation of IFN-γ producing Th1 cells withing the mesenteric lymph nodes, thereby limiting the infiltration of Th1 cells and proinflammatory cytokines in the colon, and inhibiting the inflammation associated with IBD (135). Jiang et al. (2017) looked at the roles that *S. cerevisiae*, *C. albicans*, and fungal cell wall mannans play in protecting against DSS induced colitis and influenza A virus in gnotobiotic mice, compared to the established protective effects of commensal bacteria. The authors found that *C. albicans* monocolonization, *S. cerevisiae* mono-colonization, and fungal mannan administration recapitulated the protective effects of commensal bacteria (136). This suggests that *C. albicans* on its own may not be problematic, and that it is the cooccurrence with bacteria that may exacerbate IBD. In a dextran sulfate sodium (DSS) induced colitis model, co-colonization of fungi with bacteria exacerbated colitis, whereas when mice were colonized with fungi alone inflammatory markers of colitis were similar to untreated controls (95).

C. albicans also colonizes pigs (40), and although it is unclear if poor intestinal health in pigs can be attributed to candidalysin. However, pigs may be able to serve as a model for human candida induced disease, as pigs do not require antibiotic treatment to promote *Candida* colonization as is the case in mice (137).

1.7.2 The role of fungi in obesity

Obesity is another disease where the fungal community has been implicated as a contributing factor. In mice treated with fluconazole to eliminate yeast and promote an increase in other fungi such as *Cladosporium*, there were increased proinflammatory immune markers in adipose tissue and reduced glucose tolerance (138). In the same study, germ free animals treated with fluconazole did not have the same response, suggesting that it is the alteration in the microbial community that is responsible for the disease state (138). However, another study found that *Candida parapsilosis* in combination with a high fat diet was able to induce obesity in mice (139). This study also found that the mechanism behind the induced obesity was the secretion of lipase by *C. parapsilosis*, evidenced by the finding that colonization of fungi free mice with mutant lipase-negative *C. parapsilosis* failed to produce obesity (139). Lipase can also be secreted by *C. albicans*, and the presence of genes that encode for lipase production may enhance the ability of *C. albicans* to survive and disseminate to other body sites than the one originally infected (140). Pigs may be able to serve as an obesity model for humans. Additionally, it is desirable for pigs to deposit lean muscle mass over fat and understanding the mechanisms behind obesity and the mycobiome may help us to improve pig growth.

1.8 Conclusion

The mycobiome is a complex network of fungi that interact with the host as well as other members of the intestinal microbiome. In pigs, the microbiome represents a relatively unstudied area. There are several knowledge gaps that will be addressed in this thesis. The first knowledge gap that will be addressed is surrounding the impact of antibiotics on the fungal community. Next this thesis will address the knowledge gap surrounding how the mycobiome changes over one production period and compare the mycobiome of pigs raised with intensive rearing

practices to their feral counterparts. Finally, this thesis will address the knowledge gap surrounding the role of *K. slooffiae* in piglet health and development. Addressing these knowledge gaps may help us to develop supplementation strategies that can improve pig health and production performance.

1.9 Objectives and hypothesis

Chapter 2 will explore the impact of antibiotics on the mycobiome. The objectives of this chapter were to 1) determine the impact of commonly prescribed infant antibiotic treatments on the microbial structure of fungal and bacterial communities in the piglet gastrointestinal tract. 2) determine the impact of commonly prescribed infant antibiotic treatments on microbial load of the piglet gastrointestinal tract. We hypothesized that antibiotics would differently impact fungal community structure and increase fungal load compared to control animals.

Chapter 3 will explore the composition of the porcine mycobiome over one production cycle. The objectives of this chapter were to 1) profile the mycobiome of pigs on 11 days of age, the day prior to weaning, 7 days after weaning, and 119 days after weaning, including the maternal sows. 2) characterize and compare the mycobiomes of pigs raised in a experimental barn to feral pigs and to pigs raised in a commercial facility. We hypothesized that maternal *K. slooffiae* levels would influence mycobiome composition throughout the production cycle and that feral pigs would have a mycobiome comprised predominately of soil associated fungi.

Chapter 4 will investigate the role of *K. slooffiae* in piglet development. The objectives of this chapter were to 1) investigate the role of *K. slooffiae* in bacterial succession 2) investigate the role of *K. slooffiae* in intestinal development, 3) investigate the role of *K. slooffiae* in immune system development, and 4) investigate the role of *K. slooffiae* on the host metabolite

pool. We hypothesized that *K. slooffiae* would alter immune system and intestinal development, alter bacterial succession, and have little to no impact on the host metabolite pool.

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Chapter 2: Maternal mycobiome but not antibiotics alter fungal community structure in neonatal piglets

2.1 Introduction

During early life, microbes in the gastrointestinal tract (GIT) play essential roles in host health and development. During this critical period of microbial exposure, microbes aid in the development of the host immune system (1). Aberrant microbial exposure in early life has been associated with long-term adverse outcomes such as autoimmune disease, obesity, and asthma (2). The GIT harbours a vast array of microbes including bacteria, fungi, archaea, protozoa, and viruses (3). While the impact of bacteria on immune development has been well characterized and studied, fungi (the mycobiome) have received less attention.

Fungal colonization of both the human and pig GIT occurs shortly after birth (4, 5) and fungi can be acquired vertically, horizontally, and environmentally (6, 7) . In humans, the mycobiome changes in both composition and abundance during the first two years of life and resembles adult mycobiota at approximately 2 years of age (8). In pigs the mycobiome changes most dramatically during the weaning transition and looks similar to the mycobiome of adult pigs in the post weaning period as early as 24-35 days of age (9). Human bacterial ecosystems change in both richness and diversity most dramatically in the first year of life and stabilize around 3 years of age (10). Likewise, pigs experience drastic changes in bacterial richness and diversity around the weaning transition, with bacterial stability occurring 2-3 weeks post-weaning (11). However, fungi populations show more inter-individual and intra-individual variation than bacterial populations (11).

Early life antibiotic exposure is associated with the development of asthma (12), type-1 diabetes (13), and inflammatory bowel disease (14) later in life. It is thought that disruptions in bacterial colonization during the critical early life period alters immune response, leading to the development of disease (15). In a piglet model, we have identified that early life amoxicillin results in altered pancreatic development, and divergent responses to immune and metabolic challenges compared to placebo treated piglets (16-18). However, the role of antibiotics on fungal communities has not been investigated, with the exception of *Candida albicans,* where antibiotics have been shown to alter the intestinal metabolite pool in a way which promotes *C. albicans* growth (19). It should be noted that fungi are immunomodulatory (20) and changes in the mycobiome may be a contributing factor in disease development.

Pigs serve as a good human model as they are similar in both intestinal structure and immune system function (21). In the present study, we used a piglet model to investigate the role of antibiotics on fungal community dynamics in early life, with potential implications for both human and pig health. The objectives of this study were to characterize the mycobiome in response to 3 different antibiotic regimes that are commonly prescribed in early childhood, and to quantify changes in the mycobiome in response to antibiotic administration in conjunction with changes in bacterial community structure. The 3 antibiotic regimes used for this study included amoxicillin, amoxicillin + clavulanic acid, and gentamicin + ampicillin. Amoxicillin with/without clavulanic acid were chosen because they are commonly prescribed to children for respiratory tract and ear infections (22, 23), and amoxicillin treatment has been shown to result in altered phenotypes in our piglet model (16-18). Both regimens have been shown to increase the incidence of infection with *Candida*, a common yeast of the GIT (24). The combination of gentamicin and ampicillin was chosen because this combination is recommended for neonates

with bacterial sepsis (25), and exposure to aminoglycoside antibiotics, such as gentamicin, is a risk factor for invasive *Candida* infections (26). In the context of this paper the term antibiotics refers only to those compounds which target bacteria and not fungi. It is worth noting that antibiotics themselves do not kill fungi, but by altering the bacterial community decrease competition and allow fungi to flourish and persist in the gut (27). Here we show the impact of these antibiotics on the bacterial and fungal communities as well as discuss the role of the maternal mycobiome in piglet mycobiome development.

2.2 Materials and methods

2.2.1 Animals and housing

This animal study was approved by the Animal Care and Use Committee of the University of Alberta and conducted in accordance with the guidelines of the Canadian Council on Animal Care at the Swine Research and Technology Centre (Edmonton, AB, Canada) under animal use protocol number AUP00000922. A total of 32 crossbred piglets Duroc × (Large White/Landrace) and 4 Large White/Landrace sows were used in this study. On postnatal day (PND) 1, 4 litters of piglets were weighed and 8 piglets from each litter were selected for the study based on sex and weight (4 piglets above and 4 piglets below median litter weight, litters labelled A-D). Piglets were balanced for sex and weight and remained with their mother for the duration of the study. Two piglets from each litter were then assigned to one of 4 treatment groups (n = 8): A (amoxicillin, 30 mg/kg/day orally every 12 h on PND 1-8); AC (amoxicillin + clavulanic acid, 30 mg/kg/day orally every 12 hours on PND 1-8); GA (gentamicin + ampicillin, gentamicin 5 mg/kg/day once daily by intramuscular injection, ampicillin 100mg/kg/day twice daily by intramuscular injection, on PND 5-6), or P (flavoured placebo, 30 mg/kg orally every 12

hours on PND 1-8). All oral preparations included an artificial maple flavouring and were identical except for the addition of antibiotics. Piglets in the GA group also received the placebo treatment on PND 1-4 and 7-8 to account for handling stress. An injection control was not used as all the piglets in other groups continued to receive oral treatments on PND 5 and 6, and therefore experienced a similar amount of handling stress. Fecal swabs and samples were collected on PND 3 and PND 8 from each piglet. Fecal samples were collected from defecating sows one day after farrowing. Neither piglets nor sows received any antibiotics or other medications outside of the study treatment groups. Creep feed was not provided to piglets and all sow diets were the same. PND 3 was chosen as the first timepoint as obtaining enough feces to get good quality reads from a younger piglet was not possible. A study duration of 7 days was chosen because we have previously shown that amoxicillin can cause changes in the bacterial component of the microbiome as early as PND 3 and that these changes began normalizing by PND 7 (17). Samples were stored at -80°C until further processing. Piglets were weighed on PND 1 and PND 8 and were scored for health (Table 1.1) and diarrhea daily throughout the study, as previously described (28).

2.2.2 DNA extraction

Total genomic DNA was extracted from fecal swabs using the DNeasy PowerSoil Pro Kit (Qiagen®, CA, USA) as per manufacturer's instructions with no modifications. Bead beating was performed on a FastPrep-24™ (MP Biomedicals, OH, USA) homogenizer at 5 m/s for 45 seconds. DNA concentration was quantified using a Quant-iT PicoGreen dsDNA kit (Invitrogen, CA, USA).

2.2.3 Fungal sequencing

Internal transcribed spacer (ITS) 2 sequencing was performed at Microbiome Insights (University of British Columbia, BC, CAN). The following primers were used: forward (ITSF) 5'-CCTCCGCTTATTGATATGC-3' and reverse (ITSR) 5'-CCGTGARTCATCGAATCTTTG-3'. A paired-end sequencing run was performed on the Illumina MiSeq platform (Illumina, CA, USA) using 2 x 300 cycles.

Sequencing analysis was performed using Quantitative Insight into Microbial Ecology (QIIME) 2 (v2021.4) (29). Only forward reads were utilized to account for variation in the length of the ITS2 region and reads were not truncated. The Divisive Amplicon Denoising Algorithm, version 2 plugin (30) was used to perform demultiplexing, quality filtering, denoising, and for filtering out chimeras. Amplicon sequence variants (ASVs) were aligned using mafft (31). Taxonomy was assigned to the resulting ASVs using the classify-sklearn naïve Bayes taxonomic classifier (via q2-feature-classifier plugin) (32) against the UNITE database version 8.3 (33). The R package phyloseq (v1.34.0) was used to analyze microbial community structure and diversity (34). Alpha diversity was measured using Shannon index at a sampling depth of 1000 reads and analyzed using Kruskal-Wallis. Alterations in overall fungal community composition was measured using Bray-Curtis dissimilarity and Permutational Multivariate Analysis of Variance (PERMANOVA), which was visualized using Principal Coordinates Analysis (PCoA) (R, v4.0.5). Homogeneity of dispersion was measured using Betadisper function in phyloseq (34).

2.2.4 Bacterial sequencing

Amplicon libraries of the V3-V4 region of the 16S rRNA gene were constructed in-house according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol. DNA

concentration was determined using the Quanti-iT™ Pico Green dsDNA Assay kit (Invitrogen, CA, USA). The following amplicon primers were used: forward, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and reverse, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. Sequencing was performed on an Illumina Miseq platform using 2 x 300 cycles (Illumina Inc., San Diego, CA). Due to declining quality in the reverse read only forward reads truncated at 208 bp in length were utilized. Sequence assembly was performed as listed for fungi, except taxonomy was assigned using the SILVA database version 138.1 (35) and Shannon index was measured at a depth of 11000 reads.

2.2.5 Quantitative PCR (qPCR)

Total fungal load was measured using qPCR, which was performed on a StepOnePlus Real-Time PCR system (Applied Biosystems, CA, USA). Each reaction was performed in duplicate and consisted of 5 µL PerfeCTa SYBR Green Supermix (Quantabio, MD, USA), 0.8 µL (10 µM/L) forward primer, NL1 (5'- GCATATCAATAAGCGGAGGAAAAG -3') (36) and reverse primer, LS2 (5'- ATTCCCAAACAACTCGACTC -3') (37), 1.4 µL of nuclease free water, and 2 µL template DNA. The following cycling parameters were used: 10 min at 95°C; 40 cycles of 95°C for 15 sec, 59°C for 15 sec, 72 °C for 15 sec (38). A standard curve was generated using DNA extracted from *Kazachstania slooffiae* in the manner outlined above for DNA extraction. The *K. slooffiae* was isolated from a pig in the same barn where the present study took place. *K. slooffiae* DNA was quantified using the Quanti-iT™ PicoGreen dsDNA Assay kit (Invitrogen, CA, USA).

Total bacterial load was measured via qPCR as described above. The forward primer, SRV3-1 (5'- CGGYCCAGACTCCTACGGG -3') and the reverse primer, SRV3-2 (5'- TTACCGCGGCTGCTGGCAC -3') (39) were used. The following cycling parameters were used: 95°C for 3 min and 40 cycles of 95°C for 10 sec, 60°C for 30 sec. A standard curve was generated from the PCR amplicon of pooled genomic DNA which was quantified using the Quanti-iT™ PicoGreen dsDNA Assay kit (Invitrogen, CA, USA).

2.2.6 Statistical analyses

Unless otherwise stated, all statistical analyses were done using GraphPad Prism 9.2.0. Differentially abundant taxa between treatments and litters were determined using ANCOM in QIIME 2021.4. Identified taxa were subsequently compared between litters using a Kruskal-Wallis test. Piglet average daily gain was analyzed using a one-way ANOVA. Distance from maternal sow to her piglets versus that of a sow of another litter was determined based on Bray–Curtis dissimilarity using a Mann–Whitney U test. qPCR data was analyzed using PROC MIXED with blocking by litter, followed by a Bonferroni post hoc test, using SAS software (SAS OnDemand, SAS Institute Inc., NC, USA).

2.3 Results

2.3.1 Piglet performance was not different among treatment groups or litters

Average daily gain did not differ between piglets based on treatment ($P = 0.198$) or litter ($P = 0.848$) at day 8 (Table 2.1). All piglets remained healthy with a health score of 0 (Table 2.2), and no incidence of diarrhea was observed throughout the study.

Table 2.1. Mean average daily gain by treatment and litter.

Treatment	Mean ADG (kg/day)	Litter	Mean ADG (kg/day)
P	0.23	A	0.25
A	0.28	B	0.23
AC	0.23	C	0.24
GA	0.23	D	0.24

Table 2.2. Health scoring criteria.

Score	Criteria
0	Bright, alert, and reactive; Rectal temperature 38.5 to 39.5 °C
1	Minor depression, decreased alertness, lethargy; Rectal temperature 39.6 to 40.5 °C
2	Low motility, resistant to attempts to force movement, occasional vomiting and/or diarrhea; Rectal temperature 40.6 to 41.9 °C
3	Immobile and refuses to stand, vomiting, persistent severe watery diarrhea; Rectal temperature equal to or greater than 42.0 °C or below 38.0 °C

2.3.2 Litter but not antibiotics drove mycobiome composition

Antibiotic treatment did not alter fungal β-diversity (R^2 = 0.08, P = 0.565, β-dispersion P = 0.116, Figure 2.1a) or α-diversity (P = 0.834, Figure 2.1b) on PND 8. It was noted however that fungal community composition differed by litter. On PND 3 α-diversity differed between litters (P = 0.015; Figure 2.2b) but β-diversity did not change between litters (R^2 =0.112, P = 0.352, β-dispersion P = 0.054; Figure 2.2a). By PND 8 both α-diversity (P < 0.001; Figure 2.2d) and β-diversity (R^2 =0.246, P < 0.001; β-dispersion P = 0.705; Figure 2.2c) differed between litters. Differences in fungal community structure between litters may be explained by differences in the relative abundance of *Kazachstania* (P < 0.001; Figure 2.3a) and *Nakaseomyces* (P < 0.001; Figure 2.3b) on PND 8. *Kazachstania* made up to 99.9% of the fungal community in some piglets by PND 8, whereas other piglets had no *Kazachstania* (Figure 2.4).

Kazachstania was more abundant in litters A and B than in litters C and D on PND 8 (Figure 2.4). On PND 3 *Kazachstania* abundance was lower, ranging from 0.4 to 11.2% (Figure 2.5). Piglets without *Kazachstania* had more unclassified fungi and a more diverse mycobiome on both PND 3 and 8. *Kazachstania* abundance also differed drastically in sows, with a range of 0 to 98.0% (Figures 2.4 and 2.5). Piglets from sows with high *Kazachstania* abundance had increased *Kazachstania* by PND 8 compared to PND3 (Figure 2.4). On PND 8 the fungal community structure of piglets was more similar to their dams than to that of the other dams based on Bray-Curtis dissimilarity ($P < 0.05$; Figure 2.6).

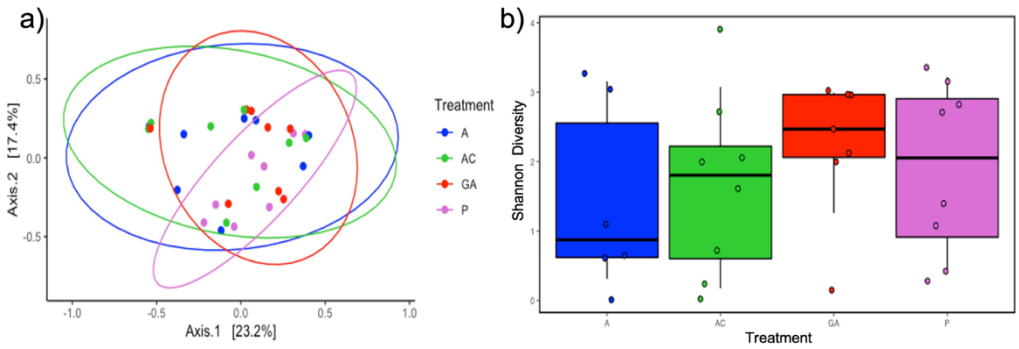

Figure 2.1. Fungal diversity following antibiotic treatment. **(a)** Fungal β-diversity using Bray-Curtis metrics (PERMANOVA, $R^2 = 0.08$, $P = 0.565$; β-dispersion, $P = 0.116$) and **(b)** Shannon diversity index on PND 8 after antibiotic/placebo treatment: amoxicillin (A) (n=6), amoxicillin + clavulanic acid (AC) (n=8), gentamicin + ampicillin (GA) (n=7), or flavoured placebo (P) (n=8) (Kruskal-Wallis, $P = 0.834$). Significance was defined as $P \leq 0.05$.

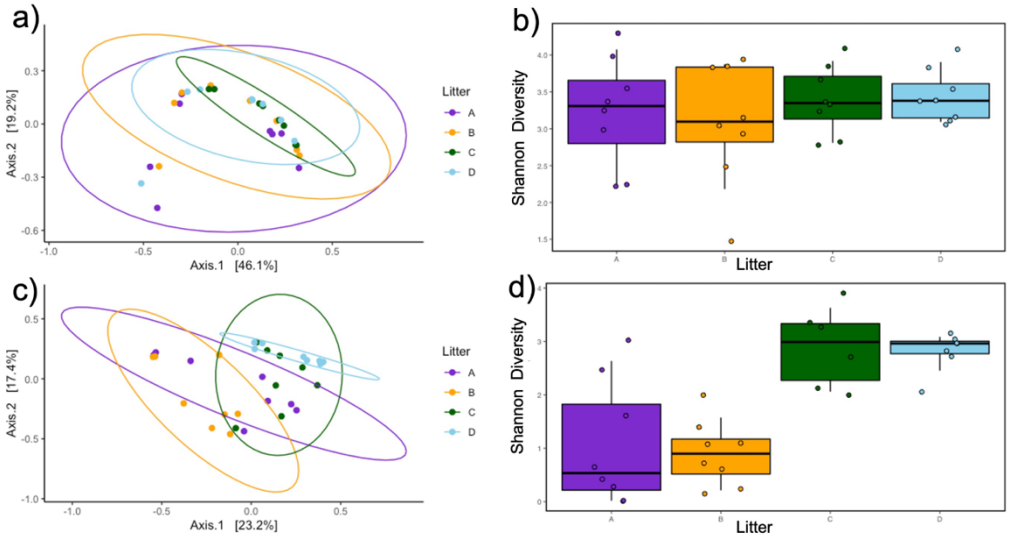

Figure 2.2. Fungal diversity by litter. (a) PND 3 fungal β-diversity by litter as measured by Bray-Curtis dissimilarity (PERMANOVA, $R^2 = 0.112$; $P = 0.352$; β-dispersion, $P = 0.054$) **(b)** PND 3 α-diversity by litter as measured by Shannon diversity index (Kruskal-Wallis, $P = 0.015$) **(c)** PND 8 fungal β-diversity by litter as measured by Bray-Curtis dissimilarity (PERMANOVA, $R^2 = 0.246$; $P < 0.001$; β-dispersion, $P = 0.705$) **(d)** PND 8 α-diversity by litter as measured by Shannon diversity index (Kruskal-Wallis, $P < 0.001$). Litters were represented by A-D (n=6-8 per litter). Significance was defined as $P \leq 0.05$.

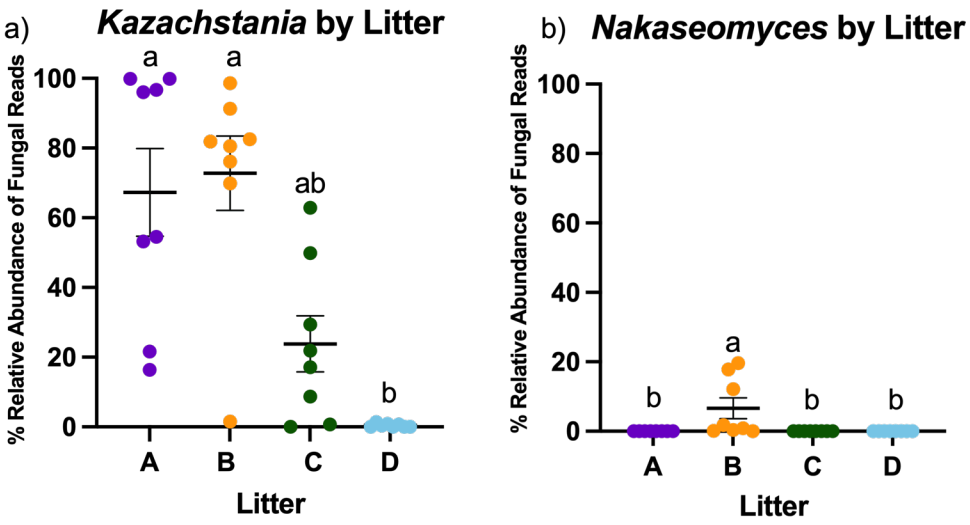

Figure 2.3. Differential fungal taxa between litters. (a) *Kazachstania* (Kruskal-Wallis test, $P < 0.001$) and **(b)** *Nakaseomyces* (Kruskal-Wallis test, $P < 0.001$) by litter on PND 8 (n = 8 per litter). Litters which do not share a letter indicate significance. Significance was defined as $P \leq 0.05$. Error bars represented mean with standard error of the mean.

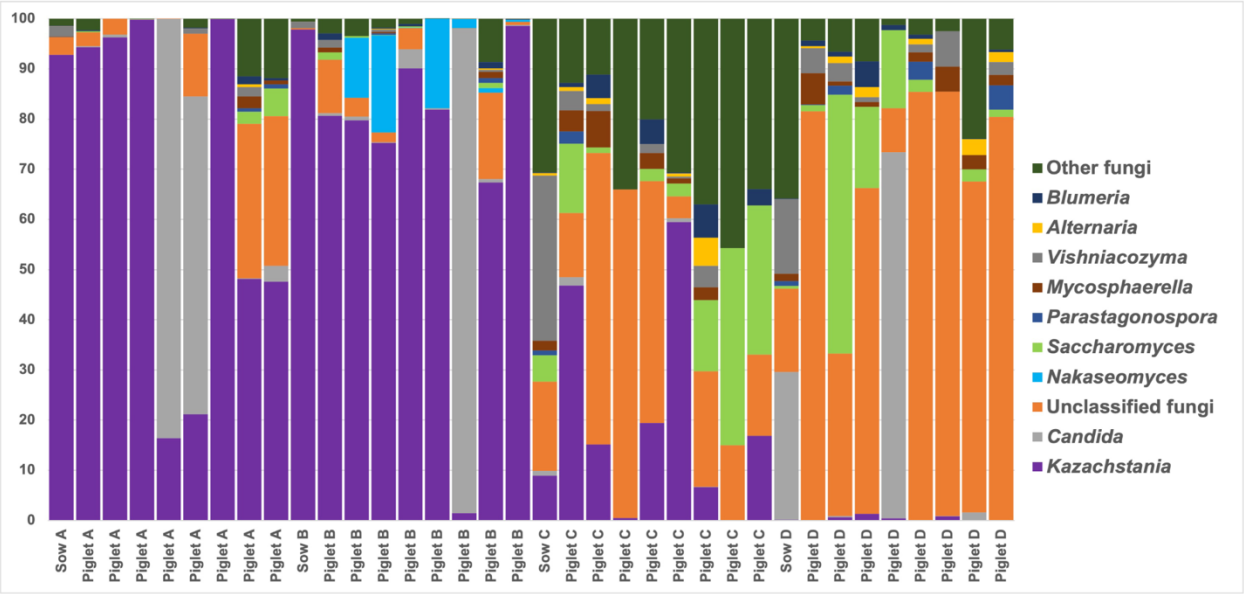

Figure 2.4. Top 11 fungal genera on PND 8. Relative abundance of top 11 fungal genera on PND 8 in piglets by litter (n = 8 per litter). Bars representing sows showed fungal relative abundance one day post farrowing. A-D indicated sow and corresponding litter.

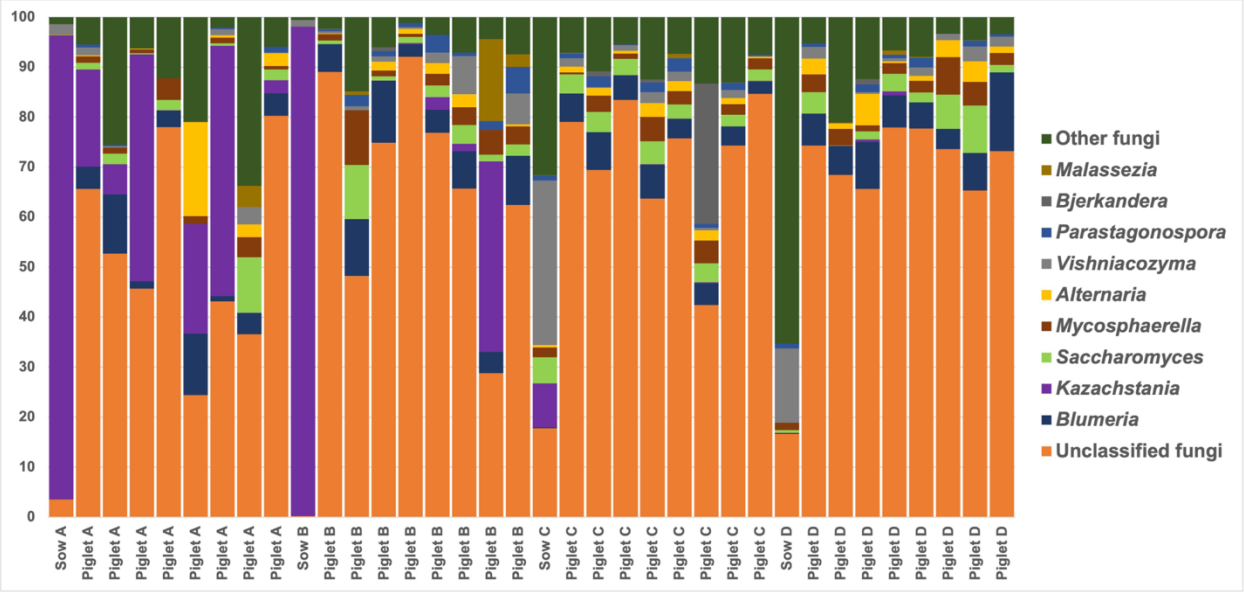

Figure 2.5. Top 11 fungal genera on PND 3. Relative abundance of top 11 fungal genera on PND 3 in piglets by litter (n = 8 per litter). Bars representing sows showed fungal relative abundance one day post farrowing. A-D indicated sow and corresponding litter.

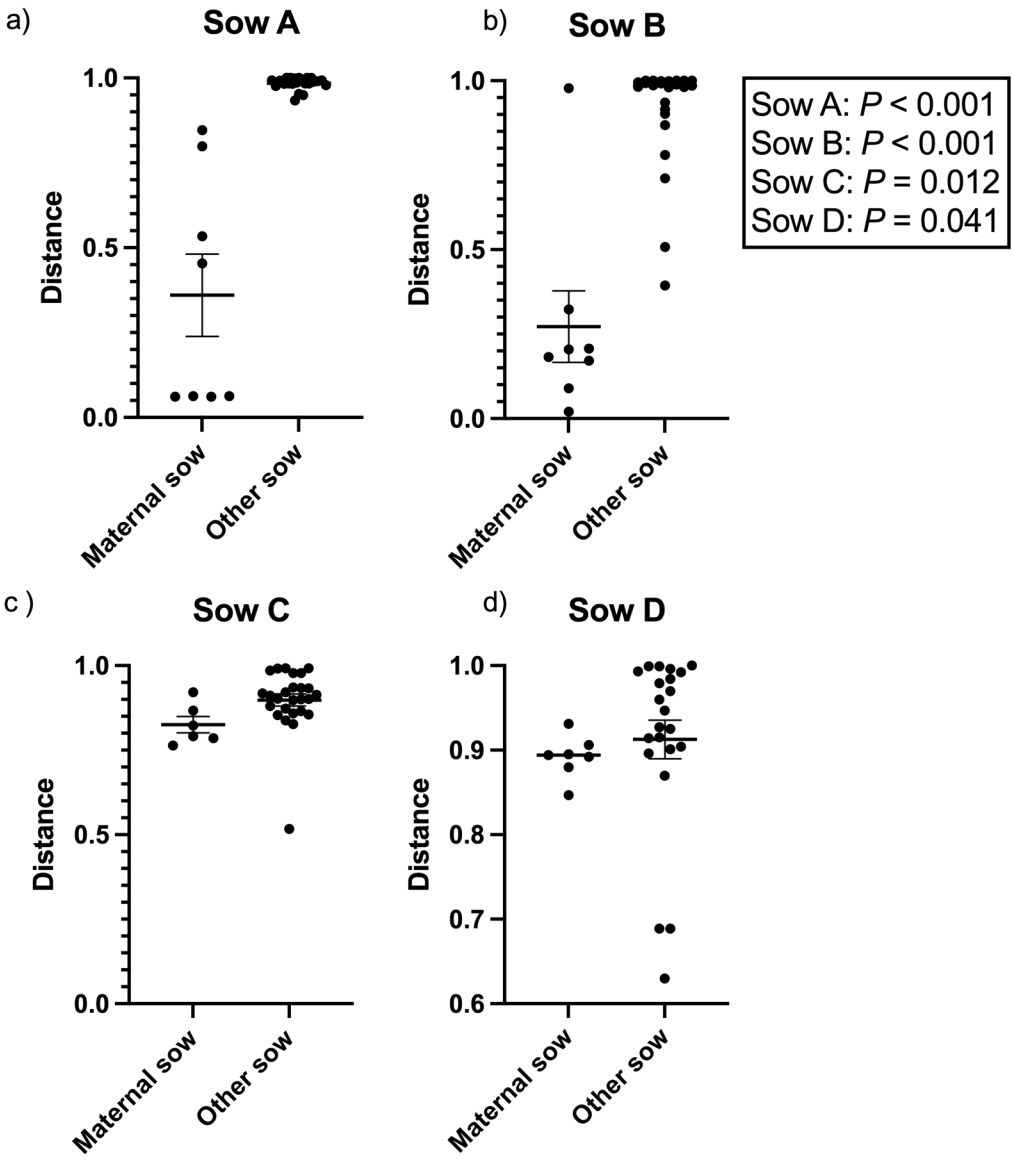

Figure 2.6. Similarity of piglet fungal community to maternal community. Distance from maternal sow vs. distance from a sow of a different litter based on Bray-Curtis dissimilarity of fungal communities on PND 8. Statistical analyses were analyzed by Mann–Whitney U test. Significance was defined as $P \leq 0.05$. Error bars represented mean with standard error of the mean. All sows had 6-8 piglets representing all treatment groups.

2.3.3 Antibiotic treatment impacted bacterial community structure

Antibiotic treatment altered bacterial β-diversity on PND 8 ($R^2 = 0.228$; $P < 0.001$; β-dispersion, $P = 0.706$; Figure 2.7a). However, bacterial α-diversity did not differ following antibiotic treatment on PND 8 ($P = 0.313$; Figure 2.7b). Animals treated with AC had decreased abundance of *Lactobacillus* ($P < 0.001$; Figure 2.8). On PND 3, litter influenced bacterial β-diversity ($R^2 = 0.201$: $P < 0.001$: β-dispersion, $P = 0.187$; Figure 2.9a) but did not impact α-diversity ($P = 0.287$; Figure 2.9b). On PND 8 litter again impacted β-diversity ($R^2 = 0.159$; $P = 0.015$; β-dispersion, $P = 0.521$; Figure 2.9c) but not α-diversity ($P = 0.789$, Figure 2.9d). On PND 8 differences in community structure between litters may be explained in the relative abundance of *Akkermansia* ($P < 0.001$, Figure 2.10). In litters A and B, piglet microbiomes were no closer to their maternal sow than they were to the other sows in the study ($P > 0.05$, Figure 2.11a and b). In litter D piglet microbiomes were closer to their maternal sow than to the sows of other litter ($P = 0.032$; Figure 2.11c). Sequencing results from sow C did not pass quality control and therefore were not included. Unlike mycobiome composition, the bacterial community composition was strongly influenced by antibiotic treatment, though litter effects were still observed.

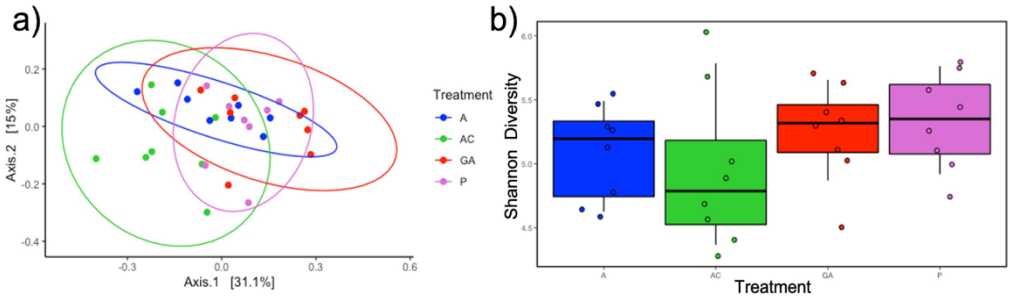

Figure 2.7. Bacterial diversity following antibiotic treatment. Bacterial **(a)** β-diversity (PERMANOVA, $R^2 = 0.228$; $P < 0.001$; β-dispersion, $P = 0.706$) and **(b)** α-diversity as measured by Shannon index on PND 8 after treatment with the antibiotics/placebo including amoxicillin (n= 8) (A), amoxicillin + clavulanic acid (n= 8) (AC), gentamicin + ampicillin (n= 8) (GA), or flavoured placebo (n=8) (P) (Kruskal-Wallis, $P = 0.313$). Significance was defined as $P \leq 0.05$.

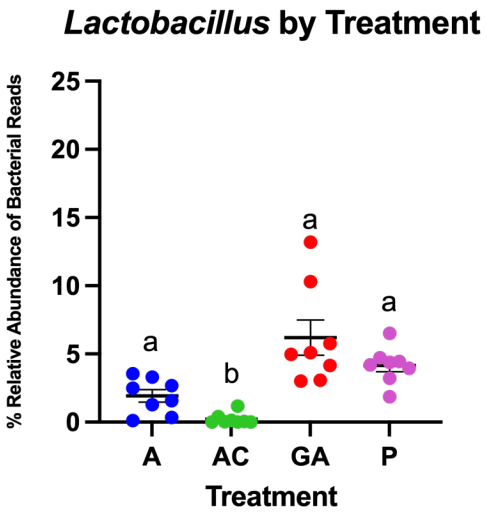

Figure 2.8. Relative abundance of *Lactobacillus* on PND 8 after treatment with antibiotics. Amoxicillin (A), amoxicillin + clavulanic acid (AC), gentamicin + ampicillin (GA), or flavoured placebo (P) (Kruskal-Wallis, $P < 0.001$). Significance was defined as $P \leq 0.05$. Error bars represented mean with standard error of the mean. a, b: Litters not sharing a letter were significantly different.

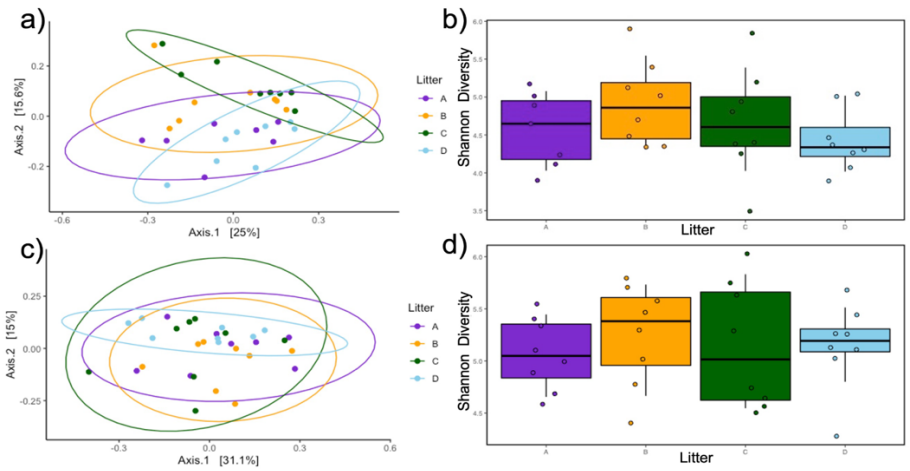

Figure 2.9. Bacterial diversity by litter. **(a)** PND 3 bacterial β-diversity by litter as measured by Bray-Curtis dissimilarity (PERMANOVA, $R^2 = 0.227$; $P < 0.001$; β-dispersion = 0.501) **(b)** PND 3 bacterial α-diversity by litter as measured by Shannon index (Kruskal-Wallis, $P = 0.287$) **(c)** PND 8 bacterial β-diversity by litter as measured by Bray-Curtis dissimilarity (PERMANOVA, $R^2 = 0.159$; $P = 0.015$; β-dispersion = 0.521) **(d)** PND 8 α-diversity by litter as measured by Shannon index (Kruskal-Wallis, $P = 0.789$). Significance was defined as $P \leq 0.05$. Litters were defined by A-D and all litters had 7-8 piglets.

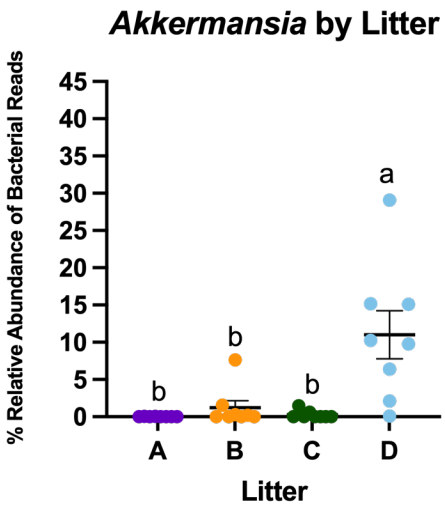

Figure 2.10. Differential bacterial genus by litter. Relative abundance of *Akkermansia* on PND 8 by litter (n= 8 per litter) (Kruskal-Wallis, $P < 0.001$). Significance was defined as $P \leq 0.05$. Error bars represented mean with standard error of the mean. Litters that do not share a letter were significantly different.

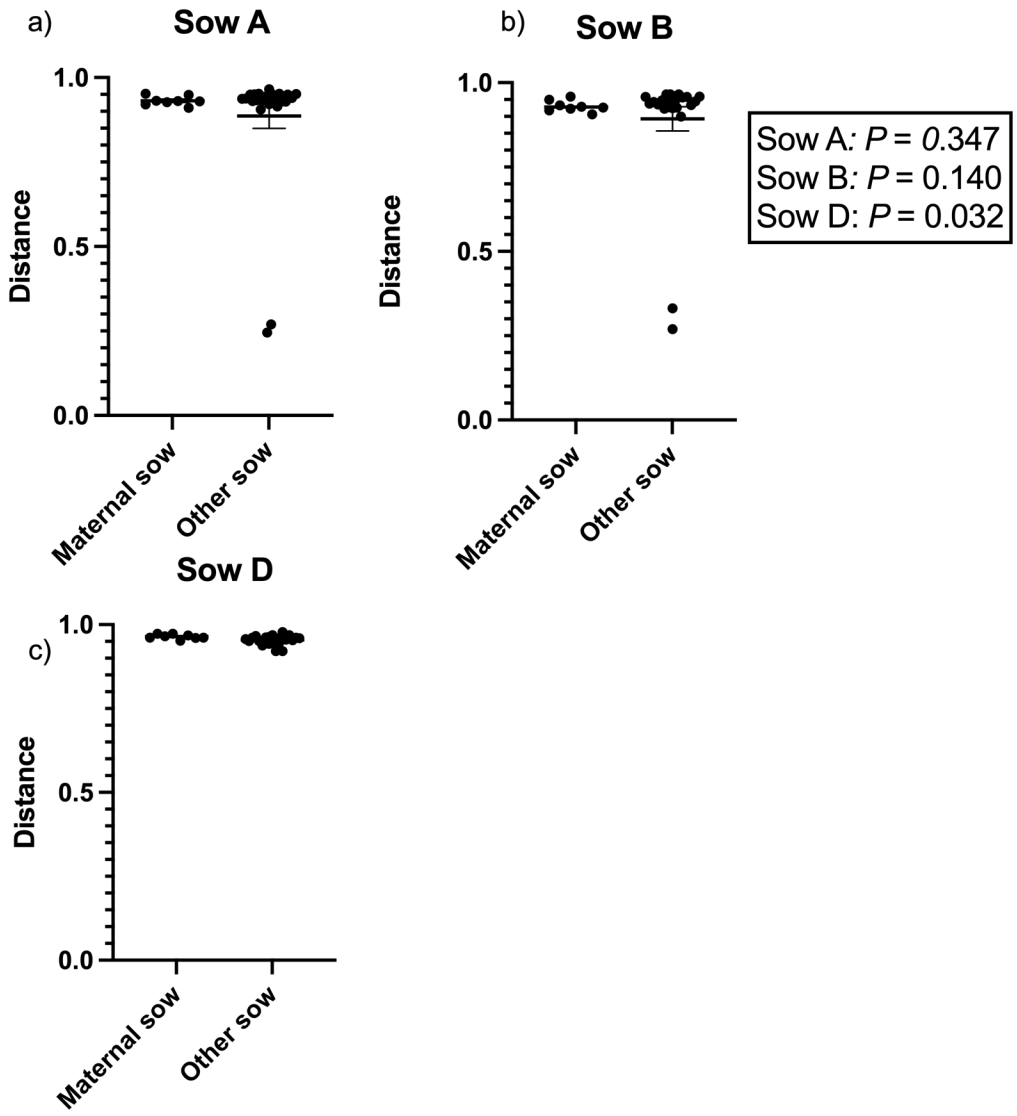

Figure 2.11. Similarity of piglet bacterial community to fungal community. Distance from maternal sow vs. distance from a sow of a different litter based on Bray-Curtis dissimilarity and measured using a Mann–Whitney U test, of bacterial communities on PND 8. Significance was defined as $P \leq 0.05$. Error bars represented mean with standard error of the mean. All sows had 6-8 piglets representing all treatment groups.

2.3.4 Antibiotic treatment increased ratio of total fungi to total bacteria

Total bacterial load did not change with antibiotic treatment ($P = 0.325$; Figure 2.12a), however, total fungi tended to be impacted by antibiotic treatment ($P = 0.083$; Figure 2.12b). As a result, antibiotic treatments caused a significant change in the ratio of total fungi to total bacteria ($P = 0.001$, Figure 2.12c). Post-hoc tests revealed that treatment with AC increased the ratio of total fungi to total bacteria compared to placebo ($P = 0.027$; Figure 2.12c).

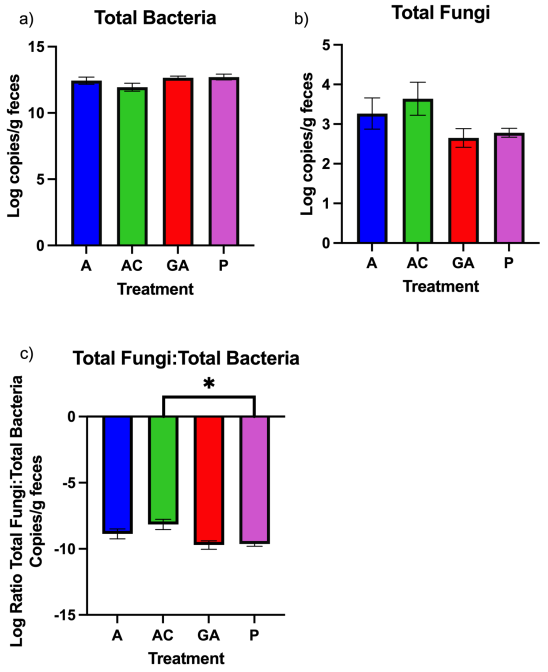

Figure 2.12. Fungal and bacterial loads. (a) Total bacteria qPCR by treatment (PROC MIXED, $P = 0.325$). **(b)** Total fungi qPCR by treatment (PROC MIXED, $P = 0.083$). **(c)** Ratio of total fungi to total bacterial load (PROC MIXED, $P = 0.001$). A significant reduction in the ratio of total fungi to total bacteria was detected between P and AC (Bonferroni, $P = 0.027$). Significance was indicated by * and was defined as $P \leq 0.05$. P (n = 5), A (n = 7), AC (n = 8), GA (n = 8). Error bars represented mean with standard error of the mean.

2.4 Discussion

In this study we sought to determine the impact of common early life antibiotic treatments on both fungal community composition and load. Although antibiotic treatments altered bacterial community composition on PND 8, we did not observe any changes to fungal community composition (Figure 2.1a). While it has been well documented that antibiotic treatments can cause *Candida* overgrowth, thus leading to an altered fungal community composition, there appears to be a great deal of individual variation in susceptibility to *Candida* overgrowth (40). This is due to a variety of factors including genetics (41), intestinal metabolite profile (19), and host immune status (42). One recent study noted that the human mycobiome was altered by antibiotic administration to the greatest degree one month post antibiotic treatment, which suggests that there may be a delayed response following antibiotic treatment (43). Therefore, it is possible that the pigs used in the present study were either not susceptible to fungal dysbiosis following antibiotic treatments or that there was a delayed change in fungal community composition that was outside of the study window. While pigs share some similarities in microbes, such as *Candida*, it may be possible that strain-level differences between humans and pigs may account for the lack of community change in response to antibiotic treatment.

The numerical reduction of total bacteria and trend of increasing total fungi following treatment with AC resulted in an increase in the ratio of total fungi to total bacteria in AC-treated animals (Figure 2.12c). Amoxicillin + clavulanic acid is a commonly used antibiotic to treat respiratory tract infections during the first year of life (44). Amoxicillin is a β-lactam antibiotic which is effective against both Gram-positive and Gram-negative bacteria, including *Escherichia*

coli and *Salmonella* species (45). However, over time many strains of bacteria have developed resistance to *β*-lactams through the production of *β*-lactamase. To overcome this resistance, clavulanic acid is frequently added to amoxicillin as it acts as a *β*-lactamase inhibitor (46). While we did not see a statistically significant difference in the abundance of total bacteria following treatments with any of the antibiotics in the present study, there does appear to be a slight decrease in the abundance of total bacteria in pigs treated with AC (Figure 2.12a). This may suggest that *β*-lactam resistance was present. The combination of gentamicin + ampicillin is commonly used to treat neonatal sepsis and is commonly prescribed for a two-day period (25). However, the short treatment duration may be responsible for the lack of reduction in bacterial load in this treatment group. In the present study, the lack of significant reduction in total bacteria may be the reason that we didn't see an increase in total fungi as expected. Additionally, we saw a decrease in the abundance of *Lactobacillus* following treatment with AC. The presence of *C. albicans* has been shown to result in a long-term reduction of *Lactobacillus* in the gut (47). Since only a few of the piglets in the present study had *Candida* present we were not able to say whether *Candida* increased following antibiotic treatment, however it is possible that the increased ratio of total fungi to total bacteria is promoting an environment in which *Lactobacillus* is suppressed.

Interestingly, litter effect was the main driver of fungal community composition in the current study rather than antibiotic exposure. Using Bray-Curtis dissimilarity, it was noted that on PND 8 piglets had mycobiomes that more closely resembled their maternal sow mycobiome than the mycobiome of other sows. This suggests that maternal fungal colonization is the driving factor in piglet mycobiome development. These results are contrary to what is seen in human infants, with one study observing that infants in their first month of life were no more similar to

their own mothers than to a randomly selected mother, suggesting external environment plays a larger role versus maternal mycobiome in mycobiome composition (47). Compared to human infants, piglets have more exposure to maternal feces due to the environment in which they live. Piglets engage in coprophagy of these feces, with one study showing a rate of consumption of sow feces of approximately 20g/day (48). Coprophagy has been shown to increase piglet feed intake, weight gain, and white blood cell count compared to piglets deprived of maternal feces in the first 7 post-natal days (49). These differences may be due to the acquisition of microbes from the sow via coprophagy. Therefore, it is possible that compared to humans, piglets are obtaining more of their mycobiome from their mother's feces. However, maternal bacterial colonization did not drive piglet bacterial colonization in the same way in the present study.

On PND 8 *Nakaseomyces* was present in litter B in all 8 piglets, ranging from 0.009% to 19.4% of the fungal community, but was absent from other litters (Figure 2.3b). Sow B did not have any *Nakaseomyces* present. *Nakaseomyces* is the name given to a clade of pathogenic *Candida* species which includes *Candida glabrata*, *Candida nivariensis*, and *Candida bracarensis* (50).The drivers behind *Nakaseomyces* detection in litter B remain unclear, and no clinical illness was observed in these piglets. The other fungal species responsible for differences in community structure on PND 8 was *Kazachstania* (Figure 2.3a). *K. slooffiae* is one of the most abundant fungi in pigs, and is present in pigs reared under varying conditions and in different locations (51, 52). *K. slooffiae* is thought to be commensal and several benefits to the host have been noted including increased short-chain fatty acid production, symbiotic relationships with beneficial bacteria such as *Lactobacillus*, and it possesses a favorable amino acid profile for pig growth (5, 53-55). Piglets' levels of *Kazachstania* colonization reflected the levels of *Kazachstania* in their respective sows, which was highly variable. Given the potential

benefits of *Kazachstania* colonization, future work should focus on the long-term health outcomes in animals with low colonization levels during the early life period as well as the impact of maternal *Kazachstania* colonization on piglet health outcomes.

A litter effect was also present in bacterial communities. In this case, on PND 8 it appeared to be driven by *Akkermansia*. It is worth noting that *Akkermansia* was the most abundant in litter D, which was also the litter with very low *Kazachstania* colonization. One previous study has noted a potential interaction between *Akkermansia* and yeast fermentate (56). Ducray et al. (2019) found that supplementing rats with a yeast fermentate prebiotic from *Saccharomyces cerevisiae* prevented a heat stress associated rise in *Akkermansia*. However, it is unclear what effect live yeast would have on *Akkermansia* colonization and merits further exploration.

2.5 Conclusion

In conclusion, antibiotic treatment altered bacterial but not fungal community composition. However, AC was found to increase the ratio of total fungi to total bacteria load in fecal content. It was found that the maternal mycobiome played a major a role in shaping the piglet mycobiome and a strong litter effect was observed on fungal communities on PND 8. This research indicates that the mycobiome of piglets is highly variable and dependent on litter of origin, and that future research of the piglet mycobiome should account for variations between litters.

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Chapter 3: Tracking the fecal mycobiome through the lifespan of production pigs and a comparison to the feral pig

3.1 Introduction

The gastrointestinal tract (GIT) is home to a vast array of microbes including bacteria, archaea, viruses, protozoa and fungi (1). While fungi make up less than 0.1% of total microbial reads in the human GIT (1), fungi contribute to host health and development and have been shown to be immunomodulatory (2, 3). The collection of fungi in the GIT is termed the mycobiome and although it has gained increased attention in recent years is largely understudied.

In both humans and pigs, yeast are the most common fungi in the GIT (4, 5). In pigs, the mycobiome has been well profiled up to 35 days of age (5-7). However, studies tracking the fungal community of a pig through the production cycle have not been completed. We have previously found that the maternal mycobiome drives piglet mycobiome assembly (8). Specifically, we found that differences in fungal community structure between litters were driven by sow *Kazachstania slooffiae* abundance (8). *K. slooffiae* is a commensal yeast found in pigs across geographic locations and under different types of production systems (9-12). Previous studies have found that *K. slooffiae* is most abundant in post-weaning pigs (5, 7, 13), although we have previously noted the presence of *K. slooffiae* in pigs as young as 3 days of age (8). *K. slooffiae* may contribute to pig health by altering intestinal bacteria, increasing short chain fatty acids in feces, and acting as a source of amino acids (especially lysine) and vitamin C (14, 15).

There is also a recent growing interest in "wild" microbiomes as a result of their potential to protect against disease. The feral pig therefore represents an interesting comparison for the identification of organisms that may have been lost as a result of high levels sanitation in

conventional pig production. Domestic wild boar were originally imported to Canada in the 1980s for livestock diversification where most animals were cross-bred with domestic pigs and in some cases with mini-pigs and pot-bellied pigs (16). Since then, they have escaped or have been released from the farms and these feral pigs are now free-ranging across over one million km^2 of Canada (17, 18). Feral pigs now roam the Canadian prairies where they wreak havoc on crop land and pose a very concerning disease risk to domestic pigs (19-21). While sanitation practices help to prevent disease among pigs, there may also be alterations in the bacterial and fungal communities. Indeed, one study has shown that exposure to soil is able to accelerate maturation of the bacterial community (22). It is not currently known how domestication affects the pig mycobiome.

Drawing on previous research, we can assume that *K. slooffiae* provides at least some benefit to the pig. While *K. slooffiae* is an important member of the mycobiome, there are other fungi that may also contribute to host health. However, it is unclear how the mycobiome changes over a production cycle, nor is it clear how the domestic pig may differ when compared to feral pigs in terms of the mycobiome. In this study we seek to understand these questions by: 1) tracking the pig mycobiome through the lifespan (production cycle), including the sows; 2) profiling the mycobiome of feral pigs; and 3) profiling the mycobiome of sows raised in a commercial facility.

3.2 Materials and methods

3.2.1 Animal use and care

This animal study was approved by the Animal Care and Use Committee of the University of Alberta (Edmonton, AB, CAN) and conducted in accordance with the guidelines of

the Canadian Council on Animal Care under AUP00002214. Feral pig capture and handling protocols were approved by the University of Saskatchewan Animal Research Ethics Board under AUP20150024 and the Saskatchewan Ministry of Environment under permit 17FW027. A total of 36 pigs were used from the Swine Research and Technology Center (SRTC) at the University of Alberta (Edmonton, AB, CAN). The pigs at SRTC represent pigs raised under experimental farm conditions, although they were raised with commercial production practices. Sows (n=12; Large White/Landrace) had fecal samples taken 3 days after farrowing. Piglets (n=24; Large White/Landrace x Duroc) had fecal swabs taken at 11 days of age (D11), 1 day prior to weaning (W-1), 7 days post weaning (W+7), and 119 days post weaning (W+119). W+119 represents a pig at market weight and the end of a production cycle. As we previously documented the pig mycobiome up to 8 days of age (8), we opted to focus on time points after 8 days of age. 2 piglets, whose weights were close to the median litter weight, were selected per sow to follow through the trial. Piglets were housed with their mother and littermates until 21 days of age when weaning occurred. Following weaning, piglets were housed with only their litter mates until 4 weeks following weaning when they were housed in groups separate from any of their litter mates. 5 pigs in the study received antibiotics (penicillin) prior to D11 due to an outbreak of scours in the farrowing room. Piglets were introduced to creep feed at 14 days of age. Fecal samples were taken from additional sows (n=8; Large White) from a commercial farm 3 days after farrowing for mycobiome analysis. Feral pigs were captured from 2 locations: Moose Mountain and Melfort, Saskatchewan, Canada. Pigs were located via helicopter and captured with a net gun. Following capture, the pigs were euthanized via captive bolt, and intestinal contents collected. Ileum content was collected 5 cm from the ileocecal junction and cecal content was collected from the tip of the cecum. A total of 12 ileum samples and 12 cecal

samples were selected for mycobiome analysis, of which 4 pigs had both ileum and cecum samples sequenced. The selection of feral pig samples was limited by availability as remaining samples had been utilized for a previous study.

3.2.2 DNA extraction

Total genomic DNA was extracted using the DNeasy PowerSoil Pro kit (Qiagen®, CA, USA) with no modifications. Bead beating was performed on a FastPrep-24™ (MP Biomedicals, OH, USA) homogenizer at 5 m/s for 45 seconds.

3.2.3 Fungal sequencing

Internal transcribed spacer (ITS) 2 sequencing was performed at Microbiome Insights (Richmond, BC, CAN). Sequencing was done using the following primers: forward (ITSF) 5'-CCTCCGCTTATTGATATGC-3' and reverse (ITSR) 5'-CCGTGARTCATCGAATCTTTG-3'. Paired-end sequencing was done on the Illumina MiSeq platform (Illumina, CA, USA) using 2 x 300 cycles.

Sequencing analysis was performed using Quantitative Insight into Microbial Ecology (QIIME) 2 (v2022.11) (23). Only forward reads were used and reads were truncated at 240 base pairs. The Divisive Amplicon Denoising Algorithm (v2) plugin was used to perform demultiplexing, quality filtering, denoising, and for filtering out chimeras (24). Mafft was used to align Amplicon Sequence Variants (ASVs) (25). Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomic classifier (via the q2-feature-classifier plugin) (26), and the UNITE database (v8.4) was used (27).

3.2.4 Statistical analysis

K. slooffiae level in sows was broken into low, medium, and high by assigning levels at natural breaks, with low being ≤ 10% total reads (n=7), medium being the lone sow at 35% and high being ≥ 70% (n=4). Random sow was found using the randomize function in Excel. Differences in the percentage of *K. slooffiae* and the distance to maternal sow versus a random sow was calculated using a Mann-Whitney U test in GraphPad Prism 9.5.1 based on Bray-Curtis distance. Phyloseq (v1.34.0) was used in R to analyze microbial community structure and diversity (28). Differences in fungal community composition were measured using Bray-Curtis dissimilarity and Permutational Multivariate Analysis of Variance (PERMANOVA), and was visualized using Principal Coordinates Analysis (PCoA) (R, v4.0.5). Homogeneity of dispersion was measured using Betadisper function in phyloseq (28). α-diversity was calculated in QIIME (v2022.11) and was statistically analyzed using Kruskal-Wallis in in GraphPad Prism 9.5.1 with Dunn's multiple comparisons. Prior to α-diversity analysis reads were rarefied to 1700 reads. Differential abundance was calculated using Analysis of Composition of Microbiomes (ANCOM) in QIIME (v2022.11) and were compared using a Kruskal-Wallis test with Dunn's multiple comparisons in GraphPad Prism 9.5.1. Differences in average daily gain (ADG) were compared using a Kruskal-Wallis test with Dunn's multiple comparisons in GraphPad Prism 9.5.1. Correlations between pig *K. slooffiae* and weight were computed using a Spearman's correlation in GraphPad Prism 9.5.1.

3.3 Results

3.3.1 Sow *K. slooffiae* colonization level shapes piglet mycobiome during early and later life, but not around the weaning transition

Levels of *K. slooffiae* varied amongst sows. All but one of the sows fell into one of two categories: ≤ 10% *K. slooffiae* (n=7) or ≥ 70% *K. slooffiae* (n=4). One sow had 35% *K. slooffiae*.

On D11, piglets tended to cluster depending on if the sows were colonized with high, medium or low levels of *K. slooffiae* (Figure 3.1a, $P = 0.087$, β-dispersion $P = 0.181$). By W-1 piglets no longer clustered based on their sows *K. slooffiae* status (Figure 3.1b, $P = 0.297$, β-dispersion $P = 0.726$). On W+7, sow *K. slooffiae* status also made no difference to the mycobiome (Figure 3.1c, $P = 0.859$, β-dispersion $P = 0.509$). However, by W+119, sow *K. slooffiae* status influenced pig mycobiome composition (Figure 3.1d, $P = 0.046$, β-dispersion $P = 0.278$). There was an effect of day on the percentage of *K. slooffiae* present in the mycobiome ($P < 0.001$). On day 11, piglets had higher mean *K. slooffiae* than on W+7 (Figure 3.2, $P < 0.001$) and sows had more *K. slooffiae* than piglets on W+7 (Figure 3.2, $P = 0.013$).

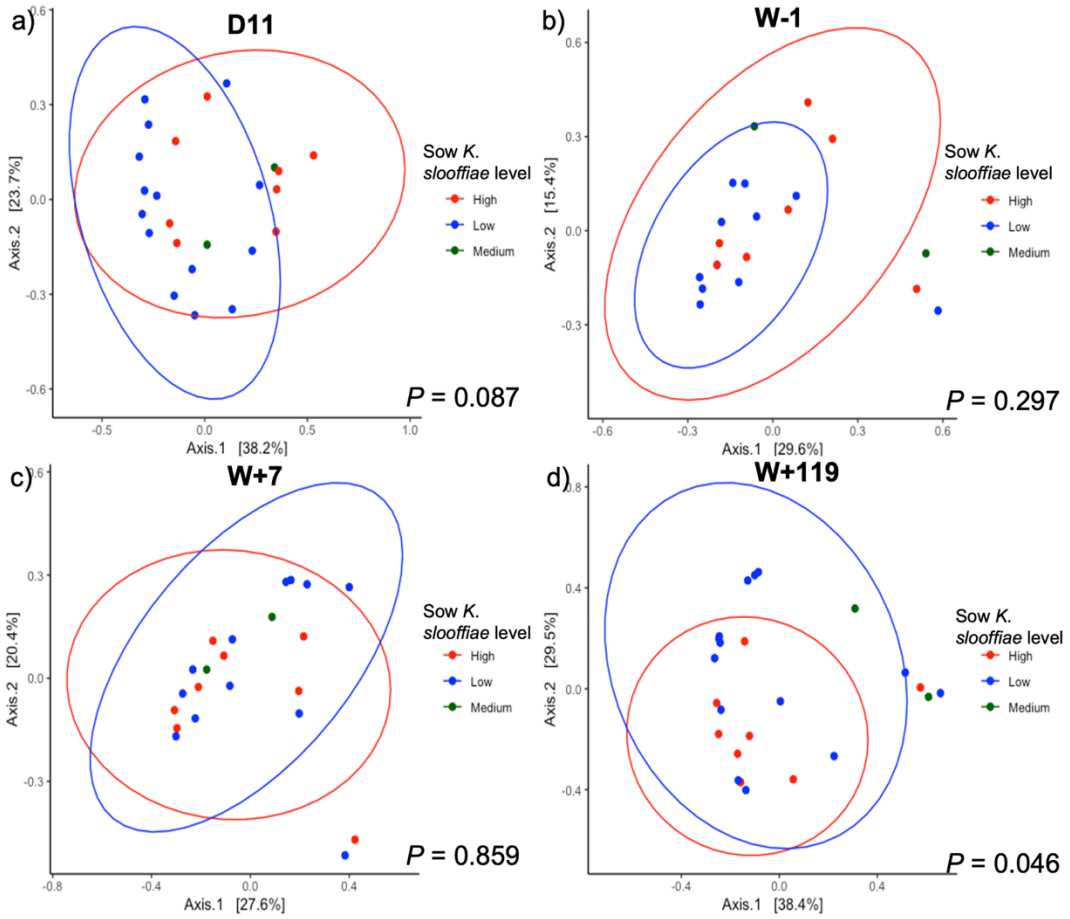

Figure 3.1. β-diversity based on Bray-Curtis dissimilarity of the mycobiome based on sow *Kazachstania slooffiae* level. **(a)** Piglets 11 days of age (D11) showed a trend in clustering by sows *K. slooffiae* status ($P = 0.087$, β-dispersion $P = 0.181$). **(b)** Piglets on the day before weaning (W-1) did not cluster by sows *K. slooffiae* status ($P = 0.297$, β-dispersion $P = 0.726$). **(c)** Pigs 7 days following weaning (W+7) did not cluster by sow *K. slooffiae* status ($P = 0.859$, β-dispersion $P = 0.509$). **(d)** At 119 days following weaning, i.e. the end of a production cycle, pigs clustered by their mothers *K. slooffiae* status ($P = 0.046$, β-dispersion $P = 0.278$). Trends were defined as $P < 0.1$, and significance was defined as $P \leq 0.05$.

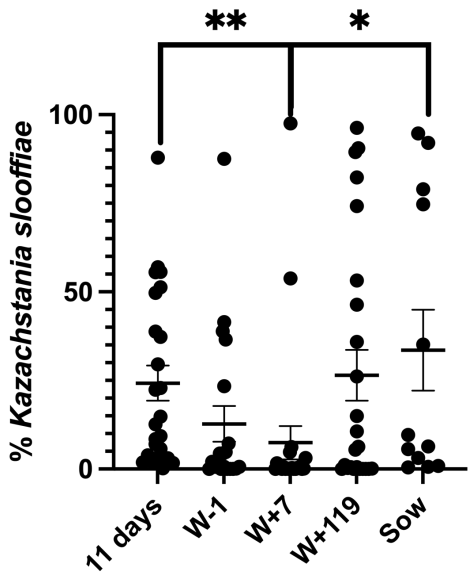

Figure 3.2. Percentage of *K. slooffiae* at different time points across a production cycle. Data is presented as the mean at each time point +/- SEM. There was a significant impact of time on percentage of *K. slooffiae* ($P < 0.001$). * indicates $P \leq 0.05$ and ** indicates $P < 0.001$. Significance was defined as $P \leq 0.05$.

The amount of *K. slooffiae* a sow was colonized with did not correlate with the weight of the piglets on D11 ($P = 0.903$, r = -0.0263), W-1 ($P = 0.720$, r = -0.0852) W+7 ($P = 0.783$, r = -0.0606) or W+119 ($P = 0.945$, r = -0.0148). The amount of *K. slooffiae* a pig was colonized with did not associate with pig weight on D11($P = 0.703$, r = -0.0821), W-1 ($P = 0.163$, r = -0.3149), W+7 ($P = 0.140$ r = -0.3171) or W+119 ($P = 0.574$, r = -0.121).

3.3.2 Piglets cluster by litter except on the day before weaning

Piglets clustered by litter on D11 (Figure 3.3a, $P = 0.006$). Around the weaning transition (W-1), piglets no longer clustered by litter (Figure 3.3b, $P = 0.184$). By W+7, piglets tended to cluster by litter (Figure 3.3c, $P = 0.087$). On W+119, piglets clustered by litter (Figure 3.3d, $P =$

0.007). On D11 piglets were closer to their maternal sow than a random sow (Figure 3.4a, $P < 0.001$), however, on W-1, W+7, and W+119 there was no difference between the distance to the maternal sow and a random sow (Figure 3.4b, $P = 0.418$; Figure 3.4c, $P = 0.116$; Figure 3.4d, $P = 0.511$).

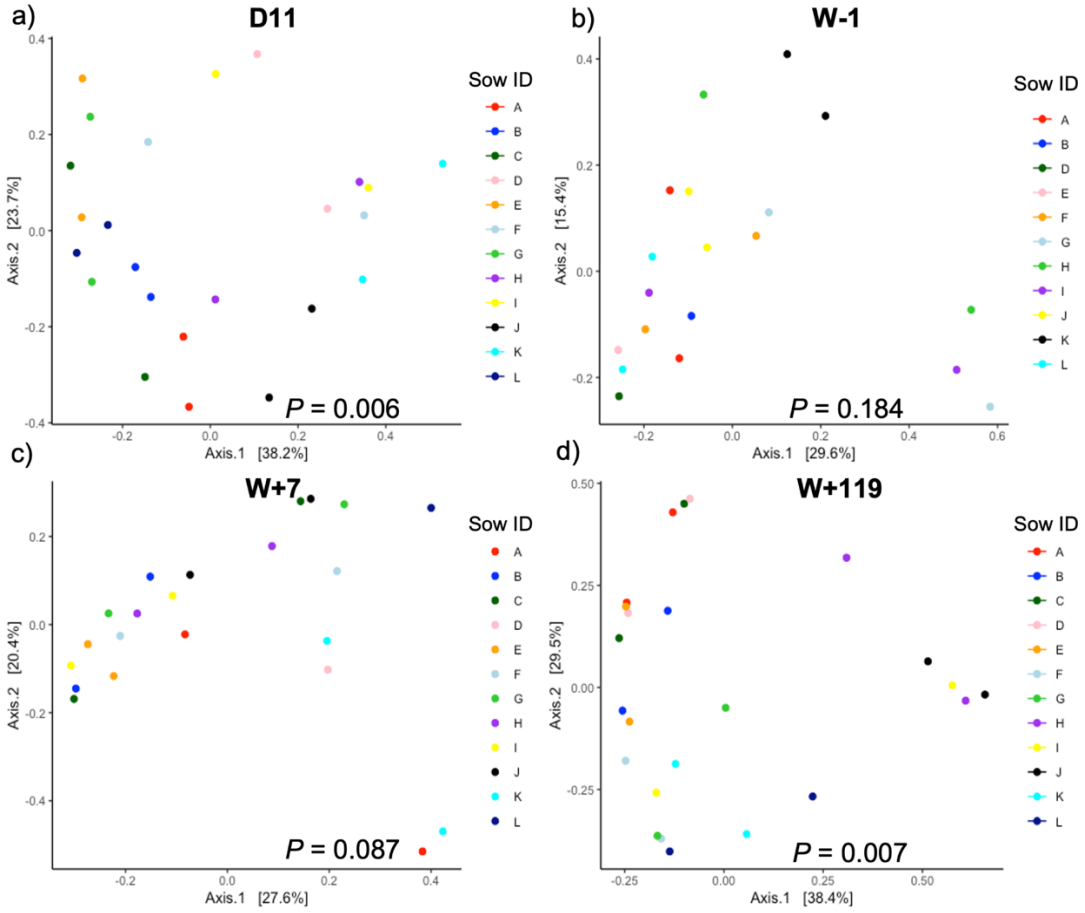

Figure 3.3. β-diversity of pigs by sow based on Bray-Curtis dissimilarity. **(a)** Piglets at 11 days of age (D11) clustered with their littermate ($P = 0.006$, β-dispersion $P < 0.001$). **(b)** Pigs on the day prior to weaning (W-1) no longer clustered by sow ID ($P = 0.184$, β-dispersion $P = 1.0$). **(c)** Pigs at 7 days following weaning (W+7) tended to cluster by sow ID ($P = 0.087$, β-dispersion $P < 0.001$). **(d)** Pigs 119 days following weaning (W+119) clustered by sow ID ($P = 0.007$, β-dispersion $P < 0.001$).

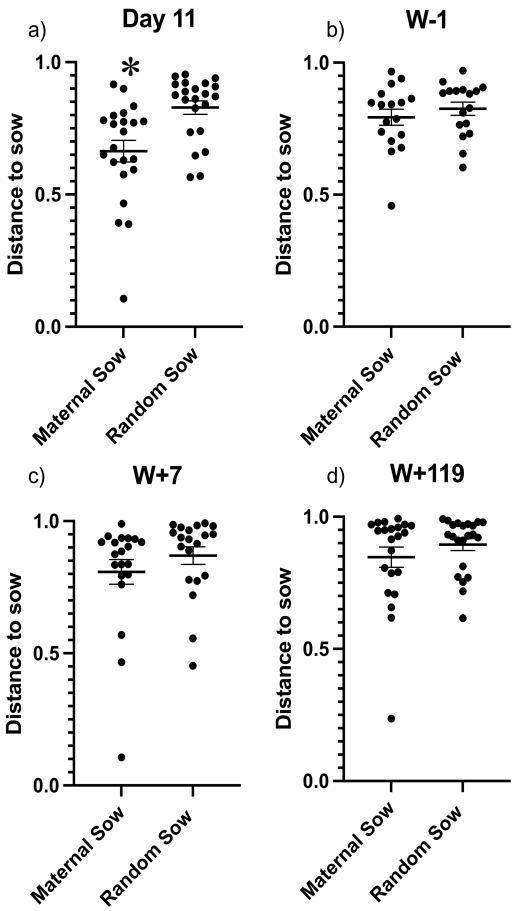

Figure 3.4. Distance of piglets to their maternal sow versus the distance to a randomly selected sow. (a) On D11 piglets were closer to their maternal sow than they were to their a randomly selected sow ($P < 0.001$). **(b)** On the day before weaning piglets were no closer to their maternal sow than to a randomly selected sow ($P = 0.418$). **(c)** 7 days after weaning, pigs were no closer to their maternal sow than to a randomly selected sow ($P = 0.116$). **(d)** At 119 days following weaning, pigs were no closer to their maternal sow than to a randomly selected sow ($P = 0.511$). Data is presented as mean +/- SEM. Significance was defined as $P \leq 0.05$. * indicates $P < 0.001$.

3.3.3 Both α-diversity and β-diversity were altered by pig age

Mycobiomes clustered based on time point (Figure 3.5a, $P = 0.001$, β-dispersion $P = 0.253$), however, there was no clear distinction based on younger or older pigs, with no clear break at weaning. α-diversity decreased with time (Figure 3.5b, $P < 0.001$). For the 5 pigs that receive antibiotics no apparent effect on mycobiome β-diversity at any timepoint was observed ($P = 0.359$).

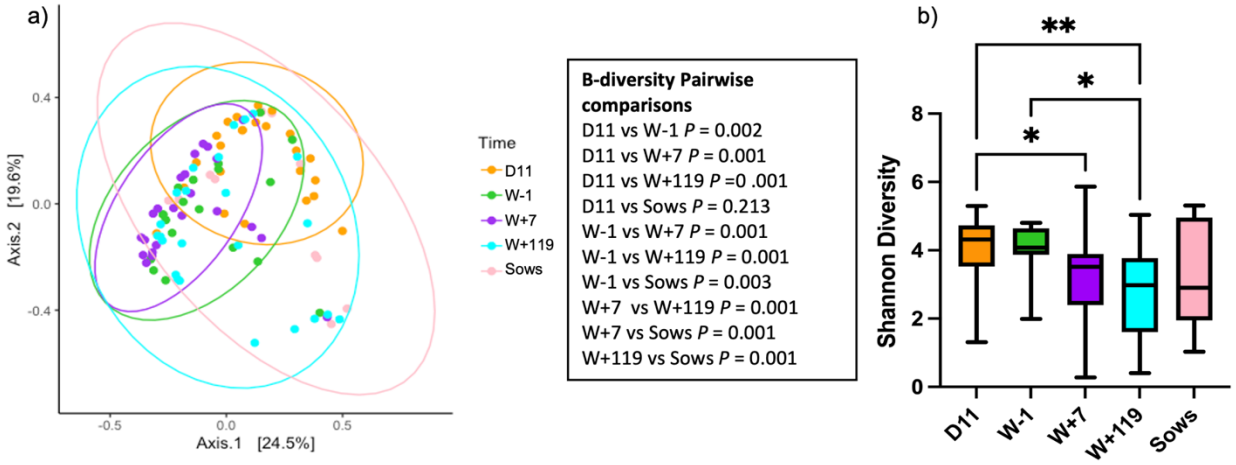

Figure 3.5. β-diversity and α-diversity of the mycobiome over time. (a) β-diversity of pigs at all time points as measured by Bray-Curtis dissimilarity. Pigs clustered based on time point ($P = 0.001$, β-dispersion $P = 0.253$). **(b)** α-diversity of pigs at different timepoints as measured by Shannon diversity index ($P < 0.001$). * indicates $P \leq 0.05$ and ** indicates $P < 0.001$. Box and whisker plot shows mean with 95% confidence interval. Significance was defined as $P \leq 0.05$.

3.3.4 K. slooffiae is more prevalent in intensively raised pigs than feral pigs

K. slooffiae was among the 10 most dominant fungi at all time points in the experimental piglets and in experimental sows (Figure 3.6a-d and Figure 3.7a). In commercial sows, *K. slooffiae* was the most dominant fungi (Figure 3.7b). While *K. slooffiae* was present in all feral pigs in both gut sections, it was not among the 10 most dominant fungi (Figure 3.7c and d). The mycobiome of feral pigs was more complex than that of both experimental and domestic pigs. Following singleton removal, there were a total of 224 genera in experimental pigs, 123 genera in commercial pigs, and 280 genera in feral pigs. Additionally, feral pigs clustered separately from commercial and experimental pigs (Figure 3.8c, $P = 0.002$, β-dispersion $P = 0.01$). ANCOM revealed several differential genera between experimental, commercial, and feral pigs: *Kazachstania*, *Saccharomyces*, *Aspergillus*, *Monilia*, *Kalmanozyma*, *Xeromyces*, *Naganishia*, *Hyphopichia*, and *Diutina*. *Kazachstania* and *Saccharomyces* were more abundant in both commercial and experimental pigs than feral pigs (Figure 3.8a, $P < 0.01$) and there was no difference between experimental and commercial pigs (Figure 3.8a, $P = 0.90$). *Aspergillus* was more abundant in commercial pigs than in feral pigs (Figure 3.8b, $P < 0.001$) and not in commercial versus experimental or in experimental versus feral (Figure 3.8b, $P = 0.052$ and $P = 0.08$). *Monilia* was only present in commercial pigs and was therefore significantly higher in this group than in experimental or feral pigs (Figure 3.8b, $P < 0.001$). *Kalmanozyma* and *Xeromyces* were only found in commercial and experimental pigs and not in feral pigs, and was therefore more abundant in these groups (Figure 3.8b, $P < 0.001$). *Naganishia* was more abundant in commercial pigs than in feral pigs and in commercial versus experimental pigs (Figure 3.8b, $P < 0.01$) but not in experimental versus feral pigs (Figure 3.8b, $P = 0.327$). *Hyphopichia* was more abundant in commercial pigs than in experimental or feral pigs (Figure 3.8b, $P < 0.001$) but not

in experimental versus feral (Figure 3.8b, $P > 0.999$). Finally *Diutina* was more abundant in commercial pigs than in experimental or feral pigs (Figure 3.8b, $P < 0.001$ and $P = 0.01$) but not in experimental versus feral pigs (Figure 3.8b, $P = 0.222$).

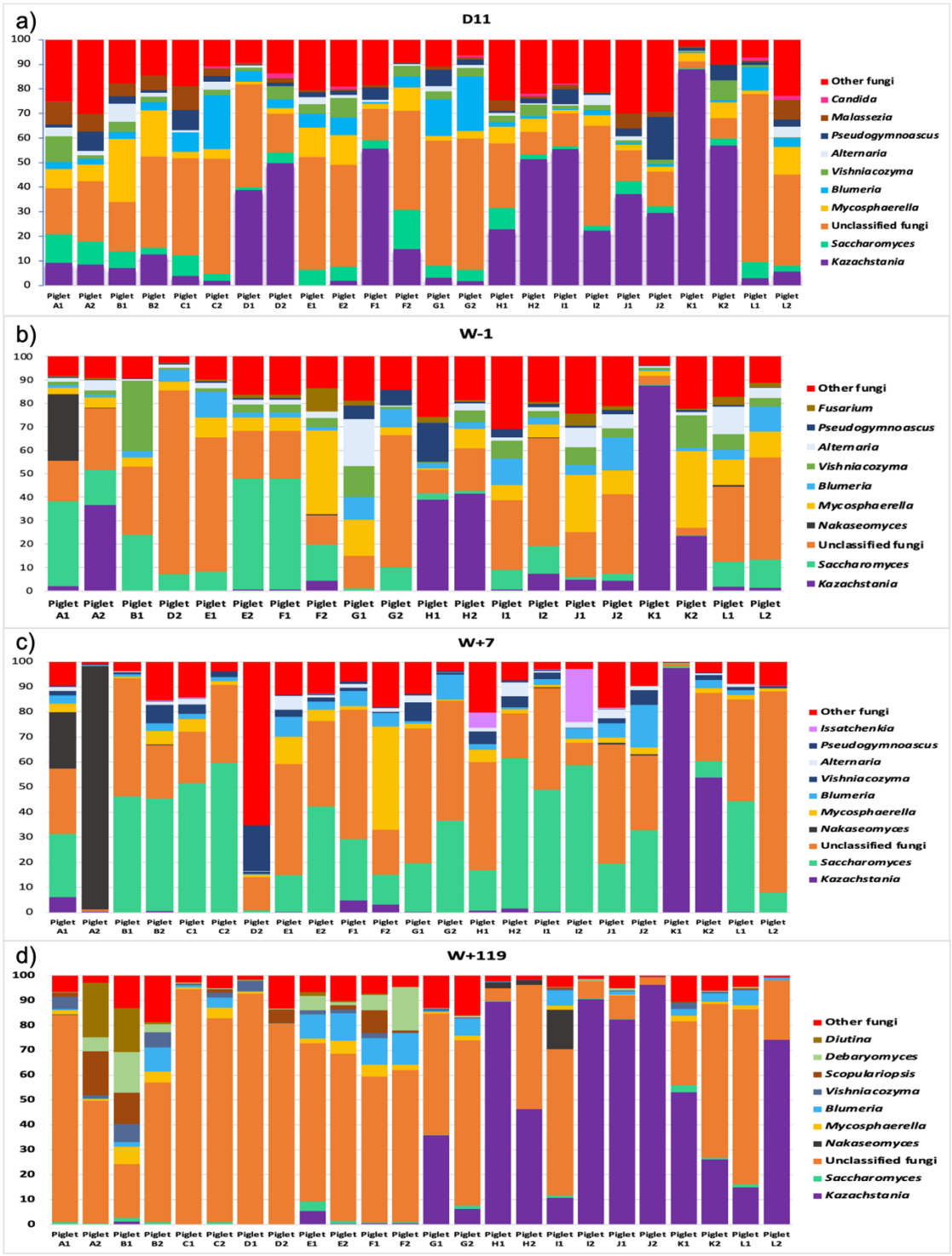

Figure 3.6. Top 10 genera across time points. (a) Top 10 genera in experimental pigs at 11 days of age. **(b)** Top 10 genera on the day prior to weaning. **(c)** Top 10 genera at 7 days post weaning. **(d)** Top 10 genera at 119 days post weaning.

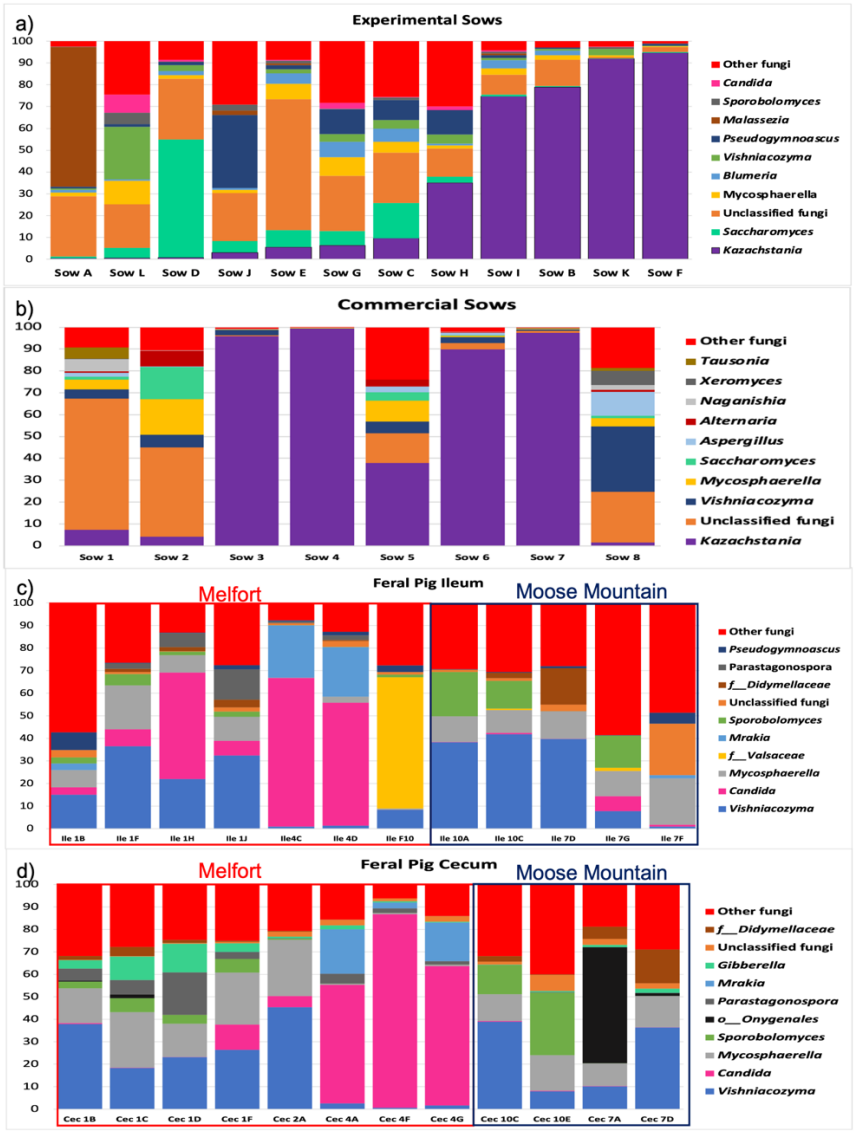

Figure 3.7. Top 10 taxa in sows from experimental and commercial farms and feral pigs. (a) Top 10 most abundant genera in experimental sows. **(b)** Top 10 most abundant genera in commercial sows. **(c)** Top 10 most abundant taxa in the ileum feral pigs. All taxa are genera unless otherwise noted. The prefix *f_* indicates a fungal family. Feral pigs are from two different locations – Melfort and Moose Mountain Saskatchewan Canada. **(d)** Top 10 most abundant taxa in the cecum of feral pigs. All taxa are genera unless otherwise noted. The prefix *f_* indicates a fungal family and the prefix *o_* indicates a fungal order. Feral pigs are from two locations – Melfort and Moose Mountain Saskatchewan Canada.

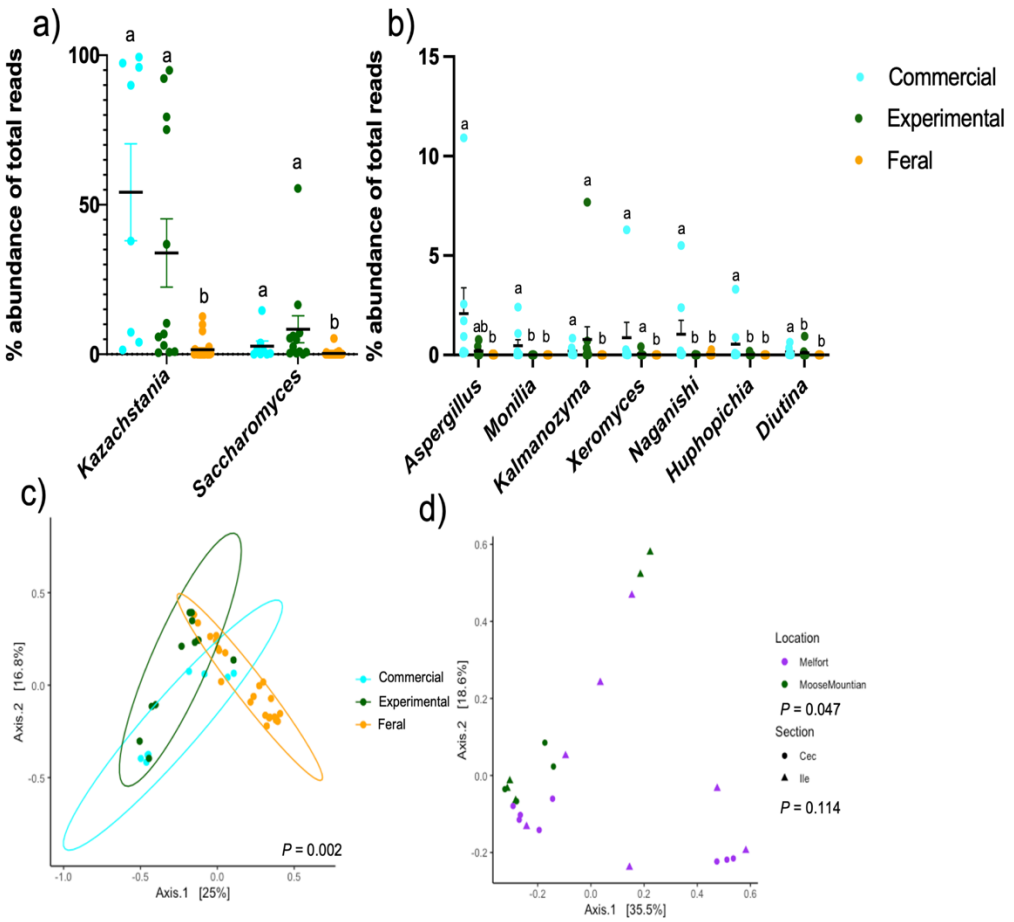

Figure 3.8. Comparison of commercial, experimental, and feral pigs. (a) *Kazachstania* and *Saccharomyces* as identified by ANCOM as being differential. **(b)** All other differential taxa as identified by ANCOM. **(c)** β-diversity based on Bray-Curtis dissimilarity of the mycobiome of commercial, experimental and feral pigs. **(d)** β-diversity based on Bray-Curtis dissimilarity of the mycobiome of feral pigs by location and intestinal section. Significance was defined as $P \leq 0.05$.

3.3.5 The feral pig mycobiome differed based on location of pig

Feral pigs clustered based on their geographical location (Figure 3.8d, $P = 0.047$, β-dispersion $P = 0.059$) but not based on the ileum versus the cecum (Figure 3.8d, $P = 0.114$, β-

dispersion $P = 0.379$). ANCOM analysis revealed the genus *Gibellulopsis* as the only differential taxa between the two locations.

3.4 Discussion

This study brings new advances to our understanding of the pig mycobiome throughout life. We found that fungal exposure in early life shapes mycobiome composition later in life, a finding which may have consequences for production performance and disease risk. Additionally, we showed that the feral pig mycobiome is more complex than the mycobiome of intensively raised pigs, and that the feral pig mycobiome contains the key taxa *K. slooffiae*, albeit at a lower level. This provides further evidence that *K. slooffiae* is a core microbe of the pig.

We found that pigs had differences in their mycobiomes based on their age. This trend is similar to what has previously been described for bacterial populations (5, 7). However, unlike the aforementioned studies, which saw a clear break between pig mycobiome surrounding the weaning transition, this break was not present in our study; likely due to *K. slooffiae* colonization in pre-weaning piglets. In the present study *K. slooffiae* made up a larger percentage of the young pig mycobiome, converse to studies indicating that it becomes detectable in large numbers after weaning (5-7, 12, 13).

The *K. slooffiae* levels of the maternal sow drove piglet *K. slooffiae* levels early and late in life but did not impact the level of *K. slooffiae* around the weaning transition. Indeed, W+7 was the time point with the lowest mean *K. slooffiae* levels suggesting perturbation of the mycobiome around the weaning transition. Additionally, piglets clustered by litter, and were therefore more similar to their littermate on D11 and W+119 with disruption right before and after weaning. This suggests that what piglets are exposed to in the farrowing pen can have long-

term impacts. A similar trend has been seen in terms of bacterial composition, where what piglets are exposed to in the farrowing pen impacts their bacteriome later in life (29). This is particularly important because in humans composition of the mycobiome has been shown to contribute to several different diseases including inflammatory bowel disease, multiple sclerosis, colon cancer, and asthma (30-33). While it is not clear how the mycobiome affects pig health and performance long-term, it is possible that there is some impact, as the bacterial community has been previously implicated in pig performance (34, 35). It also remains unclear why some pigs have large amounts of *K. slooffiae* and others do not. The overgrowth of other fungi such as *Candida* has been shown have a genetic component (36) as well as a host metabolome component (37). Therefore, it is possible that there are a number of host factors which may help determine the level to which *K. slooffiae* is able to colonize and that dissimilarity between mycobiomes may have a genetic component.

We have previously shown that piglets have mycobiomes that are more similar to their maternal sows than to that of other sows at 8 days of age (8). In the present study we show that this result is consistent, with piglets having mycobiomes that are more similar to their maternal sow than to that of a random sow at 11 days of age. However, by the day prior to weaning they no longer have mycobiomes that are closer to their maternal sow than to a random sow, despite still being housed in a pen with their mother. This is contrary to what is seen in humans, where the infant fecal mycobiome is no more similar to their own mothers mycobiome than that of a randomly selected mother (38). The reason for this difference likely comes down to the environment in which piglets live, where they are in contract with the maternal feces, and are therefore able to obtain microbes from their mothers through repeated exposure. However, as piglets age and begin to consume solid feed, their mycobiomes become less like their mothers,

suggesting that the diet contributes to mycobiome composition, a finding which has been previously noted (5).

The weaning transition is a health-challenging time for piglets (39). During the weaning transition, piglets will transition to solid feed, encounter stress from social-mixing, and develop transient intestinal malabsorption (40-42). We found that piglets on W-1 and W+7 showed the most variability in that they did not cluster with their litter mate and did not cluster by sow *K. slooffiae* level. Microbial dysbiosis during the weaning transition has previously been noted in bacterial communities as piglets begin to transition to a solid diet high in cereal grain (43). Given that piglets return to clustering with their litter mate and by sow *K. slooffiae* level later in life, we can assume that something similar is happening to the mycobiome. In our model, piglets were introduced to creep feed at 14 days of age. The amount of creep feed consumed is variable among piglets (44), therefore differences in creep feed consumption may explain the variability in mycobiome compassion among littermates at W-1.

Sows from commercial barns displayed a similar pattern of *K. slooffiae* colonization of *Kazachstania* to experimental sows, with some sows having a high abundance and others having a low abundance. This suggests that in intensively raised pigs, *K. slooffiae* is a commonly abundant microbe, which multiple studies have previously noted (5-7, 12, 13, 45).

The mycobiome of feral pigs differed from that of domestic pigs. Perhaps the most glaring difference was the presences of *Vishniacozyma* as the most dominant genera of fungi in the ileum and second most dominant in the cecum. While this microbe is also present in the experimental pigs and in commercial sows, *Kazachstania* is instead the most dominant genera in intensively raised pigs. This is likely because feral pigs are in contact with more soil as they are

free roaming and feed on crops and crop stubble (46). Indeed, many members of the feral pig mycobiome are microbes regularly found on plants or in soil. This is similar to what has been described in Tibetan pigs fed a high forage diet, where the most prominent genus was *Russula* (47), which is often found in soil (48). While *Russula* was not a common finding in the feral pigs in the present study, it was present in two individuals in small amounts. *Vishniacozyma* may not actually colonize the intestine but instead may be a passenger that simply travels through the GIT, as is potentially the case for the *Cladosporium* found in another study (7, 49). *Vishniacozyma victoriae,* the species of *Vishniacozyma* found in the present study, was originally found in Antarctica, although it has also been isolated from living environments in other locations, and was found to grow between 4 °C and 20 °C (50). Likewise, it has been found that *Vishniacozyma victoriae* is incapable of growth at human body temperature (37 °C) (51). It remains unclear what impact *Vishniacozyma* has on the pig GIT.

Not all the fungi present in the feral pig are unable to colonize. *Candida* was the most abundant genera in the cecum of feral pigs, and *Candida* species have been previously identified in the feces of pigs (12, 45, 47). *Candida* species are able to grow at body temperature (52) and are usually considered opportunistic pathogens (53). In healthy humans *Candida* coexists with commensal bacteria, where *Lactobacillus* has an antagonistic relationship and causes the loss of pathogenicity factors (54). However, *Candida* is also able to overgrow and cause sepsis, typically in hosts who are immunocompromised (55). In pigs, *Candida tropicalis* has been shown to cause mucosal disease of the GIT (56) and invasion of the oral cavity and stomach (57), although the vast majority of pigs appear unaffected. Interestingly, the commercial pigs as a whole have much less *Candida* than the feral pigs, however most pigs have at least some *Candida*, and a couple of individuals have up to 96.7% of their mycobiomes made up by

Nakaseomyces, a clade made up of pathogenic *Candida* species (58). This suggests that there is some individual susceptibility to high levels of *Candida* colonization, which has previously been shown in humans and is influenced by genetics, the host metabolite pool, and antibiotic use (36, 59).

3.5 Conclusion

We found that the amount of *K. slooffiae* piglets were exposed to in the farrowing pen dictated their *K. slooffiae* status up to 119 days post-weaning. Additionally, we found that piglets had more comparable mycobiome compositions to their litter mates in early life as well as at the end of one production cycle. Data from this work indicates a potential transient disruption to the mycobiome during the weaning, where littermate groupings no longer exist, paralleling the dysbiosis that has been documented in bacterial taxa. Overall, this study provides evidence of the importance of early life exposure on long-term mycobiome composition in pigs and how conventionally raised pigs mycobiomes differ significantly in composition and complexity from their feral counterparts. This suggests that early life may be an influential time to change the mycobiome in the long-term.

3.6 Literature cited

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Chapter 4: *Kazachstania slooffiae* alters the metabolome, immune system, and intestinal development in a gnotobiotic pig model

4.1 Introduction

Piglets are born with an undeveloped immune system (1) and gastrointestinal tract (GIT) (2). One critical component driving both immune system and intestinal development is the colonization of the GIT with microbes (3, 4). The GIT is home to a vast array of bacteria, archaea, fungi, protozoa and viruses (5), which together can be termed the microbiota. While the role of bacteria in these processes has been well documented, the role of fungi (the mycobiome) in both immune system and intestinal development is much less well characterized. However, fungi have been shown to be immunomodulatory (6). In mice colonization with fungi has been shown to shape immune system development in early life (7), although the mechanism through which this occurs is unclear. However, to the authors knowledge this is the only study examining the impact of fungi on immune system development performed in a controlled environment.

During initial colonization at birth, facultative anaerobes such as *E*scherichia dominate (8). This is because the neonatal intestine is initially aerobic, and the intestine will become anaerobic over a period of days as colonization succession progresses (9). Within days, the piglet microbiota will start to harbor more *Lactobacillus* and *Bacteroides* as the piglet consumes a milk based diet. At 7 days of age, the microbiota is dominated by *Lactobacillus* (8). Although it should be noted that succession patterns studies vary across studies, and are influenced by factors such as diet and rearing environment (10, 11). One day after birth, piglets are dominated by *Cladosporiaceae*, *Malasseziaceae*, *Dipodascaceae*, and *Nectriaceae* (12). By 7 days of age, piglet mycobiome is dominiated by *Sporidiobolaceae*, *Filobasidiaceae*, and *Trichosporonaceae*,

although it should be noted that the mycobiome is quite variable in pigs this age (12). The mycobiome does not shift to *Saccharomycetaceae* dominance (which includes *Kazachstania*), until after weaning. (12)

Fungi, typically in the form of yeast, have long been fed to livestock for a variety of purposes. Feeding *Saccharomyces cerevisiae* to pigs has been shown to improve growth performance, decrease diarrhea, and modulate immune response (13). Additionally, feeding live *S. cerevisiae* has been shown to increase villus height and villus to crypt depth ratio in the duodenum and jejunum of piglets (14), suggesting that yeast is able to alter intestinal development. In rats, feeding the fermentate of *S. cerevisiae* protected intestinal barrier integrity during heat stress (15). Microbial metabolites can impact the host in a variety of ways and feeding *S. cerevisiae* to beef steers has been shown to increase the amount of plasma indoleacrylic acid (16), a metabolite that is able to suppress inflammatory responses and enhance epithelial barrier function (17). However, *Saccharomyces* makes up less than 1% of the commensal mycobiota (18), and studies which focus on supplementing endogenous pig microbes are lacking.

In the pig, *Kazachstania slooffiae* is the most abundant yeast (18). *Kazachstania* has been isolated from pigs in multiple geographic locations and production systems (12, 19, 20). *K. slooffiae* is thought to be beneficial to pigs as it increases short chain fatty acids (SCFA) in the intestine and has been found to alter the intestinal microbiota (21). Positive interactions have been noted between *K. slooffiae* and *Prevotella* and *Lactobacillus* (18). *In vitro K. slooffiae* shows inhibited growth in the presence of supernatant from *Enterococcus faecalis* (22), suggesting that complex interactions exist between *K. slooffiae* and other microbes in the GIT. We have previously found that *K. slooffiae* abundance is highly variable among pigs, with some

pigs having no *K. slooffiae* and others having upwards of 90% of their mycobiomes composed of *K. slooffiae* (23). Additionally, we found that piglets from sows who were high in *K. slooffiae* also tended to be high in *K. slooffiae* and those from sows who were low in *K. slooffiae* were also low (23). It remains unclear what the impact is of having a *K. slooffiae* dominated mycobiome is on host health and development.

In this study, we investigate the role of *K. slooffiae* in bacterial succession, metabolite production, and intestinal and immune system development, using a gnotobiotic pig model.

4.2 Materials and methods

4.2.1 Derivation of germ free piglets

This animal study was approved by the Animal Care and Use Committee of the University of Alberta (Edmonton, AB, CAN) and conducted in accordance with the guidelines of the Canadian Council on Animal Care under AUP00002777. A caesarean section was performed on a sow on day 112 of gestation. Following removal from the uterus, piglets had their umbilical cords clamped and were immediately passed through a dip tank containing PREPODYNE® GEN (West Pentone, QC, CAN) and into a sterile transfer isolator. A total of 15 piglets were obtained. Piglets were divided into one of two treatment groups: Defined Bacterial Community only (DBC; n=6), Defined Bacterial Community + *K. slooffiae* (DBCK; n=6). Piglets were balanced for sex and size. Three additional piglets that were not balanced for sex or size were maintained germ free to confirm germ free rearing.

4.2.2 Defined bacterial community and colonization

Bacteria were isolated from feces or cecal content from pigs 7 days or older residing at SRTC (Edmonton, AB, CAN). For detailed culture media and gas conditions of isolates see Lantz (2022) (25). Isolates were preserved in isolate broth supplemented with 25% glycerol. The defined bacterial community consisted of the following organisms: *Bacteroides eggerthii*, *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus*, *Bacteroides xylaninosolvens*, *Blautia faecicola*, *Clostridium colicanis, Lactobacillus amylovorus*, *Lactobacillus delbrueckii*, *Lactobacillus johnsonii*, *Limosilactobacillus mucosae*, *Limosilactobacillus reuteri*, *Ligilactobacillus ruminis*, *Prevotella copri*, *Streptococcus hyointestinalis*, *Streptococcus pasteurianus*, *Turicibacter sanguinis* and *Escherichia coli*. Bacterial cultures were grown for 24-72h at 37 °C under anaerobic conditions with gas consisting of 70% N_2, 20% CO_2, and 10% H_2. 500 µL of each bacterium was pooled together with the addition of 25% glycerol and stored at -80 °C until use. *K. slooffiae* was grown in yeast extract broth consisting of yeast extract (Fisher Scientific, ON CAN, 10g/L) and glucose (20g/L) for 48h at 37 °C, then washed and resuspended in sterile phosphate buffered saline (PBS). The final concentration of *K. slooffiae* was roughly 3.0×10^4 CFU/mL.

Piglets in both the DBC and DBCK groups were given the DBC on days 1 and 3 of age and piglets in the DBCK group were given *K. slooffiae* on days 1, 3, and 5 of age. All microbes were given orally by syringe. Piglets were fed irradiated pooled sow colostrum, from sows residing at SRTC, every 3 hours a*d libitum* for the first 24 hours. After 24 hours, piglets were switched to irradiated milk replacer (Brown's Feeds Piglet Milk Replacer, HiBrow ltd., AB, CAN), and fed *ad libitum* by bottle every 3h.

4.2.3 Sample collection

Piglets were humanely euthanized on post-natal day 8 for sample collection. Digesta was collected from the jejunum (1 meter distal to the stomach), ileum (5 cm proximal from ileocecal junction), cecum (from cecal tip), and colon (5 cm from rectum). Digesta was either snap frozen in liquid nitrogen and stored at -80 °C, or for bacterial and yeast culture was placed into Fastidious Anaerobe Agar (Neogen, MI, USA) + 0.05% cystine or sterile PBS respectively. Tissue was collected from the ileum and colon from the same locations and was preserved in 10% formalin for histological assessment. Blood was collected via venipuncture into EDTA tubes (Fisher Scientific, ON, CAN). Mesenteric lymph nodes (MLN) were collected into Krebs Ringer Bicarbonate buffer (KRH) consisting of NaCl (303.6 g/L), HEPES (Fisher Scientific, ON, CAN, 104.0 g/L), KCl (15.6 g/L), $CaCl_2$ (6.2 g/L), NaH_2PO_4 (5.68 g/L), $MgSO_4$ (6.74 g/L), Bovine Serum Albumin (Sigma-Aldrich, MO, USA, 5g/L) and antibiotic/antimycotic (Gibco, MT, USA,10 mL/L), then stored on ice and transported to the laboratory.

4.2.4 Culture

Total anaerobes were cultured on Fastidious Anaerobe Agar (Neogen, MI, USA) under anaerobic gas conditions for 48h at 37 °C. *K. slooffiae* was cultured on yeast extract glucose agar consisting of yeast extract (Fisher Scientific, ON CAN, 10g/L), glucose (20g/L), and agar (15g/L) under aerobic conditions for 48h at 37 °C.

4.2.5 Quantitative polymerase chain reaction (qPCR)

Total bacterial load was measured via qPCR using the StepOnePlus Real-Time PCR system (Applied Biosystems, CA, USA). Each reaction was performed in duplicate and consisted

of 5 µL PerfeCTa SYBR Green Supermix (Quantabio, MD, USA), 0.5 µL (10 µM/L) forward primer, SRV3-1 (5'- CGGYCCAGACTCCTACGGG -3') and 0.5 µL (10 µM/L) reverse primer (5'- TTACCGCGGCTGCTGGCAC -3') (26), 2 µL nuclease free water and 1 µL template DNA. A standard curve was generated from the PCR amplicon of pooled genomic DNA. The following cycling parameters were used: 95 °C for 3 min followed by 95 °C for 25 sec and 60 °C for 30 sec for 40 cycles.

4.2.6 Bacterial community characterization

Amplicon libraries of the V3-V4 region of the 16S rRNA gene were constructed according to the Illumina 16S Metagenomic Sequencing Library Preparation protocol (Illumina Inc., CA, USA). DNA was quantified using the Quanti-iT™ Pico Green dsDNA Assay kit (Invitrogen, CA, USA). Amplicon primers used were as follows: forward, 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and reverse, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC -3′. Sequencing was performed on the Illumina Miseq platform using 2 x 300 cycles (Illumina Inc., CA, USA). The forward read was truncated at 260 base pairs and the reverse read was truncated at 220 base pairs. The Divisive Amplicon Denoising Algorithm (v2) plugin for QIIME2 was used to perform demultiplexing, quality filtering, denoising, and chimera filtering (26). Mafft was used to align Amplicon Sequence Variants (ASVs) (27). Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomic classifier (28). Sequences were classified using the SILVA database version 138.1 (29).

4.2.7 Histology

Approximately 1 cm of tissue was collected from the ileum 5 cm proximal to the ileocecal junction and approximately 1 cm of tissue was collected from the colon 5 cm from the rectum. Tissue was immediately placed in 10% neutral buffered formalin at room temperature for 24h then placed into 70% ethanol. Fixed tissue was then embedded in paraffin wax and sectioned into 5 μm slices and stained with hematoxylin and eosin. Sections were visualized using the EVOS FL Auto Imaging System (Thermo Scientific, ON, CAN). Villus height was measured from the villus base to the villus apex. Crypt depth was measured from the base of the crypt to the crypt opening. 5 measurements were made per slide and the average was used for statistical analysis.

4.2.8 Metabolomics

Metabolomic analysis was performed on digesta from the ileum and plasma at The Metabolomics Innovation Center (TMIC) (University of Alberta, Edmonton, CAN) using the TMIC Prime Assay plus butyric acid and acetic acid, which measured 143 metabolites. Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) and Direct Infusion-Tandem Mass Spectrometry (DI-MS/MS) were performed using the Agilent 1260 series ultra-high-performance liquid chromatography system (Agilent Technologies, CA, USA) along with an AB SCIEX QTRAP® 4000 mass spectrometer (Sciex Canada, Concord, ON, CAN).

Samples were thawed on ice and homogenized. The TMIC prime assay used a 96 deep-well plate with a filter plate attached. For all metabolites except for organic acids and lipids, 10 μL of each sample was loaded onto the center of the filter on the upper 96-well plate and dried in

a stream of nitrogen. Following drying, 50 μL of 5% phenyl-isothiocyanate was added to each well for 20 minutes at room temperature for derivatization. Following incubation, the spots were once again dried using a stream of nitrogen for 1.5 hours. 300 μL of extraction solvent containing methanol and 5 mM ammonium acetate was then added to each well of the plate which was then shaken at 300 rpm for 30 minutes at room temperature. The plate was then centrifuged at 50 x g for 5 minutes to collect the extracts in the bottom collection plate. Amino acids and their derivatives and biogenic amines and their derivatives were then diluted with 150 μL of LC-MS water prior to LC-MS injection. The remaining extracts were diluted with 400 μL of direct flow injection buffer for DI-MS/MS. For lipid analysis 5 μL of extracts were pipetted directly into the 96-deep-well plate followed by the addition of 490 μL of direct flow injection buffer and then shaken at 500 rpm for 15 minutes prior to DI-MS/MS. Isotope-labeled internal standards were utilized.

For analysis of organic acids 150 μL of ice-cold methanol and 10 μL of isotope-labeled internal standard mixture was added to 50 μL of sample overnight for protein precipitation. This mixture was then centrifuged at 13000 x g for 15 minutes. 50 μL of the supernatant was then loaded into the center of the 96-deep-well plate followed by the addition of 3-nitrophenylhydrazine. After incubation for 2 hours, 25 μL of 2 mg/mL butylated hydroxytoluene stabilizer and 350 μL of water were added for LC-MS analysis.

4.2.9 Immune Cell Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density centrifugation using Histopaque 1077 (Sigma-Aldrich, MO, USA) and were counted and diluted to 1.5 x 10^6/mL as previously described (30). Cells from MLN were obtained by passing

tissue through 100 nm nylon mesh as previously described (31) with the exception that as with PBMCs, cells were diluted to 1.5 x 10^6/mL.

4.2.10 Flow cytometry

Immune cell subsets were identified from whole blood and MLNs as previously described (32). Briefly, cells were incubated at 4° C for 30 minutes with pre-labeled monoclonal antibodies (Table 4.1) and were then washed with 5% HyClone fetal bovine serum (Cytiva, MA, USA) in PBS and fixed with 1% paraformaldehyde. The exception to this protocol was the FoxP3 antibody, where cells were first treated to permeabilize the membrane using the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Invitrogen, MA, USA) according to manufacturer instructions. The fixed cells were then analyzed within 48h by flow cytometry using a LSR-Fortessa flow cytometer (BD, NJ, USA). Further analysis was performed in FlowJo (v9) (Supplementary Figure 1)

Table 4.1. Panels used for flow cytometry.

Panel	***PerCP***	***PECy7***	***FITC***	***PE***	***APC***
1	CD3 (BD, 561478)	CD4 (BD, 561473)	CD8 (BD, 551303)	γδ T Lymphocytes (BD, 561486)	CD27 (Bio Rad, MCA5973APC)
2	CD3 (BD, 561478)	CD4 (BD, 561473)	CD8 (BD, 551303)	CD45RA (Bio Rad, MCA1751PE)	CD27 (Bio Rad, MCA5973APC)
3		CD4 (BD, 561473)	SLAII (Bio Rad, MCA2314F)	CD21 (BD, 557327)	
4	CD3 (BD, 561478)				CD335 (Bio Rad, MCA5972APC)
5			CD4 (Invitrogen, MA5-16854)	CD25(Bio Rad, MCA1736GA) + Goat anti Mouse IgG (Bio Rad, STAR117F)	FOXP3 (eBioscience, 17-5773-82)

4.2.11 *Ex-vivo* Cytokine Production

Cytokine production from PBMCs and MLNs were measured following incubation for 48h at 37°C in cells which were either unstimulated or stimulated with either lipopolysaccharide (LPS) (Invitrogen, MA, USA, 2 μL/ mL) or phytohaemagglutinin (PHA) (Sigma-Aldrich, MO, USA, 5 mg/mL). Cells were diluted to a concentration of 1.5 x 10^6/mL and incubated in RPMI 1640 media (Gibco, MT, USA) supplemented with 5% HyClone fetal bovine serum, 2.5 mM 2-mercaptoethanol, 25 mM HEPES, and 1% antibiotic/antimycotic and adjusted to a pH of 7.4 (31). Cytokines were measured by multiplex enzyme-linked immunosorbent assay (13-Plex Porcine Discovery Assay®, Eve Technologies, AB, CAN).

4.2.12 Statistical analysis

All statistics were performed in GraphPad Prism (version 9.5.0) unless otherwise stated. Differences between culture data, total bacteria, bacterial taxa, histological measurements, immune cell phenotype, and cytokines were investigated using a Mann-Whitney U test. Differences in overall bacterial communities were investigated using the R package phyloseq (v1.34.0) (33). Bacterial data was visualized by Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity and analyzed for differences using Permutational Multivariate Analysis of Variance (PERMANOVA) (R, v4.0.5). Metabolomic analysis was performed using MetaboAnalyst 5.0. Data were log transformed and mean centered. Metabolites of interest were identified by fold change analysis with anything greater than 1 log2 fold change being considered as a metabolite of interest. Raw metabolite concentrations were then used for statistical analysis where they were tested for normality using a Shapiro-Wilk test and then

analyzed using either a T-test for normally distributed metabolites or a Mann-Whitney test for metabolites that were not normally distributed.

Enrichment analysis was performed in MetaboAnalyst 5.0 using the KEGG database for all metabolites of interest with the exception of lipids. Correlation analysis between metabolites and microbes or cytokines was done using a Spearman's correlation. Significance was defined as $P \leq 0.05$ and trends were considered $P < 0.1$. A principal component analysis (PCA) plot was generated in MetaboAnalyst 5.0 and was evaluated for differences using Permutational Multivariate Analysis of Variance (PERMANOVA) (R, v4.0.5).

4.3 Results

4.3.1 Colonization with *K. slooffiae* increases total anaerobes

All animals in the DBCK group had *K. slooffiae* growth, indicating that colonization with *K. slooffiae* was successful, whereas *K. slooffiae* was not detected in the DBC group. No microbes were detected in the germ free group, indicating that maintenance of sterile rearing conditions was achieved. *K. slooffiae* colonized at the lowest level (mean = log 5.6) in the jejunum ($P = 0.004$) and at similar levels in the ileum, cecum, and colon (mean = log 6.4 – 6.6) (Figure 4.1a). Total anaerobes, enumerated on FAA, were higher in the DBCK group in both the ileum ($P = 0.015$) and cecum ($P = 0.041$) (Figure 4.1b and 4.1c). Additionally, qPCR results revealed that *K. slooffiae* colonization increased total bacterial load in both the ileum (Figure 4.1d, $P = 0.037$) and cecum (Figure 4.1e, $P = 0.009$).

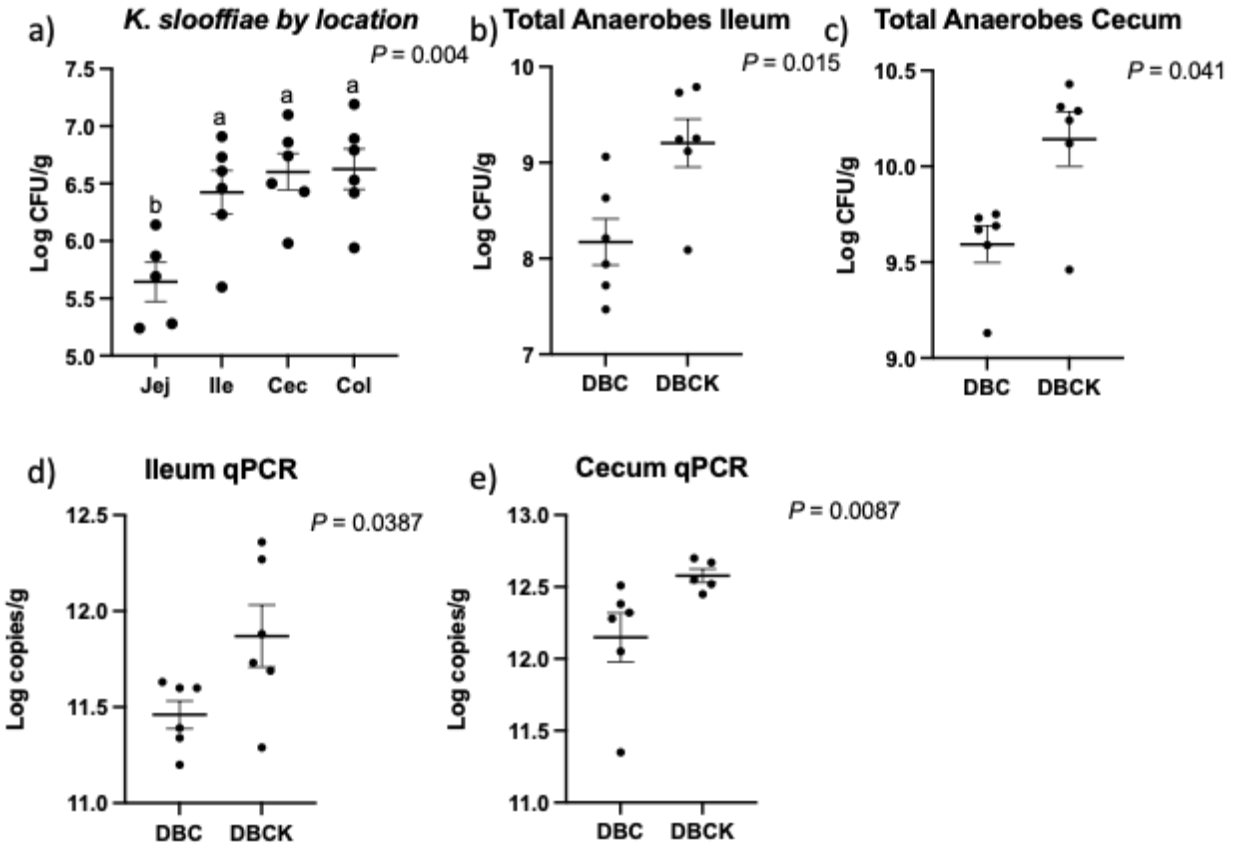

Figure 4.1. *K. slooffiae* colonization increased total anaerobes and total bacteria. Culture data showing *K. slooffiae* abundance by location in DBCK animals **(a)**, and total anaerobes in both DBC and DBCK animals in the ileum **(b)** and cecum **(c)**. Log copy number of the 16s rRNA gene (total bacteria) per g of content via qPCR in the ileum **(d)** and cecum **(e)**. Significance was defined as $P \leq 0.05$.

4.3.2 *K. slooffiae* alters bacterial community composition

The addition of *K. slooffiae* to the bacterial community altered bacterial β-diversity in the cecum (Figure 4.2c, $P = 0.048$) and tended to be altered in the ileum (Figure 4.2b, $P = 0.056$) and colon (Figure 4.2d, $P = 0.053$). There was no change in β-diversity in the jejunum (Figure 4.2a, $P = 0.857$). Additionally, the relative abundance of different taxa was altered by the addition of *K. slooffiae*. In the ileum *E. coli* was significantly higher in the DBCK group (Figure 4.3b, $P =$

0.041), while *L. reuteri* was higher in the DBC group (Figure 4.3b, $P = 0.009$). In the cecum *E. coli* was higher in the DBCK group (Figure 4.3c, $P = 0.032$) and *L. mucosae* was higher in the DBC group (Figure 4.3c, $P = 0.008$). In the colon *E. coli* was higher in the DBCK group (Figure 4.3d, $P = 0.002$) as was *S. hyointestinalis* (Figure 4.3d, $P = 0.041$), while *S. pasteurianus* tended to be higher in the DBC group (Figure 4.3d, $P = 0.065$). In the jejunum there were no differences between any of the taxa. Although, there was no difference between which bacteria colonized DBC and DBCK pigs, the following microbes were missing from all animals: *B. eggerthii*, *B. xylaninosolvens*, *B. faecicola*, *C. colicanis*, *L. amylovorus*, *L. johnsonii*, *L. ruminis*, and *P. copri*.

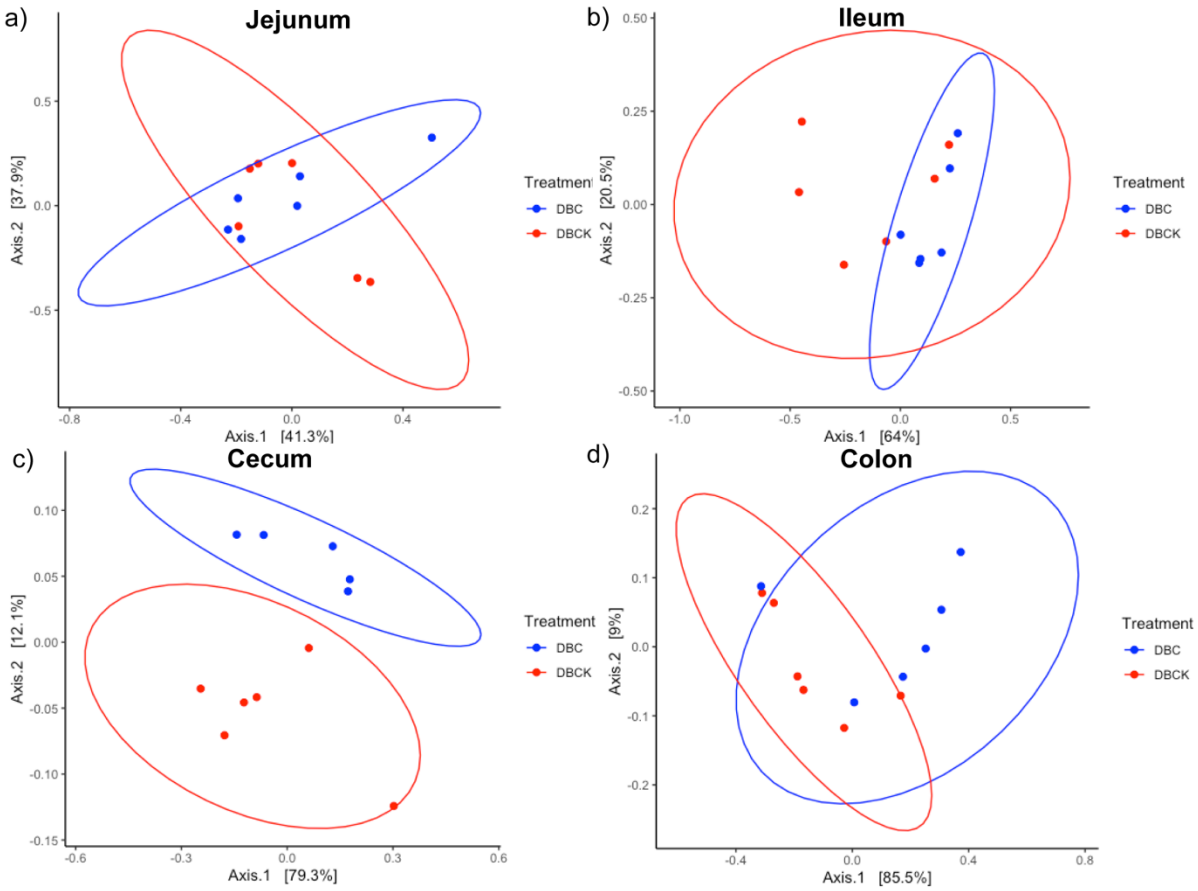

Figure 4.2. Impact of *K. slooffiae* on β-diversity. Differences in bacterial communities as shown by PCoA based on Bray-Curtis dissimilarity in the **(a)** jejunum ($P = 0.857$), **(b)** ileum ($P = 0.056$), **(c)** cecum ($P = 0.048$), and **(d)** colon ($P = 0.053$). Significance was defined as $P \leq 0.05$ and trends were defined as $P < 0.1$.

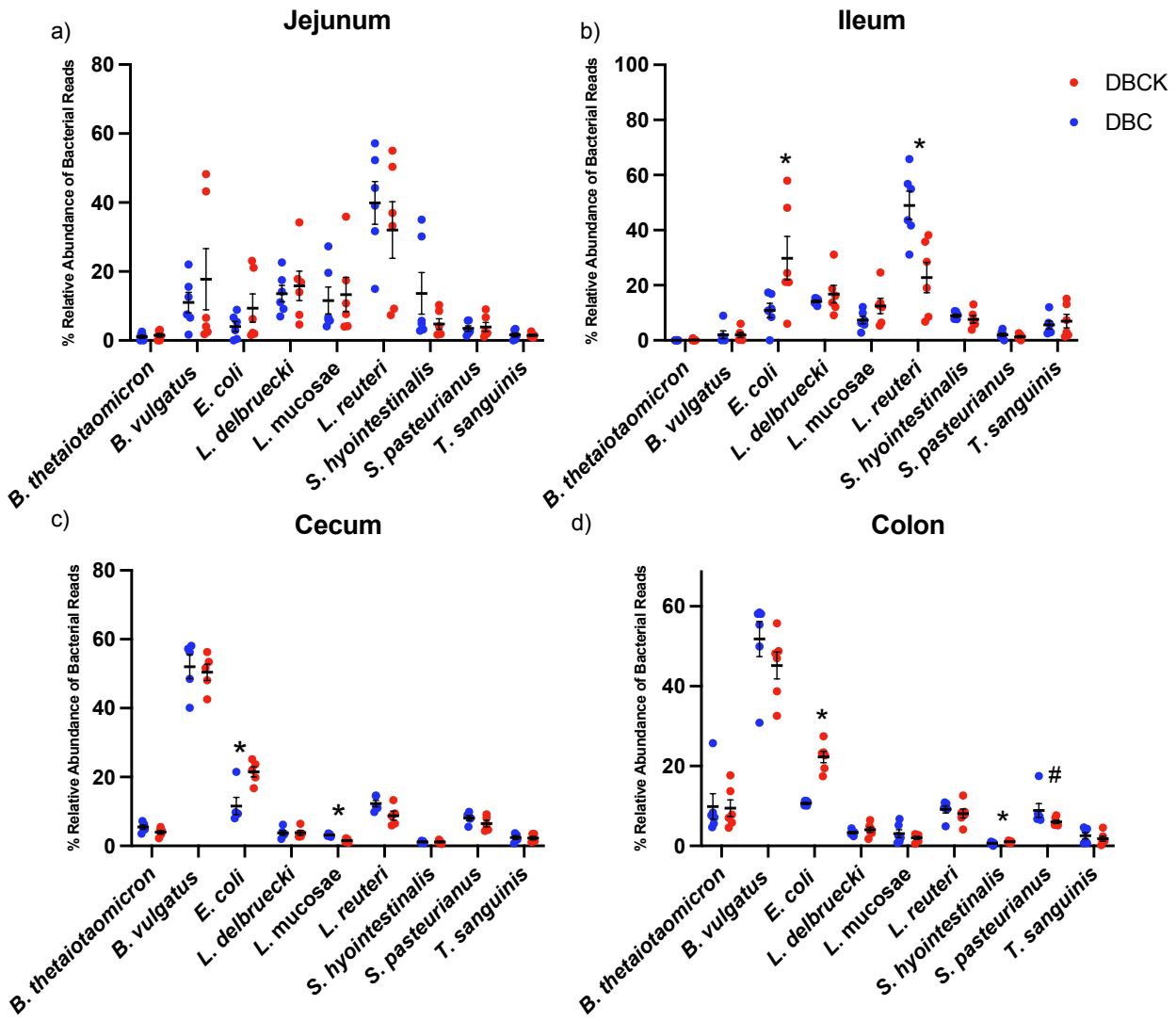

Figure 4.3. *K. slooffiae* alters the relative abundance of bacteria in the intestine. Relative abundance of bacteria in the jejunum **(a)**, ileum **(b)**, cecum **(c)**, and colon **(d)**. * indicates $P \leq 0.05$ and # indicates $P < 0.1$.

4.3.3 *K. slooffiae* increases villus height: crypt depth ratio

The villus height to crypt depth ratio was increased in the DBCK group (Figure 4.4c, $P = 0.028$). There was no change in the direct comparison of villus height between DBC and DBCK groups (Figure 4.4a, $P = 0.310$) or in crypt depth (Figure 4.4b, $P = 0.132$). In the colon there was no difference in crypt depth (Figure 4.4d, $P = 0.589$). Representative histological measurements can be seen in Figure 4.5.

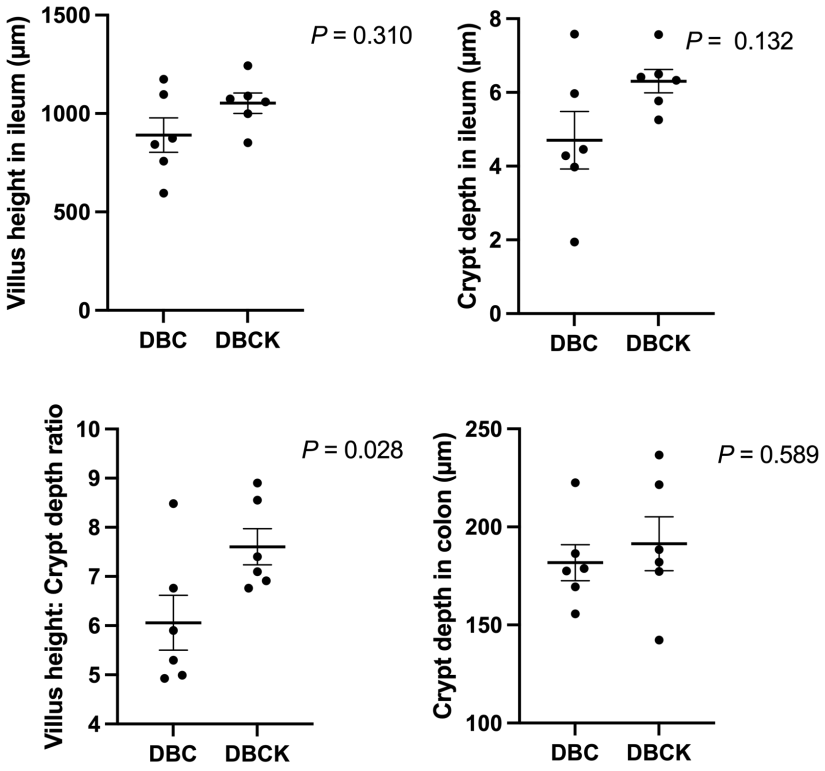

Figure 4.4. *K. slooffiae* colonization altered the ratio of villus height to crypt depth in the ileum. (a) Villus height in the ileum. **(b)** Crypt depth in the ileum. **(c)** Ratio of villus height to crypt depth in the ileum. **(d)** Crypt depth in the colon. Significance was defined as $P \leq 0.05$.

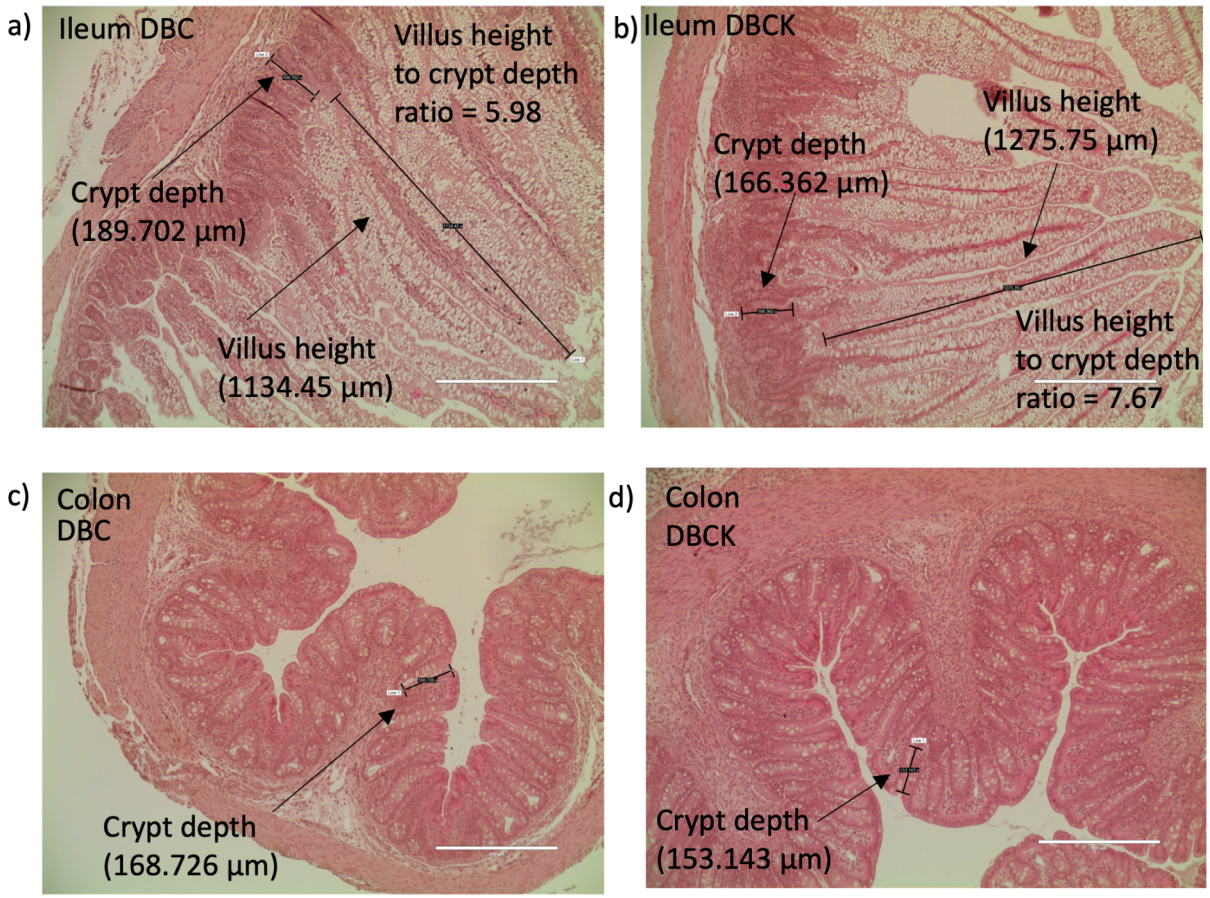

Figure 4.5. Histological assessment. Examples of histological measurements in the ileum of DBC treated animals **(a)**. The ileum of DBCK treated animals **(b)**. The villus height to crypt depth ratio is increased in the DBCK group. **(c)** shows the histological measurement of crypts in the colon of

4.3.4 *K. slooffiae* alters ileal and plasma metabolome

Colonization with *K. slooffiae* increased the concentration of butyric acid (Figure 4.6a, $P = 0.032$), fumaric acid (Figure 4.6a, $P = 0.05$), taurine (Figure 4.6a, $P = 0.015$), and putrescine (Figure 4.6a, $P = 0.004$). While the following metabolites were not significantly different between treatments despite being identified by fold change analysis: spermidine (Figure 4.6a, $P = 0.589$), citrulline (Figure 4.6a, $P = 0.137$), HPHPA (Figure 4.6a, $P = 0.309$), and SMC 18:0

(Figure 4.6a, $P = 0.132$). Conversely, pigs without *K. slooffiae* had increased levels of citric acid (Figure 4.6a, $P \leq 0.001$). *K. slooffiae* colonized animals were also enriched for the following pathways in ileal digesta: arginine biosynthesis, glutathione metabolism, arginine and proline metabolism, taurine and hypotaurine metabolism (Figure 4.6b, $P \leq 0.05$). The abundance of putrescine in ileal digesta was negatively correlated with Tumor Necrosis Factor α (TNFα) (Figure 4.6c, $P = 0.001$). However, multivariate analysis revealed that there was no separation of metabolomes based on *K. slooffiae* colonization (Figure 4.7a, $P = 383$)

In plasma, pigs without *K. slooffiae* had increased methionine (Figure 4.6d, $P = 0.003$) and betaine (Figure 4.6d, $P = 0.002$), and there was a trend in increased propionic acid in animals colonized with *K. slooffiae* (Figure 4.6d, $P = 0.093$). *K. slooffiae* colonized animals were enriched for the following pathways: glycine, serine, and threonine metabolism, and cystine and methionine metabolism (Figure 4.6e, $P \leq 0.05$). Multivariate analysis showed that there was no difference in the metabolome of animals colonized with *K. slooffiae* versus those not colonized with *K. slooffiae* (Figure 4.7b, $P = 0.323$).

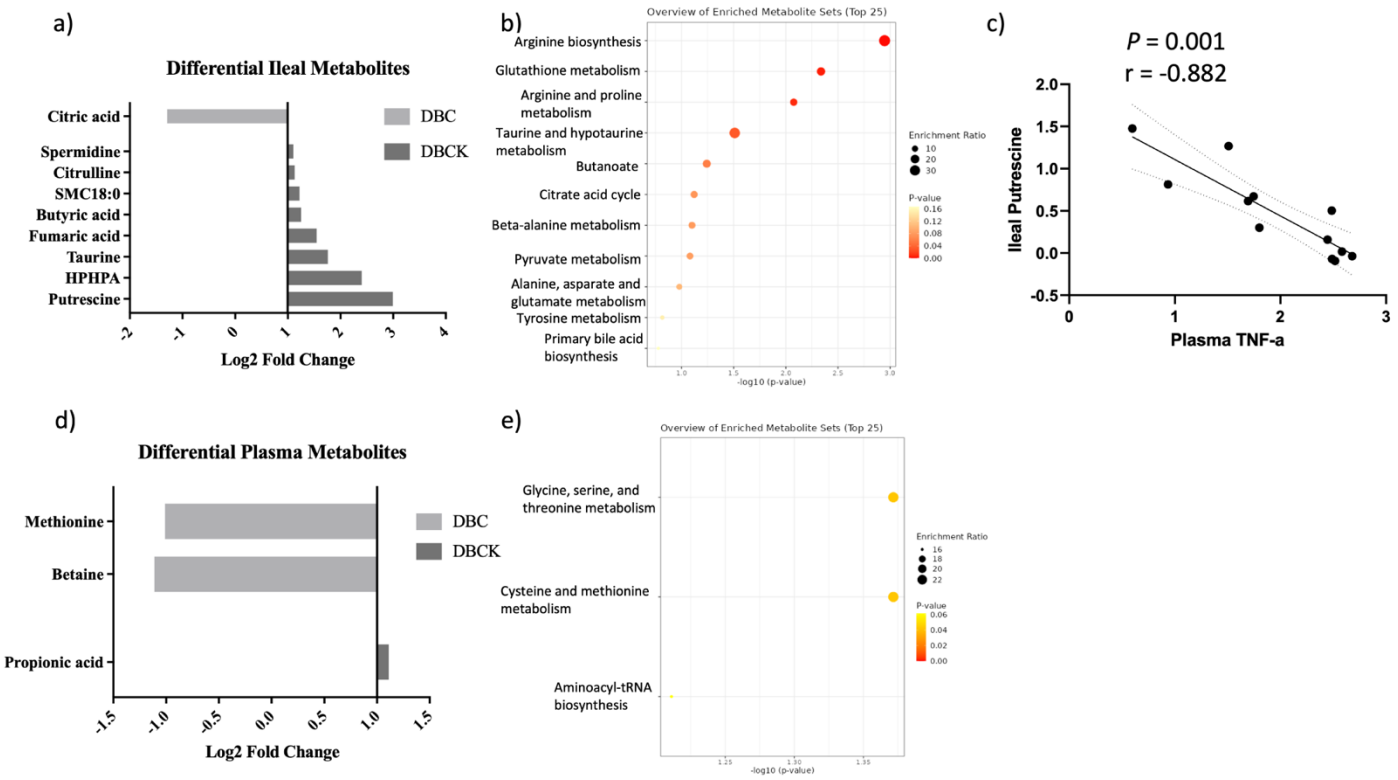

Figure 4.6. *K. slooffiae* increases key intestinal metabolites in the ileum and alters the plasma metabolome minimally (a) Log2 fold changes in ileal metabolites between DBC and DBCK animals. **(b)** Enriched pathways in the ileum. **(c)** Correlation between ileal putrescine and TNFα. **(d)** Log2 fold changes in plasma metabolites between DBC and DBCK**. (e)** Enriched pathways in plasma.

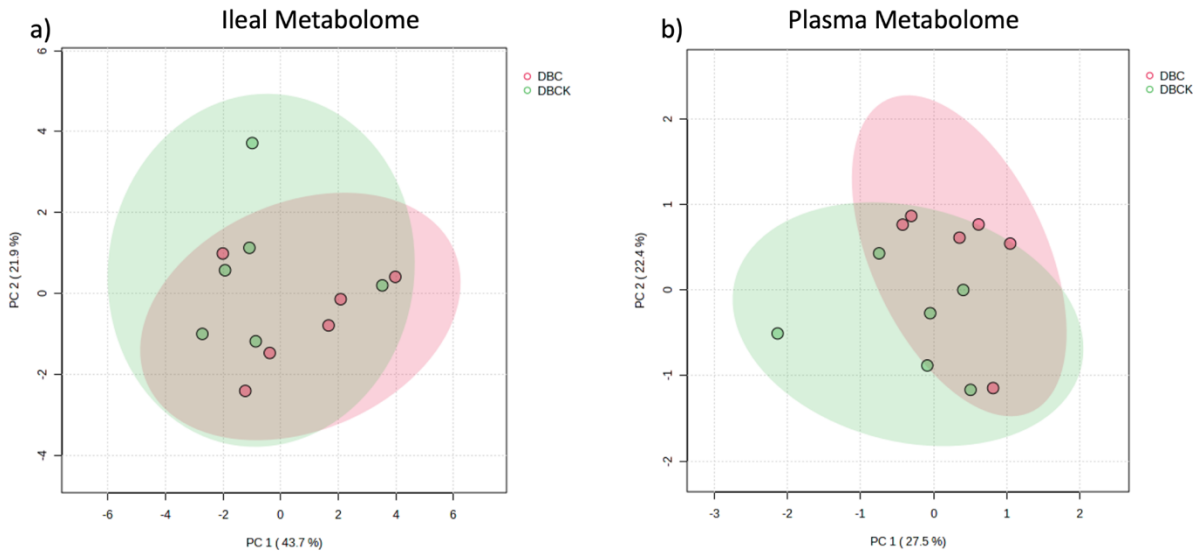

Figure 4.7. *K. slooffiae* colonization did not alter the overall ileal or plasma metabolomes. **(a)** PCA plot of the ileal metabolome between DBC and DBCK. **(b)** PCA plot of plasma metabolome between DBC and DBCK groups.

4.3.5 *K. slooffiae* alters T cell populations in mesenteric lymph nodes

K. slooffiae colonization decreased the number of total CD3+ (T cells) MLN cells (P = 0.006) as well as CD3+CD4+ (helper T cells) cells (P = 0.02). There were no changes in the number of CD3+CD8+ (cytotoxic T cells) (P = 0.70), CD3+CD4+CD8+ (double positive) (P = 0.48), γδ T cells (P = 0.13), or in CD4+CD25+Foxp3+ (regulatory T cells) (P = 0.81) (Figure 4.8a). The number of CD3+CD45RA+ (naïve T cells) tended to be higher in DBCK animals (P = 0.065) and the number of CD3+CD8+CD45RA+ (naïve killer T cells) was higher in the DBCK group. There were no differences in CD3+CD4+CD45RA+ (naïve helper T cells) (P = 0.781) or in CD3+CD4+CD8+CD45RA+ (naïve double positive T cells) (P = 0.132) (Figure 4.8b). The number of CD3+CD27+ (stimulated T cells) (P = 0.039) and CD3+CD4+CD27+ (stimulated helper T cells) (P = 0.026) were both lower in the DBCK, while there were no differences

between groups in the number of CD3+CD8+CD27+ (stimulated killer T cells) ($P \geq 0.999$) or CD3+CD4+CD8+CD27+ (double positive stimulated T cells) ($P = 0.240$) (Figure 4.8c). There were no differences in CD4-CD21+SLAII+ B cells (naïve and activated B cells) ($P = 0.31$) or CD4-CD21-SLAII+ (plasma B cells) ($P = 0.48$) (Figure 4.8d). Additionally, there were no changes in CD3-CD335+ (Natural killer (NK) cells) ($P = 0.59$) (Figure 4.8e) in MLN. In PBMCs there were no differences between groups in T cells or T cell subsets, B cells, or NK cells.

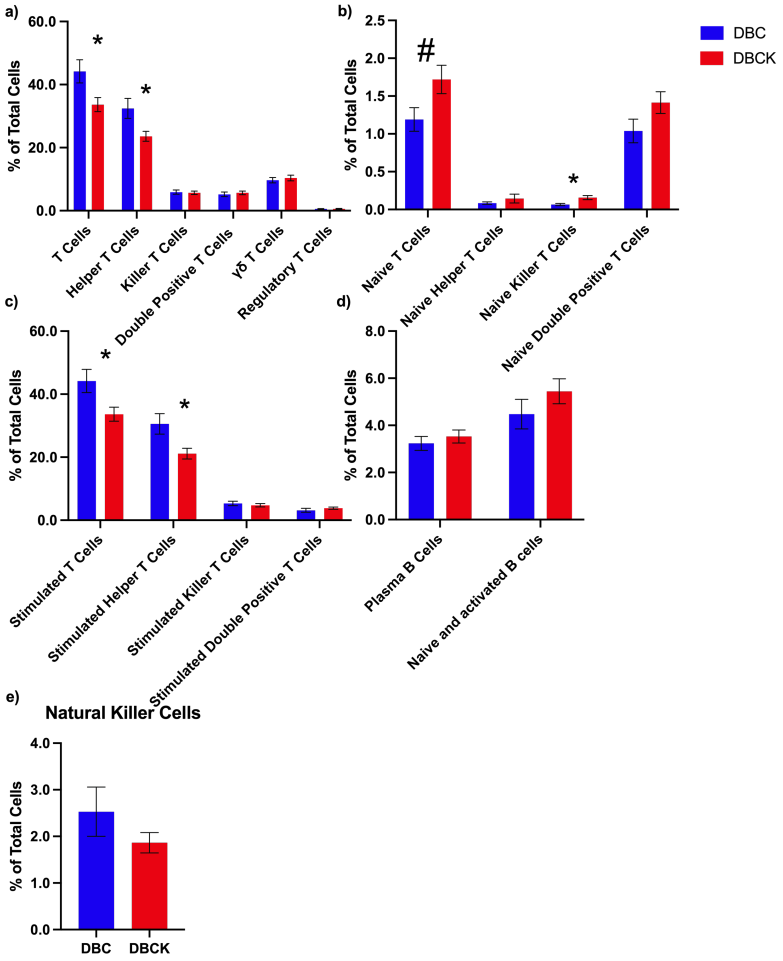

Figure 4.8. *K. slooffiae* alters immune system development. Immune cell subsets in mesenteric lymph node cells. **(a)** T cell subsets. **(b)** Naïve T cell subsets. **(c)** Stimulated T cell subsets. **(d)** B cell subsets. **(e)** Natural killer cells. Significance is indicated by * and was defined as $P \leq 0.05$. A trend is indicated by # and was defined as $P < 0.1$.

4.3.6 *K. slooffiae* alters cytokine production

Compared to DBC, animals in the DBCK group had higher levels of plasma interleukin (IL)-2 ($P = 0.02$) and IL-18 ($P = 0.02$) and lower levels of TNFα ($P = 0.002$) (Figure 4.9a). In MLN cells stimulated with LPS, DBCK animals had increased IL-18 ($P = 0.04$) and IL-10 ($P = 0.04$) (Figure 4.9b). Stimulation of MLN cells with PHA resulted in lower IL-2 ($P = 0.002$), IL-12 ($P = 0.03$) and TNFα ($P = 0.004$) in the DBCK group compared to animals in the DBC group, and higher IL-18 ($P = 0.02$) (Figure 4.9c) in the DBCK group.

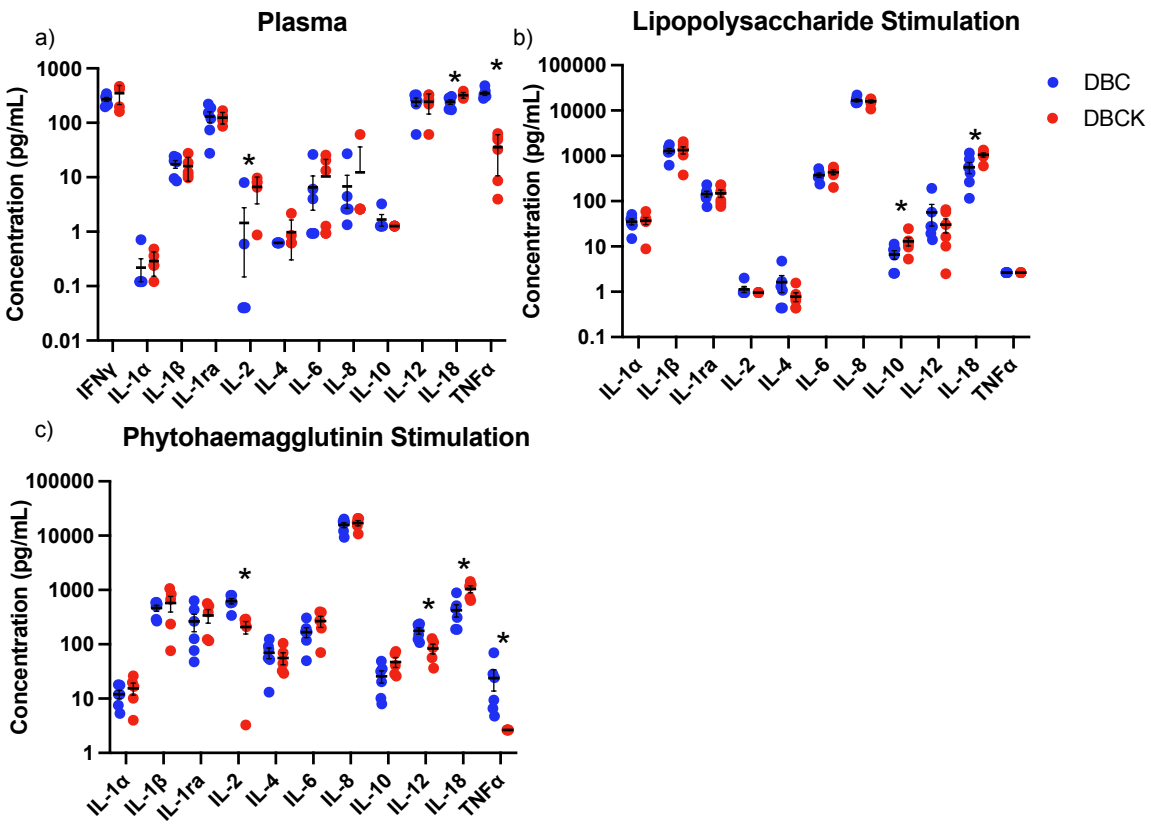

Figure 4.9. *K. slooffiae* alters cytokine production. (a) Cytokine levels in plasma **(b)** cytokine production by mesenteric lymph node cells following stimulation with lipopolysaccharide **(c)** cytokine production by mesenteric lymph node cells following stimulation with phytohaemagglutinin. Significance is indicated by * and was defined as $P \leq 0.05$.

4.4 Discussion

In this study we sought to determine the impact of *K. slooffiae* colonization on immune system and intestinal development. We found that *K. slooffiae* was able to colonize all piglets in the DBCK group, although some individual variation was present in the amount of *K. slooffiae* present. This is consistent with the greater individual variation in fungal community composition than bacterial community composition in humans (34), suggesting that some fungi may colonize

better in some individuals than others. It has been noted by other studies that *K. slooffiae* is virtually absent until the time of weaning (12, 35). However, we previously found that *K. slooffiae* was present in pigs as young as 3 days of age, if present in the dam (23). While this suggests that there is some individual variation in the time of *K. slooffiae* colonization, we felt that introducing *K. slooffiae* at 24h after birth was representative of the colonization patterns pigs in at least some environments. The abundance of total anaerobes and total bacteria were increased in *K. slooffiae* colonized animals. We speculate that this is due to *K. slooffiae* reducing the oxygen content in the intestinal tract, thereby allowing for the growth of anaerobic bacteria. Yeasts are facultative anaerobes (36). It has been suggested that other early colonizing facultative anaerobes such as *E. coli*, help to decrease the oxygen content in the GIT and thereby create a hospitable environment for strict anaerobes to colonize (37). Alternatively, yeast cell wall could be acting as a prebiotic (38), and therefore allowing for the increase in total bacteria in *K. slooffiae* colonized animals.

We determined that there were changes in the bacterial community due to *K. slooffiae* colonization. This is in consistent with multiple studies that have shown that the bacterial community can be altered by feeding *S. cerevisiae* to pigs (39, 40). Although not the focus of the current study, there are several reasons why some members of the DBC were not able to colonize. Several of the organisms, such as *Prevotella*, are strict anaerobes and may not have been able to tolerate the inoculation process (41). Additionally, microbes in the GIT exist in complex communities where cross-feeding is often an important factor for microbial survival and growth (42). Appropriate substrate for bacterial survival may have been lacking, for example, the presence of fiber is important for *Prevotella* to colonize the GIT (44). Therefore, outside of the communities in which they normally exist, some of the microbes in the DBC may have not been

able to survive and proliferate. Regarding specific differences in taxa between the DBC and DBCK groups, the increase of *E. coli* in the ileum, cecum, and colon was not expected, as yeast supplementation is typically associated with lower levels of *Escherichia* in digesta (44). However, *Escherichia* make up part of the normal piglet GIT microbial population (45), with *Escherichia* being differentially abundant in the pre-weaning intestine versus the postweaning intestine (46). The pigs in the previously mentioned study were of post-weaning age compared to the 7-day-old piglets in the present study, and it is therefore possible that the increase in *Escherichia* is unique to early life or that the increase is transitory. It is worth noting that the overall relative abundance of *Escherichia* in the present study is still generally lower than that documented in other studies in conventional pigs (45, 46).

The ratio of villus height to crypt depth is an indicator of small intestinal development and a marker of good intestinal health (47, 49). The present study found that colonization with *K. slooffiae* increased villus height: crypt depth, therefore suggesting that *K. slooffiae* may promote development of the intestine and gut health. Additionally, butyrate, which was found to be increased in the ileum of *K. slooffiae* colonized animals, has been shown to increase the villus height: crypt depth in weanling pigs (49).

The presence of *K. slooffiae* led to increases in several beneficial metabolites in the intestine. Butyrate is an important metabolite for intestinal health, as it acts as an energy source for intestinal cells (50), mediates inflammation, and helps to maintain the gut epithelial barrier (51). Putrescine can also act as an energy source in the intestine (52) and additionally can decrease the incidence of diarrhea in weanling pigs and has an anti-inflammatory function (53). We also observed a trend in increased fold change in propionic acid in plasma in *K. slooffiae* colonized animals. Circulating propionic acid has been associated with increased insulin

sensitivity in humans (54). In pigs, supplementation with live yeast has been shown to improve insulin sensitivity, which helped pigs tolerate heat stress more efficiently than pigs not supplemented with yeast (55). While measuring metabolites in the colon would have been beneficial, there was not enough digesta remaining following 16S analysis to complete metabolomics on digesta from the colon.

Immune cell phenotype was altered by *K. slooffiae*. While the overall abundance of CD3+ and CD3+CD4+ T helper cells was lower in *K. slooffiae* colonized animals, we do not believe that this represents an immunocompromised state as the percent of CD3+ and CD3+CD4+ cells are both slightly lower than the cell populations found in other studies. Pietrasina et al. (2020) found that 28 day old piglets had a mean abundance of 59.75% CD3+ lymphocytes and a mean of 32.52% CD3+CD4+ lymphocytes in blood (56). While these pigs are older than the pigs in the present study, another study found that 7 day old piglets had a mean CD3+ lymphocyte population of 57.65% and a mean CD3+CD4+ population of 18.88%, both in peripheral blood (57). Germ-free animals do not have fully developed immune systems, and even following microbial colonization display an increased susceptibility to infections, indicating that some level of immunocompromise is present (58). Therefore, it was not unexpected that the pigs in the present study had lower levels of lymphocyte subsets. We also found that *K. slooffiae* colonized animals had lower levels of CD3+CD27+ and CD3+CD4+CD27+ in MLN. CD27 expression is induced via activation of the CD3 T cell receptor (59). Increased populations of naive CD3+ and CD3+CD8+ cells were also observed in the MLN of *K. slooffiae* colonized animals. Therefore, it appears that K. slooffiae colonization induces a more naïve immune phenotype, although it remains unclear what is driving this phenotype.

Cytokine levels in plasma were altered by *K. slooffiae* colonization, with increased levels of plasma IL-2 and IL-18 as well as decreased TNF-α. Higher IL-2 levels have previously been documented in pigs fed *S. cerevisiae* compared to controls (14). IL-2 is involved in immune tolerance through the induction T cell differentiation to T regulatory cells, and it has been shown that administering IL-2 is therapeutic against multiple autoimmune and inflammatory diseases (60). *S. cerevisiae* has also been shown to decrease TNF-α gene expression in piglets challenged with enterotoxigenic *E. coli* (61). Another yeast species, *Saccharomyces boulardii*, has been shown to decrease TNF-α levels in mice using an ulcerative colitis carcinogenesis model (62). It is proposed that the anti-inflammatory effects of *Saccharomyces* species occur through either reducing the activation of nuclear factor kappa B (NF-κB) or the inhibition of mitogen-activated protein (MAP) kinases (64). Therefore, *K. slooffiae* may act in a similar way.

In MLN cells stimulated *ex vivo* with LPS, cells from animals from the DBCK group had increased secretion of IL-10 and IL-18. Since we also saw an increase in *E. coli* in the DBCK group, the response to LPS stimulation may be because the immune cells have received more exposure to LPS from *E. coli*. The presence of pro-inflammatory IL-18 suggests that immune cells from *K. slooffiae* colonized animals can mount an inflammatory response against LPS more quickly than those not colonized with *K. slooffiae*. *Ex vivo* stimulation of MLN cells with PHA caused lower TNFα and IL-12 in pigs colonized with *K. slooffiae*. This suggests that the immune cells exposed to PHA exert less of an inflammatory response upon T cell activation, as PHA is a selective T cell mitogen (64). However, IL-18 was also higher in DBCK animals, suggesting that there is still some inflammatory response, however, the overall nature of the response remains unclear.

4.5 Conclusion

This study provides evidence that colonization with commensal yeast, specifically *K. slooffiae,* can have significant impacts on the gut environment; including interactions with the bacterial community and metabolite production, resulting in altered gut architecture and immune development. While the current study would suggest a beneficial impact on the pig, future work is needed to investigate the role of *K. slooffiae* in pigs raised under commercial conditions and under states of disease challenge.

4.6 Literature cited

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Chapter 5: General discussion

The fungal community represents a largely unstudied portion of the host microbiome. In pigs the mycobiome is dominated by the yeast *K. slooffiae* (1-5). *K. slooffiae* is a commensal yeast that has been found in diverse geographic conditions and from pigs reared under different production practices (1-10). As such, *K. slooffiae* can be considered a core microbe of the pig mycobiota. *K. slooffiae* has a number of reported benefits including increasing the concentration of SCFAs in the intestine, acting as a protein source (especially the limiting amino acid lysine), and promoting intestinal glycolysis (11, 12). In chapter 2 of this thesis we report that the maternal mycobiome, especially with respect to *K. slooffiae*, is a driver of piglet mycobiome composition. With this result in mind chapter 3 explored how mycobiome composition changes over one production cycle, with special attention paid to *K. slooffiae*. We found that sow *K. slooffiae* levels shaped the piglet mycobiome in early life as well as at the end of a production cycle, with an upset around weaning. This has potential implications for pig production, as it suggests that what piglets are exposed to in the farrowing pen can have long-term impacts on their microbiome. While the long-term impacts of *K. slooffiae* colonization remain unclear, research regarding the use of *S. cerevisiae* is far more common. *S. cerevisiae* has been shown to increase serum IgG levels, increase average daily gain, reduce post weaning diarrhea, alter systemic immune system parameters and increase the ratio of villus height to crypt depth in the intestine (13-16). These studies offer some insight into the impact that yeast can have on pigs, however *S. cerevisiae* makes up less than 1% of the commensal mycobiota in pigs not supplemented with *S. cerevisiae* (2) and studies focusing on the impact of pig derived commensal organism are lacking. With the finding that *K. slooffiae* status is passed from sow to

piglet, future research should be done to determine the reason for differences in *K. slooffiae* colonization levels amongst pigs. Additionally, future research should be done to see if supplementing *K. slooffiae* to pigs early in life results in long-term colonization with *K. slooffiae*, even if they come from sows who did not exhibit high levels of *K. slooffiae* colonization. A study using cross-fostering could be performed to see if it is exposure or genetic factors which influence piglet *K. slooffiae* colonization levels.

While mycobiome research is still less common than studies focusing on the bacterial community, progress has been made into the understanding of the role of fungi in health and development through the use of gnotobiotic models (17, 18). Gnotobiotic studies allow us to look at the impacts of individual microbes while removing the background "noise" created by all of the other microbes (19). Using a gnotobiotic pig model gave us the ability to look at the role that *K. slooffiae*, a pig derived commensal organism, had on the pig, without the confounding variable of host adaptation that would have been present in a gnotobiotic rodent model. In chapter 4, we used a gnotobiotic pig model to look at the impact of *K. slooffiae* on bacterial succession, the host metabolite pool, and intestinal and immune system development. We found that *K. slooffiae* had significant impacts on the intestinal environment, including alterations in bacterial community structure, metabolite production, intestinal architecture and immune system development. These findings suggest that *K. slooffiae* is a beneficial microbe to pigs. However future research is needed to determine the mechanisms through which *K. slooffiae* acts on the host. Mono-colonization studies with *K. slooffiae* may help to distinguish between host physiologic changes due to *K. slooffiae* itself or due to changes in the bacterial community as a result of *K. slooffiae* colonization. Gnotobiotic studies using other fungi should be done to determine if the impacts discussed in this thesis are universal to fungi or unique to *K. slooffiae*.

By comparing the impact of allochthonous fungi found in the feral pig to autochthonous fungi such as *K. slooffiae*, we may be able to determine the impact of colonization versus transient fungi on host physiology. Investigating the impact of high or low *K. slooffiae* colonization as seen in chapters 2 and 3, on immune system parameters such as T cell populations and cytokine production, may have provided insight into the role of *K. slooffiae* in the host under more conventional conditions. Additionally, studies under a state of disease challenge should be done to see how *K. slooffiae* alters disease resilience by way of immune system and intestinal development.

5.1 Limitations

There were several limitations present in this thesis. In chapter 2, the duration of the study was 7 days. We found that after 7 days there was no impact of antibiotic treatment on fungal community structure. However, a study completed after the completion of our study found that there were changes to the human mycobiome at 1 month post antibiotic treatment, indicating that there may be a delayed response (20). Therefor we may have missed the window for fungal community change.

In chapter 3 we were limited by feral pig sample availability, as the samples had been used for a previous study. However, we were still able to get a representative sample that showed only minimal differences between feral pigs from different locations and no difference between the intestinal sections tested.

In chapters 2 and 3 the primers used in fungal sequencing may have limited the detection of common piglet fungi such as *L. corymbifera*, which is not amplified by the ITS2 primers used in these studies. In order to combat these limitations future studies utilizing shotgun

metagenomic sequencing should be performed in order to capture the entire porcine fungal community. Additionally, the method of DNA extraction may have selected against harder to extract fungi. The conclusions in these two studies were also both based on relative abundance data with minimal quantification, therefore it was not possible to determine if the total amount of *K. slooffiae* or other fungi differed numerically or just in terms of relative abundance to the rest of the fungal community. Chapters 2 and 3 did not investigate the role of sow milk or colostrum in fungal community composition. Sow milk has been shown to contain bacteria that may contribute to piglet microbiome development (21). However, one study showed that there was no culturable fungi in sow milk (5). Therefore, although there may be some individual variation in term of the presence of fungi in sow milk, we do not believe that sow milk is a major contributor to the piglet mycobiome.

In chapter 4 we were limited to a maximum of 6 piglets per group due to the design of the gnotobiotic isolators. Additionally, we were limited by the total number of pigs in the litter, as only one sow was used for this study. By using only one sow, we have limited availability to balance perfectly for size and sex. However, using only 1 sow allowed us to not have a litter effect as this was constant throughout the pigs.

5.2 Implications

The research presented in this thesis has several implications for the swine industry. We have shown that *K. slooffiae* is maternally transmitted and showed that what piglets are exposed to during early life can have long term implications in terms of mycobiome composition. We have also shown that *K. slooffiae* is an active participant in the intestine of young pigs and found that it may be beneficial to young pigs. With these findings in mind, it may be possible, after

further research to test efficacy, to develop *K. slooffiae* supplements for pigs that can be fed to sows so that *K. slooffiae* can be passed to the piglets. This has several benefits including decreased labor for producers, as they would not have to dose individual piglets as well as decreased costs to producers since only the sows would require supplementation.

5.3 Conclusions

In summary, this thesis provides further support that *K. slooffiae* is a core member of the porcine mycobiota and provides novel insight that early life mycobiome assembly is driven by the maternal mycobiota, especially with respect to *K. slooffiae*. Most importantly, it clearly shows that *K. slooffiae* is an active contributor to the gut environment and appears to be beneficial to the pig. Exposure in early life results in altered bacterial community structure, metabolite production, intestinal architecture, and immune system development.

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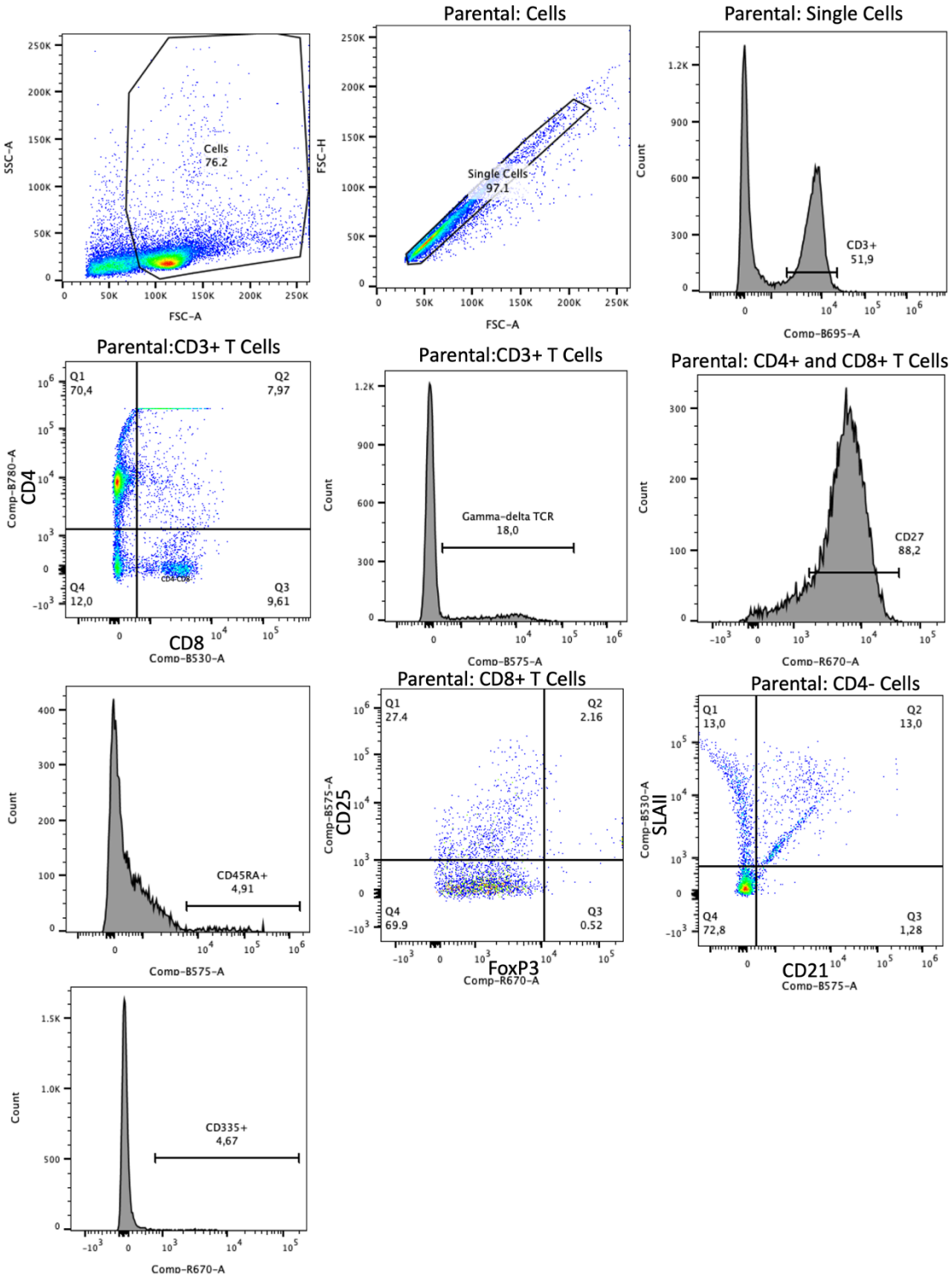
Appendix A: Supplementary figures



Supplementary Figure 1. Gating strategy for flow cytometry analysis.