Effects of environmental factors on monogastric gut microbial community and functional dynamics

by

Brandi Joann Feehan

B.S., Kansas State University, 2018
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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

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Abstract

Fundamental knowledge, for understanding establishment and disturbance of gut microbiota during both health and disease, is the composition and function of the gut microbiome. However, a healthy gut microbiota has not been defined at any profound taxonomic resolution, and even less on a functional level. Previous aims of monogastric microbiome, or comprehensive gut microbial membership, relied greatly on marker gene sequencing, which sequenced less than 0.01% of the microbial genome, limiting our knowledge on microbial functions and strain-level dynamics. The research on understanding the gut microbiota impact on the host is still at a juvenile stage, and much still needs to be learned in understanding the microbiota dynamics in healthy hosts and disturbances on the short- and long-term. To achieve such goals and understand the implications of environmental changes, associated with age development and antibiotic treatment, I utilized two distinct monogastric swine populations. With these swine, I evaluated their gut microbiota for microbial membership, function, and genetic variation.

In my first study, I elucidated the dynamics of bacteria, archaea and fungi populations in the swine gut for the duration of the host lifetime. My objective was to provide a foundational understanding of healthy gut microbiome during long-term development. I collected 234 fecal samples, across 31 time points, from 10 swine from birth through 156 days of age. Samples were collected during the three swine development stages (preweaning, nursery, and growth adult). Next, I performed bacterial 16S rRNA amplicon sequencing for bacteria and fungal qPCR for the dominating fungus of the swine gut, *Kazachstania slooffiae*. My results demonstrated a highly volatile bacteriome, with low *K. slooffiae* presence, in the young, preweaning host. Following weaning, bacterial populations became relatively established with a peak in *K. slooffiae* abundance. Finally, I determined multiple negative, competitive interactions between bacterial and *K. slooffiae* fungi

during the nursery and growth adult stages. I provided evidence for previously unknown competitive interactions which occur throughout the weaned and adult periods. This first study indicated a need for future genetic support of microbial functions pertaining to establishment and competitive dynamics.

My second objective was a thorough investigation into the functions of methanogenic archaea during the host lifetime. Archaea of the monogastric gut are historically understudied relative to bacteria. I performed shotgun metagenome sequencing on a subset of the hosts (n=7) and samples (n=112) from my first objective. I resolved 1,130 microbial genomes termed metagenome assembled genomes (MAGs). Within these genomes were 8 methanogenic archaea MAGs which fell into two orders: *Methanomassiliicoccales* (5) and *Methanobacteriales* (3). I discovered the first US swine MAGs for two archaea, while describing novel evidence of acetoclastic methanogenesis. Furthermore, I described age-associated detection and methanogenic functions. My second objective provided a comprehensive, gene-supported analysis of monogastric-associated methanogens which furthered our understanding of microbiome development and functions.

The focus of my final objective was to determine genetic variation and function of microbes following antibiotic treatments. A distinct swine population, relative to the first study, of 648 weaned swine were assigned to one of three treatments: control (no antibiotic ever), chlortetracycline (CTC) for 14 days, or tiamulin (TMU) for 14 days. Pigs were housed in pens where there were 8 pens/treatment and 27 pigs/pen (i.e. 216 pigs/treatment). Fecal samples were collected from 5 random swine from each of 2 random pens per treatment every collection. Collections occurred 7 days prior to treatment (i.e. day of weaning), and every 7 days until 14 days

past antibiotic treatment with one final collection at 28 days post treatment. Samples were pooled according to pen and collection day, followed by gDNA extraction, library preparation, and shotgun metagenomic sequencing. I curated 81 MAGs and analyzed genetic variation according to pre- and post-treatment. I found 11 MAGs with no statistical difference in detection and statistically consistently high variation in the form of genetic entropy (SDHSE [sustained detection and high sustained entropy] MAGs). The SDHSE MAGs were suggested to be multidrug resistant (MDR) due to their continued detection throughout CTC and TMU treatments. Even though I identified 22 unique antimicrobial resistance genes in SDHSE MAGs, less than a third contained genes with TMU resistance. There are likely additional TMU resistance genes contributing to the SDHSE MAGs detention throughout TMU treatment. Together, this investigation described how MDR microbial populations harbor genetic variation, with potential for additional resistance, and highlighted the need for further antimicrobial investigations into gene AMR functions.

In conclusion, this dissertation offers a comprehensive, functional understanding of the many microbiome members, including bacteria, archaea and fungi. These studies are critical for understanding how monogastric microbes act through the host lifetime and in response to antibiotic treatments, which will aid future endeavors for monogastric health as it pertains to the gut microbiome.

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Approved by:

Major Professor
Dr. Sonny Lee

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Dedication

This dissertation is dedicated to:

My partner, Phillip Haslouer

My parents, Vicki and Tom Feehan

My cats, Chunks and Marie

Chapter 1 - Introduction

Brandi Feehan and Sonny T M Lee

Division of Biology, College of Arts and Sciences, Kansas State University, Manhattan, Kansas, 66506, United States of America.

Introduction to the monogastric gut microbiome

The monogastric gut microbiome plays an important role in hosts' disease and health[1–3]. My dissertation aims to elucidate how the gut microbiome is affected by environmental factors, including aging and antibiotics. Before discussing my dissertation studies, it is essential to have a foundation of monogastric gut microbiomes, and the importance of studying this topic. Microbiome inhabitants come from diverse microbial kingdoms, such as bacteria, fungi, and archaea, with similarly diverse functions and roles, ranging from commensalistic to pathogenic[4]. The numerous interactions amongst microbiome members and its associated host perform many functions, including assisting the monogastric host by metabolizing dietary nutrients and aiding in infection prevention[1–3]. The term monogastric refers to animals, including humans and swine, containing a so-called "simple" digestive system[5,6]. The monogastric microbiome changes according to numerous factors, including: diet[7], host development[8,9], and antibiotic treatments[10,11]. With the complexities underlying these microbiome changes, determining microbial interactions associated with environmental factors is central to understanding the implications of the monogastric microbiome in health and disease[10,11].

While studies have demonstrated gut microbiome establishment starting *in utero*[12,13], the majority of microbiome development occurs following birth in the young host[14]. For example, in humans, the gut microbiome becomes relatively established by three years of age[14], whereas development is thought to be relatively stable at 28 days of age in swine[15]. Further microbiome development occurs in the adolescent and adult hosts, but generally the gut microbiota maintains a homeostatic state through these life stages[14,16]. Although, a dysbiotic microbiome can result in shifts away from gut homeostasis causing disease at any age, from birth[17] through geriatrics[18]. We must understand

what constitutes a homeostatic versus dysbiotic gut microbiome during the monogastric host lifetime so future research can evaluate dysbiotic microbiome therapeutics accordingly.

Beyond determining how and when the monogastric gut microbiome changes, our very approach to gut microbiome studies have recently evolved with shotgun metagenomic sequencing[19,20]. Rather than sequencing a single gene, as in 16S rRNA amplicon sequencing, metagenomic sequencing is a comprehensive approach targeting all genetic content[19,20]. Our scientific community is shifting the focus to microbial functions which are key components in elucidating the roles of gut microbiota in health and disease[21,22]. We can potentially determine functions, and microbial populations harboring distinct functional variants, with metagenomic sequencing. Metagenomic sequencing provides the crucial understanding of functional potential of the gut microbiome which underlies microbial interactions[21,22].

Significance of the monogastric gut microbiome in health and disease

The monogastric gut microbiome performs critical roles throughout the host lifetime. Gut microbes contribute to nutrient acquisition, host development, and gastrointestinal health, amongst numerous additional roles with implications throughout the body[1–3]. On the contrary, a dysbiotic microbiome has been associated with numerous diseases including: inflammatory bowel disease (IBD), diarrhea, obesity, and metabolic syndrome (MetS), among other ailments[23]. Diseases arising from the gut microbiome can be due to individual microbial action, such as from pathogens, or from the coordinated action of multiple microbes via microbial interactions[24]. Moreover, these interactions can span kingdoms, such as interactions between bacteria and fungi[25]. Due in part to the sheer volume of gut microbe inhabitants and differences in the microbial composition within each host, scientists are continually building our understanding of complex microbial interaction

networks within the gastrointestinal system[24,26]. Cleary, understanding the gut microbiome, especially as a complex interaction network, is paramount for elucidating the numerous intricacies impacting health and disease. Although, in order to better understand the role of the monogastric gut microbiome in health and disease, we need to understand factors which influence gut microbiome dynamics.

Environmental factors contribute to microbiome dynamics

The modulation of microbiome homeostasis and dysbiosis is related to the local gut environment which changes according to numerous external factors[10,11]. While there are countless factors influencing the monogastric gut microbiota[10], the focus in the following studies are on age-associated developmental factors and antibiotics. Our focus enabled novel insights into lifetime longitudinal microbiome development and targeted investigations into microbial genetic variation associated with antibiotic treatments.

The gut microbiome develops alongside the host from birth through adolescence and into adulthood[14,16]. Although, as mentioned previously, the majority of development occurs during infancy[14–16]. Age-associated development coincides with multiple factors which influence the gut environment including: diet[7], host development[8,9], and host environment[27–29] (i.e. housing with other hosts, cleanliness, etc.). By following the same host through aging, from birth through adulthood, we can better control individual-host associated influences[30] . Altogether, evaluating longitudinal gut microbiome dynamics allows us to gain insights into development through age-associated factors influencing the local gut environment.

On the other hand, environmental modification as a result of antibiotic use has been associated with drastic changes to the gut microbiome[c]. Antibiotics can affect both microbiome development in young hosts[31,32] while also shifting the microbiome to a dysbiotic state in older hosts[33]. Dysbiotic microbiomes resulting from antimicrobial administrations have been associated with: infections, diarrhea, and, in severe cases, death[34]. Antimicrobial resistance (AMR) is a large concern arising from antibiotic use[34]. With antibiotic administration, there is a natural selection for antimicrobial resistant microbes[35]. Moreover, resistant microbes are typically associated with increased mutation rates, termed hypermutable microbes, resulting in higher genetic variation in subsequent generations[35]. Therefore, continued use of antibiotics has resulted in AMR and multidrug resistance (MDR). Infections of MDR microbes are typically more difficult to treat[36,37] leading to increased morbidity and mortality[38,39]. In order to provide therapies to MDR infections, we need to understand the genetic variation found in monogastrics and how these populations respond to further antibiotic treatments.

I investigated, through separate studies, the influence of environment changes on the gut microbiome. Gut microbes interact in various ways, including microbe-microbe interactions, microbe-host interactions, and microbe-environment interactions[10,11,24,26,32,40,41]. By evaluating environmental influences, we can build our repertoire of knowledge surrounding the monogastric microbiome. My research provides a deeper understanding for future studies aiming to improve gut health and manage AMR.

Evaluation of microbiome membership

In order to evaluate how the gut environment shapes the microbiome, we first need an understanding of how to investigate and measure gut microbiota membership. Scientists evaluate

microbial membership with identifying who is present, with taxonomic identification, and overall diversity measures, such as compositional and alpha (α) diversity[42]. Depending on the methodology used, taxonomic identity can be resolved at a relatively high level, from phyla to genera, to a low level, including species and genetic variants within species[43]. Taxonomic resolution will be discussed further in the following section ("Movement towards metagenomics for advanced genetic analyses including microbial functional potential and functional variation"). Within the monogastric microbiome, *Firmicutes* and *Bacteroidetes* have consistently been accounted for 70-90% of microbes at the phyla level[44]. On the genus level, there are over 200 genera associated with *Firmicutes* including *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus*, and *Ruminicoccus*[44], and 125 genera[45] have been identified in *Bacteroidetes*, such as *Bacteroides* and *Prevotella*[46]. Genera are not as consistently identified across hosts due in large part due to their smaller individual abundances[47] and genera changes due environment factors, like age-factors and antibiotics as discussed previously[48,49]. Moreover, species are even more inconsistent within and between hosts in the gut microbiome environment[50]. Since monogastric microbe taxonomy differs between hosts, especially at more resolved classification levels, taxonomic identification is a standard initial report for monogastric gut microbiome studies[50]. Still, evaluation of specific microbial members does not depict overall microbial diversity, in the gut microbiome network[51], which warrants the use of compositional and α diversity measures.

Traditionally, scientists have evaluated microbial compositional and α diversity, alongside taxonomic identity, to understand gut microbiome development[52]. Compositional diversity index measures the overall taxonomic comparison amongst samples whereas α diversity quantifies how many distinct taxa are present[53]. Across similar aged hosts, of the same host species, we typically find compositional similarity[54–57]. Although, over time, studies demonstrate a changing

composition as the microbes change due to a dynamic gut environment[54–57]. Contrastingly, α diversity increases in the early lifetime until the gut environment reaches a relatively stable level of development amongst hosts[2,50]. A developed gut is expected to have a relatively higher α diversity, compared to a developing environment in the young host, indicating a diverse microbial makeup[58,59]. By evaluating the microbial composition and α diversity, scientists gain an understanding of how the gut microbiome develops during changes in the gut environment[53].

While often described in gut microbiome research, diversity and taxonomy of the monogastric gut microbiome offers limited information in terms of gut and host health[60,61]. A shift has been seen in microbiome research towards obtaining microbial functions and microbial population genetic variants[60–62]. With functions and variants, we can begin to decipher the gut microbiome network of interactions, and interaction modifications via variants[62], with implications on host health[60,61].

Movement towards metagenomics for advanced genetic analyses including microbial functional potential and functional variation

Previous monogastric gut microbiome studies mainly performed 16S ribosomal RNA (rRNA) amplion sequencing which allowed limited analyses for taxonomic and diversity investigations[19,20]. Still, the taxonomic resolution of amplicon sequencing is lacking compared to whole-genome sequencing[19,20]. Therefore, with the growing need for understanding how the gut microbiome influences host health, scientists are turning to enhanced taxonomic and functional analyses via shotgun metagenomic sequencing and metagenome assembled genomes (MAGs)[21,22]. Metagenomic sequencing aims to sequence all genetic content to allow for functions and variants of genes while improving taxonomic identity resolution[21,22]. With metagenomic sequencing reads, we can obtain MAGs which describe highly genetically similar microbial populations[21,22]. In order

to demonstrate the need for metagenome assembled genomes (MAGs), we will first review amplicon sequencing.

Historically, gut microbiome research has predominantly relied on sequencing one conserved gene: the 16S ribosomal RNA (rRNA) gene[63,64]. The 16S rRNA gene is approximately 1,550 basepairs (bps) long relative to microbial genomes which, on average, are 3 million bps[65,66]. Furthermore, PCR amplification generally targets one to three variable regions of the 16S rRNA[67], meaning less than 0.1% of the microbial genome is sequenced. Variations harbored in remaining genetic regions, including the entire genome residing independent of rRNA, are missed by sequencing only a fraction with the rRNA[21,22]. Moreover, only prokaryotes, including bacteria and archaea, contain 16S rRNA, whereas remaining eukaryotes, including fungi and other commensal and pathogenic eukaryotes, will not be discovered through 16S rRNA sequencing[68]. Relying upon a small portion of a gene only found in prokaryotes diminishes the applications of 16S rRNA gene sequencing. While the upfront cost is relatively lower of 16S rRNA amplicon sequencing compared to metagenomic sequencing and the methods to study the microbiome using 16S rRNA gene are well-established[43], there are numerous limitations in using 16S amplicon sequencing to advance gut microbiome research in the current scientific era[19,20].

Metagenomic sequencing is a robust alternative which targets the shortcomings of 16S rRNA sequencing. In sequencing the entire DNA content, metagenomic sequencing has the ability to detect eukaryotes and prokaryotes simultaneously[69]. Bioinformatic applications can utilize sequencing reads to assemble longer sequences, termed contigs[22]. Then these contigs may be clustered together, based on genetic similarity, to form metagenome assembled genomes

(MAGs)[22]. MAGs offer multiple advantages over amplicons including: enhanced taxonomic resolution, functional potential, and functional variation[22].

With a more comprehensive approach to sequencing genetic material, MAGs offer greater resolution, to species and even genetic variations within species, for taxonomy identify of gut microbes[22]. While there are multiple reasons to support enhanced taxonomic resolution, this is especially insightful as we identify specific microbes, such as those with detrimental functions, and trace their distribution across geography and time, as in phylogenetics and epidemiology[70]. One of the largest benefits of MAGs is the ability to determine functional potential from MAG genes[22]. With the rise of antibiotic resistance, MAGs have garnered interest as antibiotic resistance (AMR) genes are easily identifiable in MAGs[71]. Just as the strain resolution is high within MAGS, we also gain the resolution of functional variants[71–73]. In terms of AMR, we can now decipher distinct AMR variants and identify concerning microbial variants of the monogastric gut[71,72]. On a larger scale, with MAGs, we can also begin to understand the functions within the complex interaction network of the gut microbiome[74]. Studies have demonstrated how metabolic pathways differ according to hosts or environmental factors, such as age[75] and diet[76]. Metagenomics, and in particular genome-centric studies, further develop our understanding of the functions of the gut microbiome members in health and disease.

In summary, metagenomic sequencing provides further support for the reason underlying the vast majority of microbiome research: elucidating intricate microbial interactions as they pertain to host health[77,78]. Moreover, metagenomic sequencing, and particularly resulting MAGs, also offer enhanced taxonomic resolution relative to amplicon sequencing. MAGs provide an abundance of genetic information to enhance our understanding of the gut microbiome network.

Introduction to dissertation studies

In my studies to uncover intricacies of the monogastric gut microbiome, I evaluated microbiome dynamics in distinct investigations of longitudinal development and antibiotic treatments. I formed three studies entitled:

1. Stability and volatility shape the gut bacteriome and *Kazachstania slooffiae* dynamics in preweaning, nursery and adult pigs (Chapter 2)
2. Novel Complete Methanogenic Pathways in Longitudinal Genomic Study of Monogastric Age-Associated Archaea (Chapter 3)
3. High proportions of single-nucleotide variations associated with multidrug antibiotic resistance in gut microbial populations (Chapter 4)

The following studies share a key component in advancing gut microbiome research: investigating microbial functions. As discussed previously, microbial functions influence gut microbiome interactions and development, in turn impacting gut and host health. I discuss the interactions of bacteria, fungi and archaea, demonstrating broad inter-kingdom implications. Moreover, I determine functional potential and genetic variants in both age-associated development and antibiotic treatments. The following chapters describe novel microbes and functions previously unknown. Overall, my research is foundational to building the monogastric gut microbiome repertoire for understanding gut health.

Chapter 2 - Stability and volatility shape the gut bacteriome and Kazachstania slooffiae dynamics in preweaning, nursery and adult pigs

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Brandi Feehan[1], Qinghong Ran[1], Victoria Dorman[1], Kourtney Rumback[1], Sophia Pogranichniy[1], Kaitlyn Ward[1], Robert Goodband[2], Megan C Niederwerder[3], Katie Lynn Summers[4], Sonny T M Lee*[1]

*Corresponding author, email: leet1@ksu.edu

[1]Division of Biology, College of Arts and Sciences, Kansas State University, Manhattan, Kansas, 66506, United States of America.

[2]Department of Animal Sciences and Industry, College of Agriculture, Kansas State University, Manhattan, Kansas 66506, United States of America.

[3]Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS 66506, United States of America.

[4]Animal Biosciences and Biotechnology Laboratory, Beltsville Agricultural Research Center, Agricultural Research Center, United States Department of Agriculture, Beltsville, Maryland 20705, United States of America.

Abstract

Background: The gut microbiome plays important roles in the maintenance of health and pathogenesis of diseases in the growing host. In order to fully comprehend the interplay of the gut microbiome and host, a foundational understanding of longitudinal microbiome, including bacteria and fungi, development is necessary. In this study, we evaluated enteric microbiome and host dynamics throughout the lifetime of commercial swine. We collected a total of 234 fecal samples from 10 pigs across 31 time points in 3 developmental stages (5 preweaning, 15 nursery, and 11 growth adult). We then performed 16S rRNA gene amplicon sequencing for bacterial profiles and qPCR for the fungus *Kazachstania slooffiae*. **Results**: We identified distinct bacteriome clustering according to the host developmental stage, with the preweaning stage exhibiting low bacterial diversity and high volatility amongst samples. We further identified clusters of bacteria that were considered core, increasing, decreasing or stage-associated throughout the host lifetime. *Kazachstania slooffiae* was absent in the preweaning stage but peaked during the nursery stage of the host. We determined that all host growth stages contained negative correlations between *K. slooffiae* and bacterial genera, with only the growth adult stage containing positive correlates. **Conclusions**: Our stage-associated bacteriome results suggested the neonate contained a volatile gut microbiome. Upon weaning, the microbiome became relatively established with comparatively fewer perturbations in microbiome composition. Differential analysis indicated bacteria might play distinct stage-associated roles in metabolism and pathogenesis. The lack of positive correlates and shared *K. slooffiae*-bacteria interactions between stages warranted future research into the interactions amongst these kingdoms for host health. This research is foundational for understanding how bacteria and fungi develop singularly, as well as within a complex ecosystem in the host's gut environment.

Introduction

Host-associated microbiomes have critical roles in host health, growth and development. The digestive system contains microbes with a wide array of functions for hosts, such as aiding in nutrient availability, protecting from pathogen invasion and maintaining a healthy gut epithelial barrier[2,50,79]. An imbalance of microorganisms, or their associated functions, in this enteric, or digestive, microbiome can lead to a dysbiotic state and diseased host[2]. Diseases and symptoms associated with a dysbiotic enteric microbiome include inflammatory bowel disease (IBD), diarrhea, obesity, and metabolic syndrome (MetS), among other ailments[23]. In order to develop therapies for these illnesses, it is paramount to understand the enteric microbiome dynamics spanning microbial kingdoms, including bacteria and fungi, throughout the lifetime of swine hosts.

Foundational to evaluating microbial interactions is first determining dynamics of the gut during the host lifetime. Traditionally, scientists have evaluated microbial composition and alpha (α) diversity to understand gut microbial development[52]. Composition includes the overall taxonomic comparison amongst samples whereas α diversity quantifies how many distinct taxa are present. During the host lifetime, we expect to see compositional similarity in similar aged hosts, but distinctions over time as microbes change in abundance[54–56]. Contrastingly, we expect to see α diversity develop in the early lifetime until the gut environment reaches a relatively staple level of development amongst hosts[2,50]. A developed gut is expected to have a relatively higher α diversity, compared to a developing environment, indicating a diverse microbial makeup with similarly diverse roles within the microbiome system and for host health[58,59]. By evaluating the microbial composition and α diversity we will have a foundational understanding of how the gut microbiome develops during the host lifetime which aids investigations into bacterial and fungal interactions.

As mentioned previously, understanding microbial correlations and interactions between microbial kingdoms, including *Fungi* and *Bacteria*, are critical to elucidating diseases impacted by these microbial kingdoms. Previous research has shown a negative correlation, indicating a competitive relationship, between bacterial diversity and fungal abundance[80]. Still, the microbial mechanisms, influencing other microbes and the host alike, underlying these outcomes have not been described. We must understand bacterial-fungal interaction intricacies to provide treatments targeting specific microbes and mechanisms, especially those of bacterial-fungal dysbiotic gut microbiomes.

Current research lacks an understanding of how the dominant swine enteric fungus, *Kazachstania slooffiae,* changes in the majority of the swine lifetime, and how these changes are influenced by the bacterial communities. *Kazachstania slooffiae* is a member of the *Saccharomycetaceae* family, and the fungus is a proposed commensal in the swine gut microbiome[81]. Studies indicate *K. slooffiae* dominates the mycobiome from 70% to 90% of total yeasts, especially following weaning[82,83]. The fungus has been demonstrated to significantly alter the gut microbiota during weaning, leading to a potentially beneficial increase in short chain fatty acid (SCFA) concentration[84]. Although *K. slooffiae* is the primary fungus after weaning, we currently lack a longitudinal understanding of this fungus. Publications have only evaluated *K. slooffiae* abundance from birth until 39 days of age[82,85]. The average time to market age is 160 days, so prior publications have only evaluated *K. slooffiae* dynamics in 25% of the swine lifetime to market[86]. Moreover, previous studies have identified eight SparCC correlations between *K. slooffiae* and bacterial genera from nursery-aged hosts[87,88]. We hypothesized there were more inter-kingdom correlations occurring throughout the swine lifetime (including preweaning and growth adult as these were not studied previously) which influence microbiome establishment and host health[80].

Our study aimed to elucidate novel stage-associated bacteriome-*K. slooffiae* correlations to build a foundation for future inter-kingdom interaction studies.

This study highlights development of bacteria, fungi and host, with an investigation into bacterial-fungal correlations. We followed 10 swine from birth. We first determined the foundational gut microbiome development during the host lifetime. Studies have demonstrated various factors from biology, such as host diet and housing environment, to methodology, including DNA extraction and bioinformatic approaches, impacting resulting identification of microbes and microbial diversity[89–91]. Therefore, we aimed to first provide a baseline understanding of how our gut bacteria changed in the lifetime of the 10 swine hosts. With this knowledge, we could then further interpret how the microbial composition and α diversity pertained to microbial inter-kingdom interactions and potential implications on host health at various ages. Understanding inter-kingdom interaction, influenced by gut development, in the swine may provide insights into the intricate relationship between the host and the microbiome. Foundation to this longitudinal study, swine were grown in three stages which varied according to host development, diet and housing: preweaning (milk diet and housed with littermates and dam; birth-21 days of age), nursery (pellet diet and co-housed with other litters; 21-80 days) and growth adult (pellet diet and co-housed with other litters; 80-122 days). As discussed previously, directly following weaning into the nursery stage in swine hosts, one fungus has been consistently identified in the enteric mycobiome: *Kazachstania slooffiae*[83,88]. For this reason, our study focused on elucidating longitudinal dynamics between *K. slooffiae* and bacteria.

In our study, we determined specific host-age and -dietary stage microbiome development characteristics. These included an increasing microbial diversity, decreasing volatility and increasing fungus *K. slooffiae* in the young host (preweaning and nursery developmental stages).

The older host (growth adult stage) microbiome was relatively established with a complex correlation network amongst bacteria and *K. slooffiae*. Together, these findings indicated a dynamic microbiome development from birth until weaning with an increasing number of inter-kingdom interactions throughout the host lifetime.

Materials and Methods

Hosts and study design

We followed 10 swine over the course of their lifetime, with fecal collections, rectal temperature, weights, and general health observations collected from 2 days to 157 days of age, to understand successive shifts in microbial populations (Ch2_host_and_dam_demographics_diets_samples.xlsx; Ch2_general_observations_scoring.pdf). We started the study with 10 swine, but one pig died prematurely at 28 days of age. The experimental unit was each individual swine. The hosts were housed indoors and fed distinct diets according to their stage of life. Five dams were randomly selected from the same farrowing group, and one male and one female were randomly selected per dam. Swine were housed with their dam in the preweaning stage, in groups of 5 in the nursery stage, and all in one pen during the growth adult stage. Hosts were sampled in three stages: preweaning, nursery, and growth adult. The preweaning diet consisted of mother's milk and potentially feed as the hosts grew old enough to reach their mother's trough. Nursery diet, phase 1, transitioned from milk to pelleted feed after weaning from the mother and moving into a new barn environment. A second pelleted feed was fed during nursery phase 2, while a meal was fed for nursery phase 3. The growth adult stage also included three phase diets with an initial move into another barn environment accompanying the nursery-growth transition. Hosts did not receive antibiotics or antifungals prior to or during the study. Males were castrated during the preweaning

stage. Pigs were managed according to the Kansas State University Institutional Animal Care and Use Committee (IACUC) approved protocol #4036, and methods are reported according to ARRIVE guidelines. Additionally, the authors confirmed that all methods were performed in accordance with relevant guidelines and regulations, and all methods were approved by Kansas State University.

We performed fecal collection with a fresh set of sterile gloves using the free-catch method, prior to contact with the ground. We collected fecal samples every five days during preweaning and nursery stages, and every seven days during the growth adult stage. Immediately after collection, samples were stored in either a sterile 15mL tube or sterile bag, kept on ice, and then transported to the laboratory for subsequent storage at -80°C until genomic DNA extraction.

DNA extraction and marker gene sequencing

We used the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Inc.; Norcross, GA) to extract the microbial DNA from the fecal samples. We used the manufacturer pathogen detection protocol without bead beating and utilized only 30µL elution buffer per sample. Extracted DNA was quantified with Nanodrop and a Qubit™ dsDNA BR Assay Kit (Thermo Fisher; Waltham, MA) for sample DNA quality and concentration. DI water was utilized during quantification as a negative control. Extracted microbial DNA was stored at -80°C until library preparation. Bacterial 16S rRNA gene V4 region was amplified during library preparation via Illumina's Nextera XT Index Kit v2 (Illumina, Inc.; San Diego, CA) (primers: 515F, GTGCCAGCMGCCGCGGTAA and 806R, GGACTACHVGGGTWTCTAAT)[92]. Library preparation and subsequent sequencing also included a no template negative control. Sequencing was done on an Illumina MiSeq which generated paired-end 250bp reads.

Kazachstania slooffiae qPCR

We performed the *K. slooffiae* qPCR, with the SensiMix™ SYBR® Hi-ROX Kit (Bioline, Meridian Bioscience; Cincinnati, OH), as previously described (primers: KS-f, ATCCGGAGGAATGTGGCTTC and KS-r, AGCATCCTTGACTTGCGTCG)[82]. Master mix components and qPCR conditions are listed in Ch2_*Kazachstania_slooffiae*_qPCR.xlsx. Each qPCR run included at least one PCR-grade water with the master mix as a non-template control (NTC), with one *K. slooffiae* positive sample repeatedly used across plates as the positive control.

Bioinformatic and Statistical Analysis

We used cutadapt and DADA2 in QIIME2 v2019.7 (https://qiime2.org/) to trim and perform quality control for the sequencing reads (Ch2_raw_QIIME2_sequence_analysis.xlsx)[93,94]. Reads in which no primer was found were discarded. The reads were truncated at locations where 25-percentile of the reads had a quality score below 15. Diversity analysis was carried out at a sampling depth of 11,105 reads. The pre-trained classifier offered by QIIME2 using the SILVA version132 (https://www.arb-silva.de/documentation/release-132/) database was used for taxonomic assignment of bacteria[95–97]. We used a weighted UniFrac, generated from QIIME2, on the rarefied dataset (11,105 reads) to evaluate differential microbial composition among the samples in different stages, and we utilized a QIIME2 principal coordinate analysis (PCoA) to visualize the microbial composition structure based annotated ASVs (Ch2_scripts.pdf). The following applications were utilized in generating the PCoA composition plot with RStudio version 1.3.1093 (https://www.rstudio.com/products/rstudio/older-versions/): tidyverse version 1.3.1 (https://cran.r-project.org/package=tidyverse), qiime2R version 0.99.6 (https://github.com/jbisanz/qiime2R), plyr version 1.8.7

(https://www.rdocumentation.org/packages/plyr/versions/1.8.7), and ggpubr version 0.4.0 (https://CRAN.R-project.org/package=ggpubr)[98–102]. We calculated α diversity to represent the species diversity in each sample. We utilized Shannon index, estimated number of species (ENS), and Faith's phylogenetic diversity, all within QIIME2, to measure the number of ASVs and the uniformity of ASV abundance for diversity evaluation (Ch2_scripts.pdf)[94]. Kruskal-Wallis was used in QIIME2 to provide overall and stage pairwise statistical analyses for Shannon, ENS, and Faith's phylogenetic diversity[94]. We calculated Shannon effective number by calculating the exponential [exp(H)] of the original Shannon diversity index (H)[103]. We used PERMANOVA in QIIME2 on Bray-Curtis dissimilarity index to test if there were statistically significant differences between stages[94]. Volatility results were analyzed within QIIME2 on the first axis of the PCoA to indicate how dispersed samples were at the associated swine age[94].

We further used DESeq2 version 1.30.1 (https://github.com/mikelove/DESeq2), in RStudio, to mark the statistical differences in the bacterial populations (phyla and genera) predominance between the stages and to generate heatmaps with pheatmap version 1.0.12 (https://CRAN.R-project.org/package=pheatmap)[102,104,105]. Adjusted p-values were utilized, rather than standard p-values, as the adjusted values incorporated the Benjamini-Hochberg false discovery rate (FDR)[104,106].

16S rRNA gene amplicon genera and fungal qPCR Ct values were utilized in a SPIEC-EASI co-occurrence network analysis as previously performed in RStudio using SpiecEasi version 1.2.4 (https://github.com/zdk123/SpiecEasi), devtools version 2.4.3 (https://CRAN.R-project.org/package=devtools), phyloseq version 1.4.0 (https://bioconductor.org/packages/phyloseq/), and igraph version 1.2.11 (https://CRAN.R-

project.org/package=igraph)[87,107–109]. Specific SPIEC-EASI parameters included: Meinhausen–Bühlmann estimation method, lambda minimum ratio of 0.01, and nlambda of 20[107]. SPIEC-EASI utilized a neighborhood selection method termed Meinshausen and Bühlmann (MB)[107,110]. The MB method has been demonstrated to control FDR[111]. Correlations were performed for each stage (preweaning, nursery, and growth adult) with corresponding and fungal qPCR Ct values, according to individual samples (i.e. individual swine and single time point). Correlation plots were simplified to only correlations connected to the fungal node in each stage.

All bioinformatic scripts can be found in Ch2_scripts.pdf.

Results and Discussion

We collected a total of 234 samples across 31 time points (5 preweaning, 15 nursery, and 11 growth adult) from 10 pigs (Ch2_host_and_dam_demographics_diets_samples.xlsx). A total of 10,187,636 sequences resulted from sequencing; we recovered an average of 33,394 ASVs per sample following QIIME2 quality control. Out of the recovered ASVs, an average of 80.1% (79.7% bacteria and 0.4% archaea) populations were annotated with SILVA version 132 (Ch2_QIIME2_taxonomy.xlsx).

Volatility in the preweaning stage preceded microbial establishment and stability in later growth stages

As shown in Figure 1.1A, the weighted UniFrac PCoA illustrated a distinct clustering of bacterial community composition between the three growth stages as the pig transitioned from young host to adult (Ch2_QIIME2_weighted_unifrac_PCoA.qzv; can be uploaded and viewed at https://view.qiime2.org/). We further observed convergence amongst dietary clusters of the swine

hosts in the nursery stage, with the two latter diet-stages of nursery being more similar to the growth adult hosts. We showed in our study that a young host lacked an established, shared microbiome, but converged with age and environmental changes such as diet and shared housing. The preweaning and first part of the nursery stages had the most divergent microbial composition amongst the individual swine. After this first nursery stage, the composition was relatively similar amongst the latter two nursery and growth stage swine. These patterns suggest the microbiome could be highly influenced by their respective diets during the different stages, and was rather stable once the microbial members had established[54–56]. Previous research has illustrated distinct microbial populations in swine hosts according to stage of development[54–56].

Our α diversity results (Shannon index, Faith's phylogenetic diversity, effective number of species (ENS), Shannon effective number, and Bray Curtis dissimilarity index) paralleled the PCoA analysis, indicating an establishing microbiome in the young swine (Figures 1B, Figure 1.1C, Ch2_QIIME2_taxonomy.xlsx, Ch2_QIIME2_Shannon_diversity_index.qzv, Ch2_QIIME2_ENS.qzv, and Ch2_QIIME_Faith's_phylogenetic_diversity.qzv; each QIIME2 file can be uploaded and viewed at https://view.qiime2.org/). We found that α diversity increased throughout the lifetime. We demonstrated the increasing diversity as overall stage comparisons (preweaning [P] vs nursery [N]: N vs growth adult [G]: and P vs G) were significantly different ($p \leq 0.001$) for all α diversity measures according to PERMANOVA and Krustal-Wallis analyses. Studies have indicated this increase in microbial diversity during host development is typical across many different host species[2,50]. When we investigated the data longitudinally according to sampling day, the preweaning host demonstrated comparatively lower diversity which increased until weaning. This developmental diversity increase was followed with a relatively stable period during the nursery stage. We found distinctions in α diversity methods in the growth adult host.

Shannon index and Faith's phylogenetic diversity demonstrated small increases in the growth adult α diversity, whereas ENS and Shannon effective number illustrated a greater range of α diversity in the older swine. These distinctions in α diversity in the older swine should be further evaluated for an enhanced understanding of diversity in older swine, and how a wider range of diversity across swine could impact swine health.

Volatility results corroborated previous findings of a changing neonate microbiome which established in the weaned host (Ch2_QIIME2_volatility.qzv; can be uploaded and viewed at https://view.qiime2.org/). Our microbial composition volatility index in the preweaning host hovered near -0.5 while approaching 0 in the early nursery stage (Figure 1.1D). These volatility findings further suggested that the young preweaning host had a relatively more volatile, fluctuating microbiome. Our results were consistent with another mammalian study which demonstrated a volatile youth microbiome establishment period in children aged from birth to approximately 3 years of age[112].

Together, our PCoA, diversity indices and volatility analyses suggested that the preweaning neonate host contained a developing gut microbiome which started establishing in the nursery stage. We showed that the microbiome was converging in the early nursery host, and there were comparatively fewer changes in microbial diversity after the convergence of the microbial community in the nursery host. We suggest that the forming and establishment of microbial populations during the preweaning and early nursery stages was likely crucial to the well-being of the swine host. Previous research demonstrates the importance of early microbiome dynamics as abnormal neonate gut microbiome development can result in diabetes, IBD and obesity[23,113].

Microbial-host stage development suggested metabolic and pathogenic potential associations

Our study supported previous bacterial establishment dynamics while elucidating novel stage-associations, highlighting a need for functional determination of the enteric microbiome according to host development. We analyzed the host microbial membership and identified 23 phyla (Figure 1.2, Ch2_DESeq2.xlsx). We demonstrated a core bacterial population consisting of two phyla (*Bacteroidetes* and *Firmicutes*) which dominated throughout the lifetime of the swine host, suggesting these bacterial populations have essential implications to the host's health and well-being[55,114,115]. Our study showed that *Bacteroidetes* and *Firmicutes* were the predominating core microbes (Figure 1.2A). These results were consistent with findings from previous research demonstrating consistent domination of *Firmicutes* and *Bacteroidetes*[55,114,115]. *Firmicutes* and *Bacteroidetes* are known to metabolize carbohydrates into short chain fatty acids (SCFAs)[116,117], suggesting that the two core phyla in our results have a wide range of beneficial attributes for the swine including acting as a cellular energy source, protecting DNA, and modulating diseases[118–120]. Therefore, given the necessity for energy and continual carbohydrate availability throughout the host lifetime, it is reasonable to identify *Firmicutes* and *Bacteroidetes* throughout the host life.

DESeq2 differential phyla distinctions between stages (adjusted $p \leq 0.05$) suggested microbes were changing between preweaning and nursery stages but were relatively stable between nursery and growth adult swine. Two phyla identified in preweaning swine, compared to nursery swine, contained distinct microbes with potentially different metabolic implications: *Euryarchaeota* (log2-fold change: 3.06) and *Lentisphaerae* (log2-fold change: 4.26) (Figure 1.2B). *Euryarchaeota* is an archaeon which has been associated with improved fiber digestion[121,122]. We hypothesized that microbes within the *Euryarchaeota* phylum were working alongside and with the bacterial community to shape the host microbiome, which can influence overall host health and well-

being[123,124]. Alternatively, *Lentisphaerae* is thought to have a role in SCFA production resulting in a crucial source of energy for the swine host[125,126]. This differential identification of two carbohydrate metabolizing phyla, *Euryarchaeota* and *Lentisphaerae*, supports the different dietary sources of carbohydrates during the preweaning and nursery stages. Compared to the preweaning host, we identified three metabolic-associated phyla in the nursery host: *Deferribacteres* (log2-fold change: -31.09), *Fibrobacteres* (log2-fold change: -5.50), and *Tenericutes* (log2-fold change: -4.28). *Deferribacteres* is associated with diets containing iron[127–129]; *Fibrobacteres* is known for metabolizing non-soluble polysaccharides or carbohydrates[130]; and the function of *Tenericutes* remains elusive although the bacteria has been positively correlated with diets high in protein[131]. These three phyla remained unchanged between the nursery and growth adult swine suggesting that the microbes might perform similar metabolic roles during both developmental stages. Our observations of distinct microbial populations through the different stages of the pig paralleled the PCoA, diversity and volatility results, indicating a distinct gut microbiome composition and development during preweaning and early nursery. Considering previous research, we surmised that alongside bacteria and archaea establishment, microbial metabolic roles contributed to this stage-associated development under the influence of host factors, especially diet.

In addition, we also observed that the differential phyla indicated development of stage-dependent potential opportunistic pathogens (Figure 1.2B). Preweaning-associated potential opportunistic pathogens included (Figure 1.2B): *Fusobacteria* (log2-fold change: 6.2)[132–134], *Synergistetes* (log2-fold change: 5.6)[135,136], and *Proteobacteria* (log2-fold change: 3.8)[137,138]; nursery: *WPS-2* ([P vs N log2-fold change: -25.5][N vs G log2-fold change: 4.5])[139], and *Spirochaetes* ([P vs N log2-fold change: -1.4][N vs G log2-fold change: 0.9])[140]; and growth adult: *Fusobacteria* (log2-fold change: -10.4)[132–134] and *Synergistetes* (log2-fold change: -2.7)[135,136]. Interestingly,

Fusobacteria and *Synergistetes* were found in both the preweaning and growth host. Further investigation is needed to evaluate the pathogenicity and determine the developmental significance of these phyla in the nursery growth swine.

We identified 25 genera with an average relative abundance greater than 1% amongst the three stages (Figure 1.3 and Ch2_DESeq2.xlsx). Unlike the phyla level analysis, we did not observe a core genus but instead identified three distinct clusters, based on tree branches and detection patterns, throughout the lifetime of the host (Figure 1.3A). The first cluster consisted solely of *Bacteroides* as the bacterial population decreased post-weaning. *Succinivibrio* and *Selenomonas* appeared sporadically in the mid-nursery host followed by plateau in the growth adult stage. The final cluster, with 22 genera, generally appeared at a higher relative abundance earlier, than *Succinivibrio* and *Selenomonas*, in the preweaning or newly weaned host. Interestingly, although *Bacteroides*, *Succinivibrio*, and *Selenomonas* are all heavily reliant on carbohydrate utilization[141–143], our data suggested that these genera were absent during different developmental stages. We hypothesized this could be related to these bacteria utilizing distinct carbohydrate sources[144–146]. Future research is necessary to evaluate how these bacterial species were utilizing dietary carbohydrates and interacting among the microbes and host.

The majority of stage-associated genera were identified in the nursery host which could indicate a need for specialized microbial roles in SCFA productions during this stage. *Bacteroides* ([P vs N log2-fold change: 2.4] [N vs G log2-fold change: 6.2]) was decreasing in relative abundance throughout the pig's life stages (Figures 3A and 3B)[141]. Conversely, *Succinivibrio* ([P vs N log2-fold change: -10.2;][N vs G log2-fold change: -1.8) was increasing through the stages[146]. The remaining potential SCFA-associated genera that were associated with the nursery host were

Faecalibacterium ([P vs N log2-fold change: -12.8][N vs G log2-fold change: 4.5])[147], *Prevotella 7* ([P vs N log2-fold change: -14.4][N vs G log2-fold change: 1.5])[148], *Prevotella 1* ([P vs N log2-fold change: -8.2][N vs G log2-fold change: 2.6])[148], *Subdoligranulum* ([P vs N log2-fold change: -7.6][N vs G log2-fold change: 3.1])[88], *Prevotella 9* ([P vs N log2-fold change: -8.0][N vs G log2-fold change: 2.4])[149], *Alloprevotella* ([P vs N log2-fold change: -2.4][N vs G log2-fold change: 2.9])[150], *Prevotellaceae NK3B31 group* ([P vs N log2-fold change: -3.2][N vs G log2-fold change: 1.6])[151], *Ruminococcaceae UCG-014* ([P vs N log2-fold change: -2.2][N vs G log2-fold change: 2.3])[152], *Ruminococcaceae UCG-005* ([P vs N log2-fold change: -3.1][N vs G log2-fold change: 0.9])[152], and *Ruminococcaceae UCG-010* ([P vs N log2-fold change: -1.3][N vs G log2-fold change: 0.8])[152]. The nursery host contained the most genera with SCFA metabolizing potential, suggesting that this is related to the microbiome dynamics as the microbes were working towards establishing. The bacteria populations within these genera associated with the nursery host could have taken advantage of the perturbations during these stages to proliferate. Akin to the SCFA potential metabolism findings, potential opportunistic pathogen genera were also only identified in nursery swine: *Streptococcus* ([P vs N log2-fold change: -2.6][N vs G log2-fold change: 1.4])[153] and *Terrisporobacter* ([P vs N log2-fold change: -2.0][N vs G log2-fold change: 1.6])[154]. We observed that potential opportunistic pathogens were solely in the nursery host, suggesting that a turbulent microbiome enhanced the risk of pathogen development[59]. Although our present study provided insights into the microbial shifts during the different life stages of the swine, clearly there are complexities during microbiome establishment which warrant increased investigation. Further studies should elucidate how microbial metabolic roles and interactions influence microbiome establishment and pathogen prevalence.

Temporal dynamics of Kazachstania slooffiae and association with bacterial diversity

Our findings suggested that fungal-bacterial interactions in the swine host could influence both bacteriome and mycobiome establishment and dynamics, therefore leading to the decline in *K. slooffiae* abundance in hosts. We performed qPCR and demonstrated varied *K. slooffiae* abundance according to developmental stage (Figure 1.4). We noticed the fungus was absent in the preweaning host but its presence peaked in the nursery host from 25-46 days of age, with a steady decrease in abundance past 46 days of age. We determined fungal presence was more dispersed in the older host, as indicated by a larger 95% confidence interval. Interestingly, we found the increase in *K. slooffiae* coincided with the establishment of the microbiome near weaning. Previous studies have indicated an increase of *K. slooffiae* in the early nursery stage (swine hosts aged 21-35 days)[83,87]. *Kazachstania slooffiae* abundance past 35 days of age were previously unknown. Our findings showed that *K. slooffiae* abundance declined during the late nursery stage and plateaued in the growth adult stage, adding to the growing knowledge in the understanding of this fungi. Our fungal research suggested that *K. slooffiae* underwent stage-specific growth patterns, similar to that of the bacteriome. The factors which directly influenced *K. slooffiae* increase and decline are not yet known. Prior publications indicate associations between members within the microbiome, including between fungi and bacteria, may have implications to the well-being of the hosts[80].

We performed taxonomic correlation analyses to further investigate fungi-bacteria interactions in the gut microbiome. Our increasing correlation network complexity with host age and lack of shared *K. slooffiae*-correlating genera across stages highlighted stage-dependent microbiome development. We simplified our correlation models to depict direct correlations between *K. slooffiae* and genera according to developmental stage (Figure 1.5 and Ch2_SPECI-EASI.xlsx).

We identified 65 correlations (3 in preweaning, 30 nursery, and 32 growth adult). Previous research has indicated increasingly complex fungal-bacteria network correlations as both the microbiome and host develop from preweaning to nursery, but growth adult stage correlates were previously unknown[87]. We identified only two shared correlates between the nursery and growth adult stages: *Rikenellaceae RC9 gut group* and *Candidatus Gastranaerophilales bacterium Zag*. The significance of these genera, especially pertaining to *K. slooffiae*, are not understood and are a topic for future research. The lack of shared *K. slooffiae* correlating taxa may be related to stage-specific bacteria and stage-specific bacteriome-mycobiome interactions.

Our specific network correlation highlighted novel associations between *K. slooffiae* and the bacteriome throughout the host lifetime, suggesting the changes associated with weaning, including dietary change and stress, might have allowed for *K. slooffiae* expansion while the fungal decline may be attributed to competition with bacteria. Previous publications have identified eight correlations with *K. slooffiae*[87,88]. Our results included three out of the eight prior *K. slooffiae* correlations: *Lactobacillus* (correlation coefficient -1.2, growth adult), *Prevotella 9* (-1.2, growth adult), and *Prevotella 2* (-1.1, nursery)[88]. Previous research indicated positive correlations of *K. slooffiae* and *Lactobacillus*, *Prevotella 9*, and *Prevotella 2*, whereas our correlations were negative[88]. We surmised that negative correlation between *Lactobacillus* and *K. slooffiae* would be analogous to the inhibition of *Lactobacillus* growth by *Candida* in humans[80]. Previous research has identified genetic similarity between *K. slooffiae* and *Candida*[81,155]. Previous studies suggested that *Lactobacillus* may work alongside other bacteria to deter *Candida* growth, such as through short chain fatty acid production[80]. In fact, for our findings, the majority of our network correlations between *K. slooffiae* and genera were negative, with only three positive correlations (*Rikenellaceae RC9 gut group* (0.9), *Prevotellaceae NK3B31 group* (0.7), and *Ruminococcaceae*

UCG-005 (0.4)) were identified in growth hosts. Inverse abundances between fungi and bacteria are indicative of competition or amensalism[156], which could explain the sharp decline of *K. slooffiae* populations in the nursery host (Figure 1.4). We further hypothesized that the post-weaning increase of *K. slooffiae* might be attributed to the dietary change as *K. slooffiae* is unable to utilize milk galactose[81]. The dietary transition and host stress from preweaning to nursery might have allowed the increase in *K. slooffiae* populations, even with bacterial establishment relatively progressed[81,88]. Our correlation network results showed numerous (63 novel correlations, Figure 1.5) novel *K. slooffiae* correlations which could aid in divulging establishment dynamics within the bacteriome and mycobiome.

Conclusions

We provided a comprehensive evaluation of how bacteria and the fungus, *Kazachstania slooffiae*, developed through the different life stages of swine. The young preweaning host demonstrated comparatively low microbial diversity which increased near weaning. The growth adult host had a relatively similar microbiome overall compared to the nursery host, yet stage-specific associations, such as potential pathogens and fungal development, were noticed. We noticed the developing microbiome across hosts, even with differences in dam diet and parity status. Future research, with more swine, are crucial to determining the extent to which these dam factors and stage-associated characteristics influence microbiome development dynamics. Our findings provided a foundation for gut microbiome studies.

While microbial inter-kingdom interactions are known to have implications on host health, the intricacies of dynamics between bacteria and fungi are not well understood. We determined that distinct microbial taxa, diversity, and bacterial-fungi correlations were associated with different

stages of life. These stage-associated attributes indicated there could be further stage-associated characteristics such as illness-inducing pathogens and energy providing carbohydrate metabolizing microbes. Future research is crucial to understand the interplay amongst microbes, especially on the functional level pertaining to carbohydrate utilization and relating these findings back to host health. As we evaluated general-swine host stage, additional research is also necessary to attribute specific host growth, development, and environmental factors, such as diet and housing, to the diversity changes we identified.

Declarations

Ethics approval and consent to participate

Pigs were managed according to the Kansas State University Institutional Animal Care and Use Committee (IACUC) approved protocol #4036.

Availability of data and material

The dataset(s) supporting the conclusions of this article is(are) available in the NCBI repository, BioProject PRJNA798835, https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA798835.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Brandi Feehan (B.F), Robert Goodband (R.G.) and Sonny Lee (S.L.) designed the study. Sample collection was performed by B.F. B.F., Victoria Dorman (V.D.), Kourtney Rumback (K.R.), and

Kaitlyn Ward (K.W.) completed DNA extraction and Nanodrop and Qubit quality analysis. B.F. completed *Kazachstania slooffiae* qPCR. Qinghong Ran (Q.R.) performed the QIIME2 pipeline on 16S rRNA amplicons from sequencing. B.F. performed further DESeq2 and SPIEC-EASI analysis, attributed biological relevance, wrote the manuscript and prepared figures, and supplemental files. Katie Lynn Summers (K.S.) provided *K. slooffiae* and SPIEC-EASI insights. B.F. and S.L. performed major manuscript and figure refinement while remaining authors contributed to lighter refinement. All authors contributed to manuscript revision, read, and approved the submitted version.

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Figures

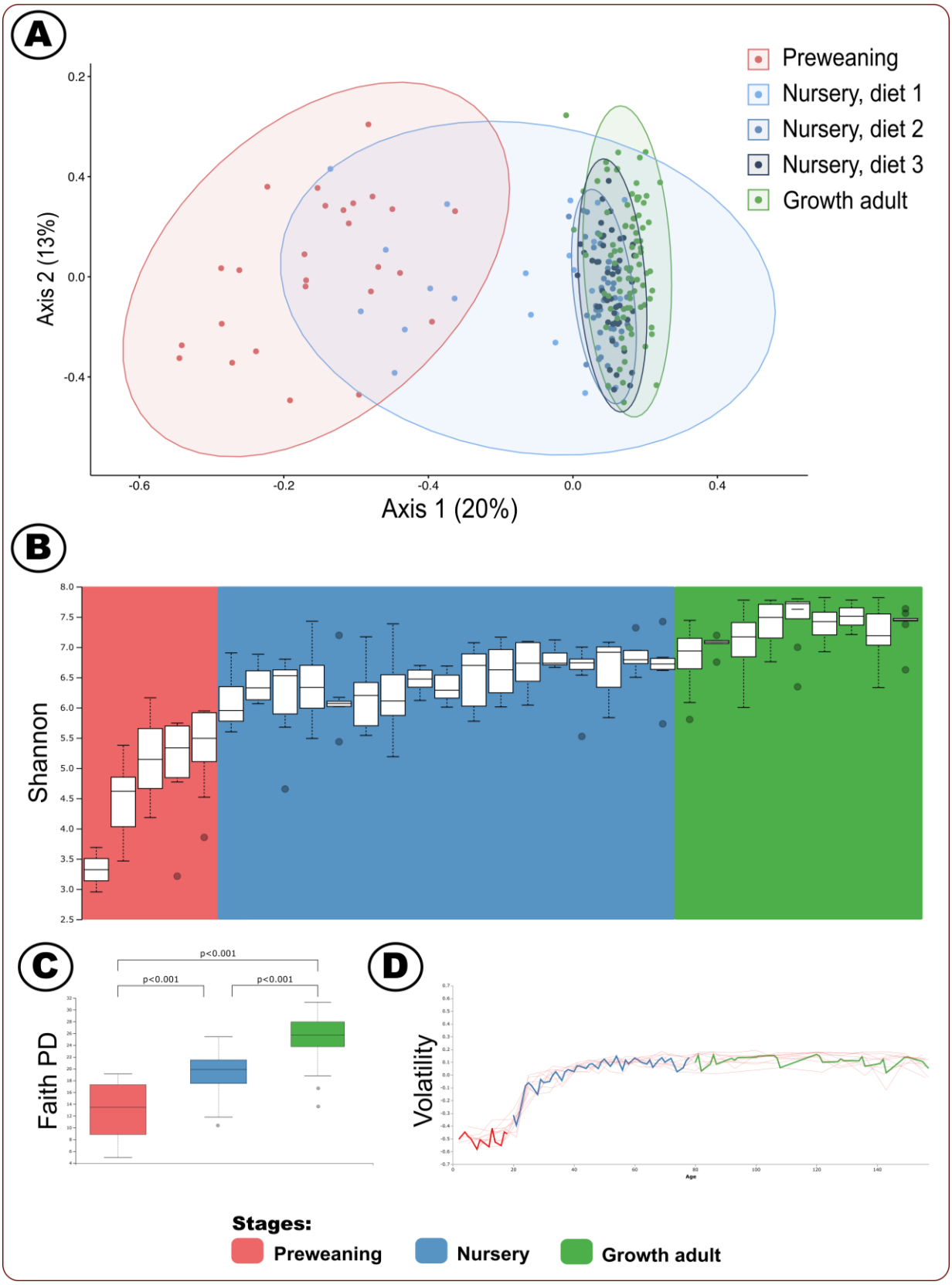
A
Preweaning
Nursery, diet 1
Nursery, diet 2
Nursery, diet 3
Growth adult
Axis 2 (13%)
Axis 1 (20%)
B
Shannon
C
Faith PD
p<0.001
p<0.001
p<0.001
D
Volatility
Stages:
Preweaning
Nursery
Growth adult

Figure 2.1: A) Weighted uniFrac PCoA plot[98–101] depicting composition; dots represent distinct samples. Nursery stage is separated according to the three diets fed during the stage. B) Longitudinal Shannon diversity with Kruskal-Wallis statistical analysis[99]. C) Faith's phylogenetic diversity[99] (PD). D) Volatility control chart of the first axis of the PCoA[99]. Figure was edited in Inkscape version 1.0.2 (https://inkscape.org/)[157].

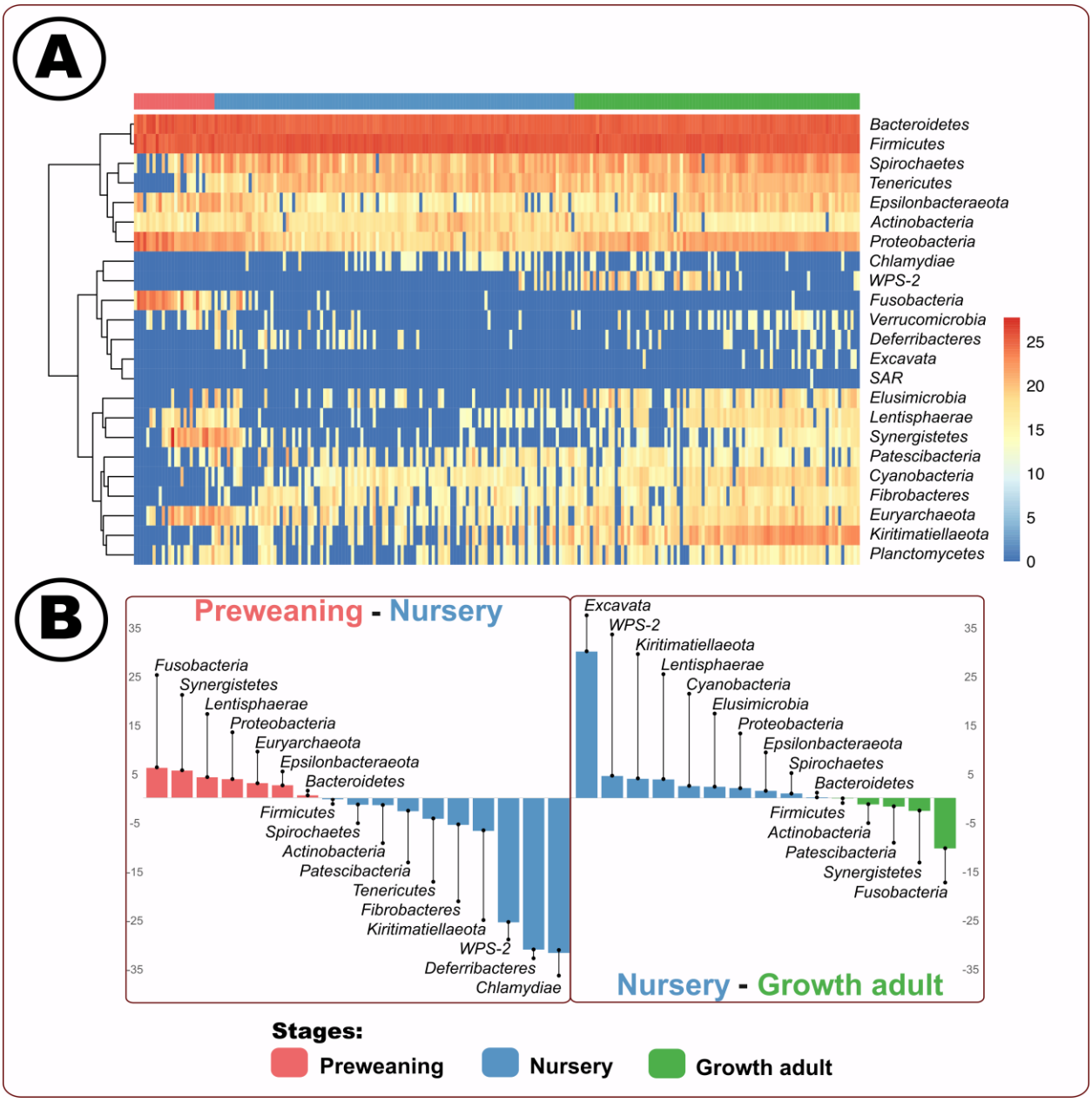

Figure 2.2: A) Longitudinal heat map of DESeq2 resulting phyla relative abundances; each column represents a distinct sample[102,104,105]. B) DESeq2 differentially identified ($p<0.05$) phyla[102,104]. Figure was edited in Inkscape version 1.0.2 (https://inkscape.org/)[157].

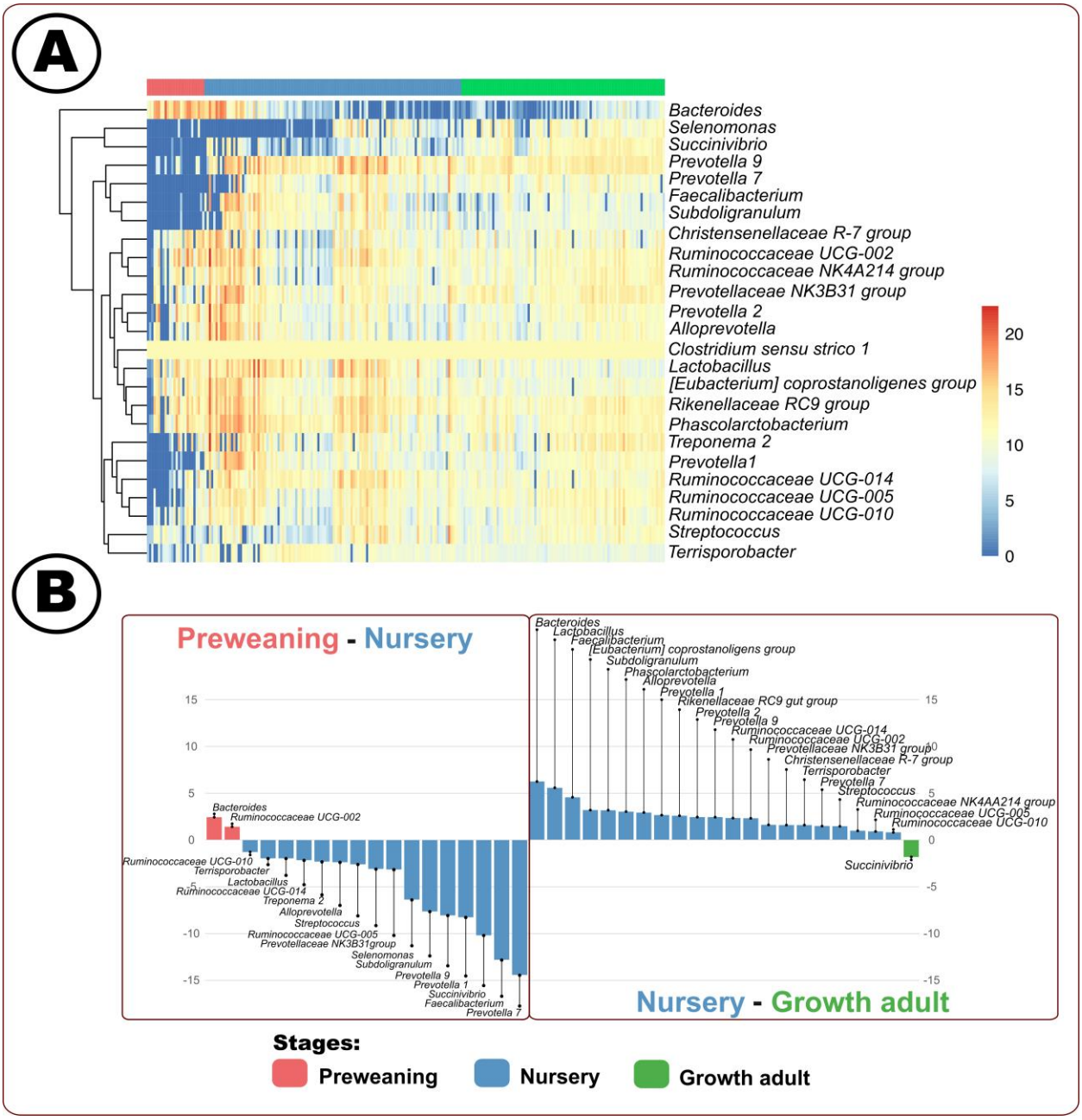

Figure 2.3: A) Longitudinal heat map of DESeq2 resulting genera relative abundances; each column represents a distinct sample[102,104,105]. B) DESeq2 differentially identified ($p<0.05$) genera[102,104]. Figure was edited in Inkscape version 1.0.2 (https://inkscape.org/)[157].

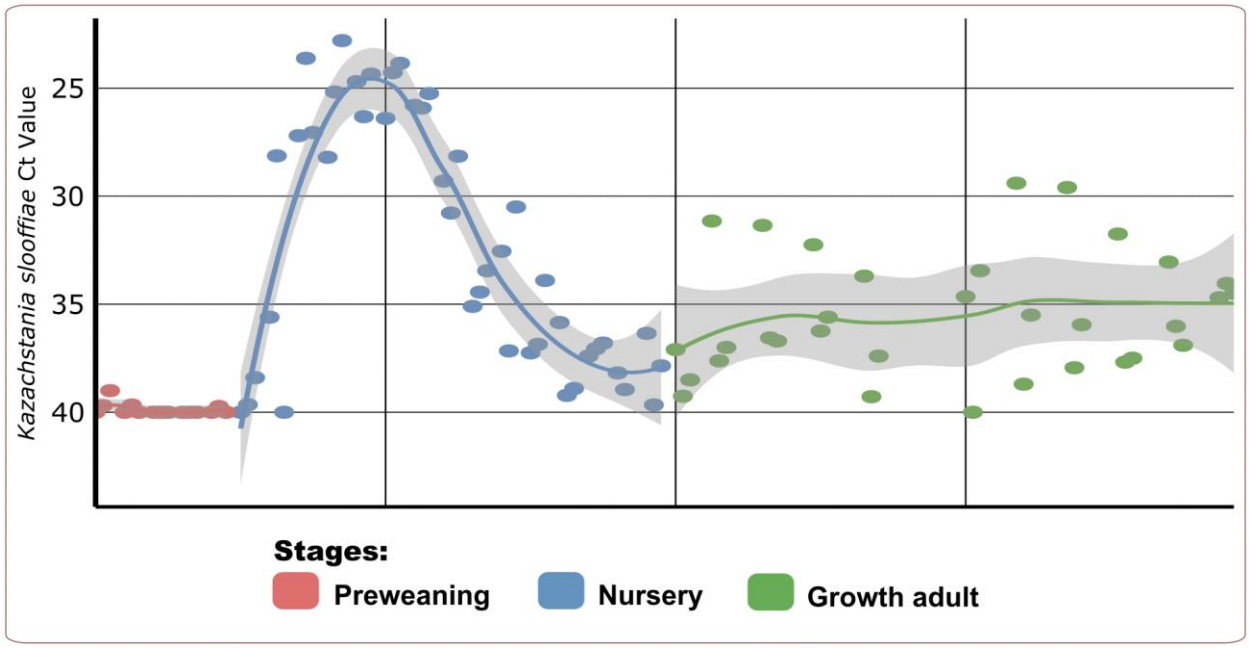

Figure 2.4: *Kazachstania slooffiae* qPCR Ct value according to day of age with line of best fit and 95% confidence interval by stage. Figure was edited in Inkscape version 1.0.2 (https://inkscape.org/)[157].

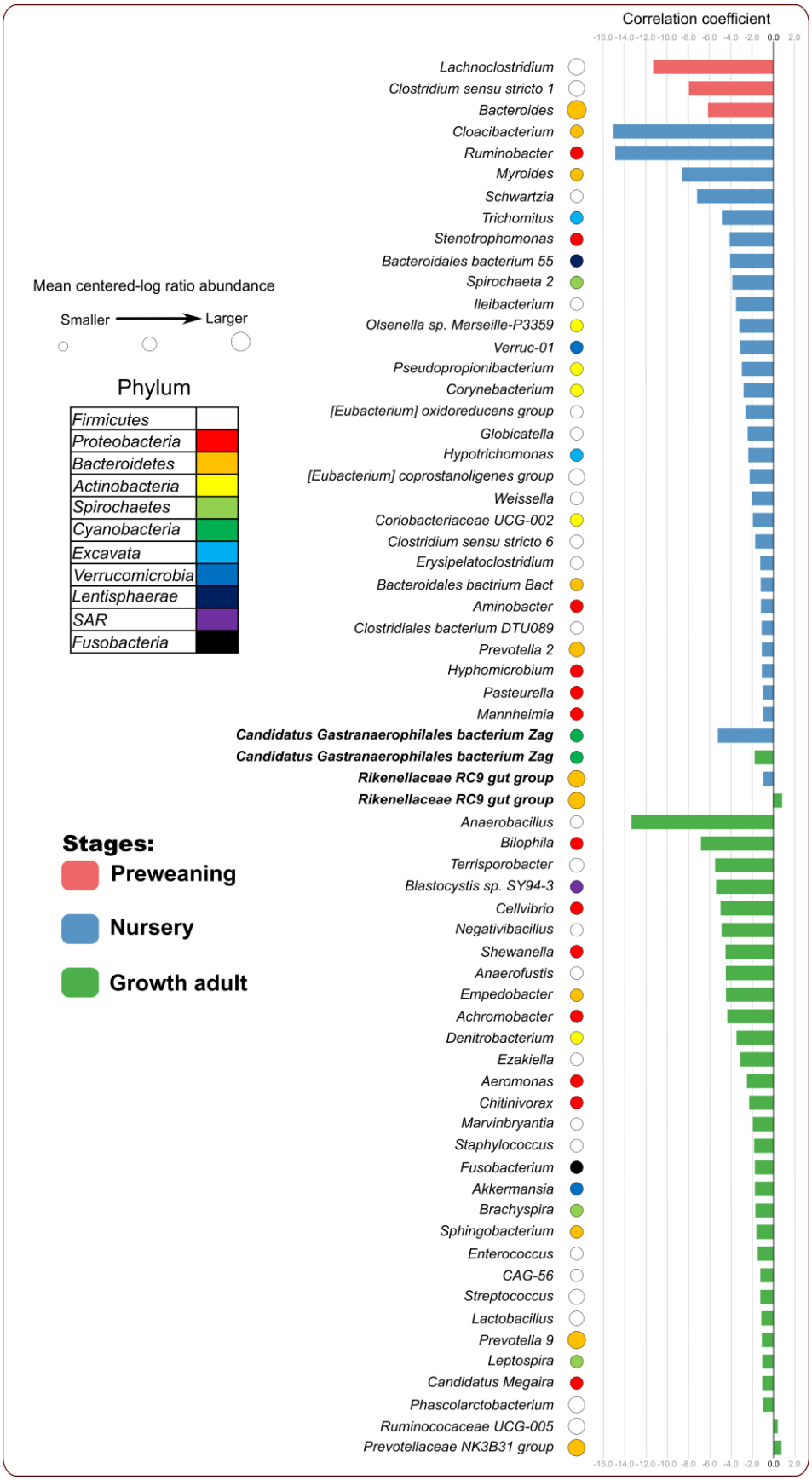
Correlation coefficient
-16.0 -14.0 -12.0 -10.0 -8.0 -6.0 -4.0 -2.0 0.0 2.0
Lachnoclostridium
Clostridium sensu stricto 1
Bacteroides
Cloacibacterium
Ruminobacter
Myroides
Schwartzia
Trichomitus
Stenotrophomonas
Bacteroidales bacterium 55
Spirochaeta 2
Ileibacterium
Olsenella sp. Marseille-P3359
Verruc-01
Pseudopropionibacterium
Corynebacterium
[Eubacterium] oxidoreducens group
Globicatella
Hypotrichomonas
[Eubacterium] coprostanoligenes group
Weissella
Coriobacteriaceae UCG-002
Clostridium sensu stricto 6
Erysipelatoclostridium
Bacteroidales bactrium Bact
Aminobacter
Clostridiales bacterium DTU089
Prevotella 2
Hyphomicrobium
Pasteurella
Mannheimia
Candidatus Gastranaerophilales bacterium Zag
Candidatus Gastranaerophilales bacterium Zag
Rikenellaceae RC9 gut group
Rikenellaceae RC9 gut group
Anaerobacillus
Bilophila
Terrisporobacter
Blastocystis sp. SY94-3
Cellvibrio
Negativibacillus
Shewanella
Anaerofustis
Empedobacter
Achromobacter
Denitrobacterium
Ezakiella
Aeromonas
Chitinivorax
Marvinbryantia
Staphylococcus
Fusobacterium
Akkermansia
Brachyspira
Sphingobacterium
Enterococcus
CAG-56
Streptococcus
Lactobacillus
Prevotella 9
Leptospira
Candidatus Megaira
Phascolarctobacterium
Ruminococcaceae UCG-005
Prevotellaceae NK3B31 group
Mean centered-log ratio abundance
Smaller
Larger
Phylum
Firmicutes
Proteobacteria
Bacteroidetes
Actinobacteria
Spirochaetes
Cyanobacteria
Excavata
Verrucomicrobia
Lentisphaerae
SAR
Fusobacteria
Stages:
Preweaning
Nursery
Growth adult

Figure 2.5: SPIEC-EASI correlation results between *Kazachstania slooffiae* and genera)[87,107–109]. Figure was edited in Inkscape version 1.0.2 (https://inkscape.org/)[157].

Chapter 3 - Novel Complete Methanogenetic Pathways in Longitudinal Genomic Study of Monogastric Age-Associated Archaea

Brandi Feehan[1], Qinghong Ran[1], Victoria Dorman[1], Kourtney Rumback[1], Sophia Pogranichniy[1], Kaitlyn Ward[1], Robert Goodband[2], Megan C Niederwerder[3,4], Sonny T M Lee*[1]

*Corresponding author

[1]Division of Biology, College of Arts and Sciences, Kansas State University, Manhattan, Kansas, 66506, United States of America.

[2]Department of Animal Sciences and Industry, College of Agriculture, Kansas State University, Manhattan, Kansas 66506, United States of America.

[3]Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506, United States of America.

[4]Swine Health Information Center, Ames, Iowa, 50010, United States of America.

Abstract

Background: Archaea perform critical roles in the microbiome system, including utilizing hydrogen to allow for enhanced microbiome member growth and influencing overall host health. With the majority of microbiome research focussing on bacteria, the functions of archaea are largely still under investigation. Understanding methanogenic functions during the host lifetime will add to the limited knowledge on archaeal influence on gut and host health. In our study, we determined lifelong archaea detection and methanogenic functions while assessing global and host distribution of our novel archaeal metagenome assembled genomes (MAGs). We followed 7 monogastric swine throughout their life, from birth to adult (1-156 days of age), and collected feces at 22 time points. The samples underwent gDNA extraction, Illumina sequencing, bioinformatic quality and assembly processes, and MAG taxonomic assignment and functional annotation. **Results**: We generated 1,130 non-redundant MAGs with 8 classified as methanogenic archaea. The taxonomic classifications were as follows: orders *Methanomassiliicoccales* (5) and *Methanobacteriales* (3); genera *UBA71* (3), *Methanomethylophilus* (1), *MX-02* (1), and *Methanobrevibacter* (3). We recovered the first US swine *Methanobrevibacter UBA71 sp006954425* and *Methanobrevibacter gottschalkii* MAGs. The *Methanobacteriales* MAGs were identified primarily during the young, preweaned host whereas *Methanomassiliicoccales* primarily in the adult host. Moreover, we identified our methanogens in metagenomic sequences from Chinese swine, US adult humans, Mexican adult humans, Swedish adult humans, and paleontological humans, indicating that methanogens span different hosts, geography and time. We determined complete metabolic pathways for all three methanogenic pathways: hydrogenotrophic, methylotrophic, and acetoclastic. This study provided the first evidence of acetoclastic methanogenesis in monogastric archaea which indicated a previously unknown capability for acetate utilization in methanogenesis for monogastric methanogens. Overall, we

hypothesized that the age-associated detection patterns were due to differential substrate availability via the host diet and microbial metabolism, and that these methanogenic functions are likely crucial to methanogens across hosts. This study provided a comprehensive, genome-centric investigation of monogastric-associated methanogens which will further our understanding of microbiome development and functions.

Introduction

The gastrointestinal system contains countless microorganisms spanning multiple kingdoms performing equally diverse functions. Archaea, bacteria, viruses, and fungi work in concert and competition to acquire nutrients and space[24]. The focus of previous gut microbiome research has predominantly been on the identification and function of bacteria[158,159]. However, archaea have been demonstrated to be equally important members of the gastrointestinal microbiome[160]. Methanogenic archaea, or archaea which carry out methanogenesis, perform crucial roles in the gut[160,161]. Yet, current research has not indicated how methanogenic gut function changes throughout the lifetime of monogastric hosts[162,163]. With limited research on archaea, and even more minimal analysis on methanogenic functions, we are lacking an in-depth understanding of gastrointestinal associated methanogens, especially our comprehension of methanogen influence on gut and host health throughout host stages of life. By investigating monogastric associated methanogens with a longitudinal approach, we are adding essential knowledge to the limited understanding of monogastric methanogens.

While some beneficial and detrimental associations of archaea to host health have been reported, overall the role of archaea in health and disease is still under investigation[161]. To date, archaea have been associated with a few illnesses including: brain abscesses[161,164], sinus abscesses[161,165], and several gastrointestinal disorders, such as constipation[161,166,167] and obesity[161,168]. Conversely, archaea have also been associated with beneficial attributes. For example, archaea metabolize trimethylamine (TMA), which is thought to decrease cardiovascular disease[160,161], has prompted further evaluation of archaea members as a probiotic for cardiovascular health[161,169]. Moreover, archaea allow continued microbial metabolism, growth and action by lowering hydrogen gut levels[161]. Archaea's role of hydrogen utilization is especially important in the gut where

microorganisms work in concert within the shared gut-microbiome system. However with limited prior research, there is a critical need to understand the role of gastrointestinal archaea in health and sickness via hydrogen metabolism.

Overall, archaea are classified into four superphyla: *Euryarchaeota*, *Asgard*, *TACK* (*Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota* and *Korarchaeota*), and *DPANN* (*Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanoarchaeota*, and *Nanohaloarchaeota*)[170]. To date, *Asgard* archaea have not been indicated as methanogens[170], and *TACK* and *DPANN* have only been identified in non-host associated environmental sites[170–172]. Therefore, currently known host-associated gut methanogens fall within the seven orders of *Euryarchaeota*: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanocellales, Methanopyrales, Methanomassiliicoccales*[173–176]. These *Euryarchaeota* orders are obligate anaerobes which perform methanogenesis to conserve energy for ATP production, where methane is a byproduct[173,177]. Actions immediately following methanogenesis generate an ion gradient which is coupled with ATP production[178,179].

Given the necessity for ATP production, it is unsurprising that historically, studies have primarily relied on the methanogenic gene methyl-coenzyme M reductase A (*mcrA*) or 16S rRNA for identification of gut-associated methanogens[163,180–182]. *McrA* has been identified in all methanogens to date, as the protein performs a critical role in the final methane production step of methanogenesis[182,183]. While prior research was heavily reliant on targeted PCR methodologies, we are missing a functional understanding, from complete genetic sequencing, of gut-associated methanogens[184]. Functional methanogen studies become even more profound when evaluated in a longitudinal approach, especially when following the same hosts. In doing so, we can determine

lifetime gut methanogen dynamics and host implications. Currently, studies which evaluate longitudinal methanogen dynamics typically involve ruminants, such as cows, sheep, goats, and deer[185]. At the time of publication, we could not find a longitudinal study of methanogen genomes (i.e. not marker studies such as 16S rRNA or *mcrA*) following the same monogastrics hosts throughout their lifetime, highlighting the crucial need for such metagenomic longitudinal evaluations[162,163]. Without this knowledge, we cannot determine lifetime dynamics of archaea, and how their methanogenic function may be related to age-associated factors, such as diet and host development.

Our study is the first description of longitudinal monogastric methanogens with genomic analysis of methanogenic function. We evaluated methanogen abundance and functions of 7 monogastric swine hosts over their lifetime at 22 timepoints from birth through adulthood (ages 1-156 days). We described how our methanogen metagenome assembled genomes (MAGs) were identified during specific host ages. Furthermore, we determined methanogenic pathways previously unknown to monogastric-associated methanogens. This study provided evidence of multiple novel methanogen characteristics, which will aid future studies as we build the monogastric methanogen repertoire.

Materials and Methods

Study design, sample collection and DNA extraction

Our study design and sample collection occurred as previously described[186]. We collected fecal samples from 7 swine over 22 timepoints, ranging in swine age from 1 to 156 days across three developmental stages: preweaning (P), nursery (N), and growth adult (G) (Figure 1, Ch3_host_and_dam_demographics_diets_samples.xlsx). Swine were born and raised at the

Kansas State University Swine Teaching and Research Center. Swine originated from the same farrowing group, and were weaned between 18-20 days of age, depending on day of birth. Pigs were managed according to the Kansas State University Institutional Animal Care and Use Committee (IACUC) approved protocol #4036, and methods are reported according to ARRIVE guidelines. The authors also confirmed that all methods were performed in accordance with relevant guidelines and regulations, and we affirmed that all methods were approved by Kansas State University.

We stored fecal samples at -80°C until DNA extraction. We extracted total genomic DNA from fecal samples utilizing the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek Inc.; Norcross, GA), following the manufacturer protocols. We then quantified the extracted genomic DNA with a Nanodrop and Qubit™ (dsDNA BR Assay Kit [Thermo Fisher; Waltham, MA]) for DNA quality and concentration. We stored extracted DNA at -80°C until library preparation and sequencing.

Metagenomic sequencing and 'omics workflow

DNA libraries were generated for a total of 112 samples with Nextera DNA Flex (Illumina, Inc.; San Diego, CA). Resulting libraries were then visualized on a Tapestation 4200 (Agilent; Santa Clara, CA) and size-selected using the BluePippin (Sage Science; Beverly, MA). The final library pool of 112 samples was quantified on the Kapa Biosystems (Roche Sequencing; Pleasanton, CA) qPCR protocol, and sequenced on the Illumina NovaSeq S1 chip (Illumina, Inc.; San Diego, CA) with a 2 x 150 bp paired-end sequencing strategy.

We utilized the 'anvi-run-workflow' program to run a combined bioinformatics workflow in anvi'o v.7.1 (https://anvio.org/install/)[187,188], with a co-assembling strategy. The workflow used Snakemake to implement numerous tasks including: short-read quality filtering, assembly, gene

calling, functional annotation, hidden Markov model search, metagenomic read-recruitment and binning[189]. Briefly, we processed sequencing reads using anvi'o's 'iu-filer-quality-minoche' program, which removed low-quality reads following criteria outlined in Minoche *et al.*[190]. The resulting quality-control reads were termed "metagenome" per sample. We organized the samples into 3 metagenomic groups based on the developmental stages (P, N, G), and used anvi'o's MEGAHIT v1.2.9 to co-assemble quality-filtered short reads into longer contiguous sequences (contigs)[187,191]. The following methods were then utilized in anvi'o to further process the contigs: (1) 'anvi-gen-contigs-database' to compute k-mer frequencies and identify open reading frames (ORFs) using Prodigal v2.6.3[187,192]; (2) 'anvi-run-hmms' to annotate bacterial and archaeal single-copy, core genes using HMMER v.3.2.1[187,193]; (3) 'anvi-run-ncbi-cogs' to annotate ORFs with NCBI's Clusters of Orthologous Groups (COGs; https://www.ncbi.nlm.nih.gov/research/cog)[194]; and (4) 'anvi-run-kegg-kofams' to annotate ORFs from KOfam HMM databases of KEGG orthologs (https://www.genome.jp/kegg/)[195].

We mapped metagenomic short reads to contigs in anvi'o with Bowtie2 v2.3.5[196], and we then converted mappings to BAM files with samtools v1.9[187,197,198]. We used the anvi'o 'anvi-profile' program to profile BAM files with a minimum contig length of 1,000 bp. Next, we combined profiles with 'anvi-merge' into a single anvi'o profile for downstream analyses. We grouped contigs into bins with 'anvi-cluster-contigs' and CONCOCT v1.1.0[199]. We manually processed bins with 'anvi-refine' using bin tetranucleotide frequency and coverage across samples[187,200,201]. Following manual processing, we labeled bins that had >70% completion and <10% redundancy (both based on single-copy core gene annotation) as metagenome-assembled genomes (MAGs). Finally, we used 'anvi-compute-genome-similarity' to calculate average nucleotide identity (ANI), using PyANI v0.2.9[187,202], for each MAG to identify non-redundant MAGs. We analyzed MAG

occurence in a sample with the "detection" metric. We considered a MAG as detected in a metagenome if the detection was >0.25, which is an appropriate cutoff to eliminate false-positive signals in read recruitment results. We used 'anvi-gen-variability-profile' with '--quince-mode' to export single-nucleotide variant (SNV) information on all MAGs after read recruitment, to identify subpopulations of the MAGs in the metagenomes[187]. We used DESMAN v2.1.1 in anvi'o to analyze the SNVs and determine the number and distribution of subpopulations in the MAGs[203]. We accounted for non-specific mapping by removing any subpopulations that made up less than 1% of the entire population that were explained by a single MAG.

Data analyses

We used the "detection" criteria (>0.25) for downstream statistical analyses. We downloaded metagenomes from swine[204], humans[205,206], mice[207], chicken[208], and cattle[209], and performed mapping to the non-redundant archaea-MAGs according to specifications above (Ch3_sequencing_and_assembly_results.xlsx). We used RStudio v1.3.1093[102] to visualize MAGs detection patterns in RStudio (https://www.rstudio.com/products/rstudio/) using: pheatmap (pretty heatmaps) v1.0.12[105], ggplot2 v3.3.5 (https://ggplot2.tidyverse.org/)[210], forcats v0.5.1 (https://forcats.tidyverse.org/)[211], dplyr v1.0.8 (https://dplyr.tidyverse.org/)[212], and ggpubr v0.4.0 (https://CRAN.R-project.org/package=ggpubr)[101].

We utilized the RASTtk Genome Annotation Service on PATRIC v3.6.12 (https://patricbrc.org/) and anvi'o COG annotations for metabolic function analyses[213,214]. We used the comparative pathway tool in PATRIC to predict the metabolic pathways of our resolved non-redundant MAGs. We obtained similar genomes that were deposited in public databases and performed phylogenetic analyses of our non-redundant MAGs in PATRIC[214]. Parameters were set as follows: 100 genes, 10 max allowed deletions, and 10 max allowed duplications. We constructed phylogenetic trees

for our MAGs with 192 closely related genomes, using the amino acid and nucleotide sequences from the global protein families database. RAxML program was used to construct the trees based on pairwise differences between the aligned protein families of the selected sequences.

Our final figures were edited in Inkscape v1.2.1[157].

Results and Discussion

Host-associated archaeal methanogens have been linked to various conditions of health and disease. Most archaea-centric intestinal microbiome studies have been conducted on a single time point in the lifetime of the host. Using molecular and cultural approaches, intestinal archaea have been identified in many hosts, including: humans, swine, horses, rats, birds, fish, and kangaroos[215]. Overall, these analyses reported that the most common methanogens in the gut are members of the *Methanobacteriales* and *Methanomassiliicoccales* orders[215]. However, little is known about the presence and distribution of archaea through the lifetime of the swine. There is also a lack of data on the functions of the archaea in the swine gut. Overall, this knowledge gap has hindered the identification of factors that influence the diversity, abundance and functions of archaea in the swine. In this study, we recovered 8 methanogenic archaea metagenome assembled genomes (MAGs) that exhibited differential colonization patterns in the host at different ages. While distribution of methanogens across multiple hosts has been previously demonstrated, we recovered the first US swine *Methanobrevibacter UBA71 sp006954425* and *Methanobrevibacter gottschalkii* MAGs[215]. Moreover, we attributed methanogenic functions to our age-associated archaea, and identified the first evidence of acetoclastic methanogenesis in monogastric archaea, found in our *Methanomassiliicoccales* MAGs, indicating a previously unknown capability of monogastric methanogens to utilize acetate in energy acquisition. Alternatively, we attributed hydrogenotrophic

methanogenesis, where carbon dioxide (CO_2) is utilized, in the *Methanobacteriales*. We surmised that the age-associated detection patterns were due to differential substrate availability, which was highly influenced by diet. Altogether, we provided a comprehensive, genome-centric investigation of monogastric-associated archaea to further our understanding of microbiome development and function.

Taxonomic classification of gut metagenome-assembled genomes

To broadly sample gut-associated microorganisms of the swine host across different age-associated growth stages, we obtained 5,840,640,191 paired-end reads from Illumina NovaSeq sequencing data of 112 swine fecal samples (Ch3_anvio_results.xlsx). After quality trimming, we generated 5,167,665,150 paired-end reads. The resulting 3 co-assemblies contained 9,431,702 contigs that described approximately ~3.6 x 10^{10} nucleotides and ~3.7 x 10^{7} genes. Using a combination of automatic and manual binning strategies resulted in 4,556 metagenome-assembled genomes (MAGs). We further removed redundancy by selecting a single representative for each set of genomes that shared an average nucleotide identity (ANI) of greater than 95%, resulting in 1,130 final non-redundant MAGs (nr-MAGs) (Ch3_anvio_results.xlsx). Among the nr-MAGs, we recovered an average of 203 ± 187 contigs, with an average N50 of 32,737 ± 35,205. The resolved nr-MAGs had completion values of 87.9% ± 8.6%. The genomic lineages for archaeal and bacterial nr-MAGs based on domain-specific single-copy core genes resolved to 20 phyla (2 archaea phyla and 18 bacterial phyla). We could also assign 88.4% of the bacterial and archaeal nr-MAGs to their genera.

Resolved archaeal MAGs phylogenetically similar to diverse hosts and geographic disbursed archaea

Among the 1,130 nr-MAGs that we resolved, our genomic collection also included 8 archaea nr-MAGs (hereafter known as archaea-MAGs; Ar-1 through Ar-8; Table 1; Ch3_anvio_results.xlsx). We observed that our resolved archaea-MAGs harbored genes which encoded for critical methyl-coenzyme M reductase (*mcrABG*) proteins required for methanogenesis, including *mcrA* which is typically utilized for methanogen classification[216,217] (Ch3_anvio_results.xlsx and Ch3_gene_annotations.xlsx). To our best knowledge, these MAGs represent the first genomic evidence of putative methanogens differential colonization pattern of the monogastric gut. The resolved methanogen MAGs had an average genome size of 1.4 Mbp, 1,573 KEGG gene annotations, 1,535 COG gene annotations, and a GC content ranging from 31% to 56% (Table 1; Ch3_anvio_results.xlsx). We resolved 7 of the methanogen MAGs to the species level with one archaea-MAG resolving to the genus level (Table 1; Ch3_anvio_results.xlsx). Our resolved archaea-MAGs were assigned to the following orders: *Methanomassiliicoccales* (5) and *Methanobacteriales* (3). Moreover, the genera were as follows: *UBA71* (3), *Methanomethylophilus* (1), *MX-02* (1), and *Methanobrevibacter* (3).

We downloaded 95 *Methanomassiliicoccales* and 97 *Methanobacteriales* genomes to investigate the phylogenetic relationship of our resolved archaea-MAGs (Figure 2; Ch3_original_phylogenetic_trees.pdf). We showed that our methanogen populations had close phylogenetic relationships with archaea from geographically distinct mammalian hosts, suggesting high similarities in gene functions in archaea among diverse host species. Given similarities amongst such diverse host species with diverse digestive systems, we hypothesize these close genetic relatives of our resolved archaea-MAGs might be more ubiquitous in a wider range of

hosts than are currently discussed. We noticed Ar-4 clustered, as expected, with 6 *Methanomethylophilus alvus* strains: 5 from human gut samples and 1 from swine (MAG221) (Figure 2A)[218–223]. Ar-7 clustered with 4 *MX-02 sp006954405*. United Kingdom strain 10[224] and Chinese strain MAG014[221] have been identified as swine-originating, whereas B5_69.fa and B45_maxbin.030.fa were from humans[225]. Interestingly, clustering on the same branch (*B5_69.fa* and *B45_maxbin.030.fa*) are archaea isolated from South African adult humans[225]. Ar-1 was in the same branch with archaea isolated from Tibetan pig MAG098[221]; Ar-2 with Chinese roe deer RGIG3983[226] strain; and Ar-3 with Tibetan pig MAG196[221]. Likewise, we observed Ar-8 was on the same phylogenetic branch with an Australian *Methanobrevibacter gottschalkii* isolate: A27[227](Figure 2B). Interestingly, Ar-6 formed an outbranch alongside this branch with a further outbranch containing two *Methanobrevibacter gottschalkii* strains[228,229]. This would suggest that our resolved archaea-MAG Ar-6 might be a *Methanobrevibacter gottschalkii*. Moreover, Ar-5 clustered amongst *Methanobrevibacter smithii* strains: Tibetan pig MAG004[221], Canadian pig SUG1019[230], and US Florida human ATCC 35061[231].

While many of our methanogen MAGs clustered with swine originating archaea populations, we also demonstrated our methanogen MAGs alongside human and deer associated methanogens, suggesting similarities in microbial genes and associated functions in the methanogens amongst these host species. The roe deer similarity is especially intriguing considering that deer contain a ruminant digestive system, with four stomach compartments, compared to the single stomach system of monogastric swine and human[182,232]. Moreover, even though our archaea-MAGs originated from the United States (in the state of Kansas) swine, our phylogenetic analyses indicated similarities to archaeal populations from Australia, South Africa, Tibet, China, United Kingdom and Canada, further supporting the global presence of archaea amongst diverse hosts.

We surmise that our resolved methanogen archaea-MAGs and these close genetic relatives might be more widespread in more hosts than we expected[215].

We recovered from our study novel archaeal genomes that were previously unidentified in US swine. We were able to resolve and obtain the genomic information, to the best of our knowledge, of the first swine-associated *Methanobrevibacter UBA71 sp006954425* and *Methanobrevibacter gottschalkii* MAGs. The methanogenic archaea family *Methanobacteriales* has been identified in many previous swine studies, with the majority of these studies utilizing 16S sequencing and/or real-time PCR identification[124,221,224,233–241]. Still, there is a lack of understanding of the *Methanobacteriales* in terms of genomic studies, and the *Methanomassiliicoccales* order collectively in general. Up to this moment, only three swine *Methanomassiliicoccales* MAGs (*Methanomethylophilus alvus*, *MX-02 sp006954405*, and *Methanobrevibacter smithii*) have been identified[221,224,241]. Thus, adding our highly resolved novel archaea-MAGs to the repertoire of swine-associated microbial populations will aid in understanding swine archaea, including functions, host associations (such as age, health status, sex, etc.), and global distribution.

Prevalence of archaeal MAGs and variants at distinct host ages

Assessing the abundance of the methanogens in different growth stages of the swine host provided an opportunity to investigate the association between the host-associated methanogens and the different conditions faced by the swine as they grow. Our genome-centric metagenome analyses revealed two dominant orders of archaea - *Methanobacteriales* and *Methanomassiliicoccales*. We showed that resolved methanogen MAGs were differentially detected at different growth stages of the swine, but does the environment affect the functional niche specificity between these two orders of archaea?

The heatmap shown in Figure 3A provides a graphical summary of the changes in detection for the archaea-MAGs. Hierarchical clustering grouped the archaea-MAGs into three clusters based on detection: A (top cluster; Ar-1 through Ar-4), B (middle cluster; Ar-5 and Ar-6), and C (bottom cluster; Ar-7 and Ar-8). We observed that Cluster A contained only *Methanomassiliicoccales* MAGs, while Cluster B contained 2 *Methanobacteriales* MAGs, and Cluster C one of each order. Cluster A archaea-MAGs were primarily identified in the final stage of growth adult hosts. Conversely, Cluster B methanogens were primarily identified in preweaning hosts. Finally, Cluster C archaea were identified throughout the host lifetime.

We noticed the majority of archaea-MAGs detection values increased closely after a stage transition (preweaning to nursery and nursery to growth adult), suggesting that stage transition changes, including diet, housing, and stress, can lead to changes in microbiome composition[89]. Although, exactly how these changes impact archaea is relatively understudied, as most research evaluates bacteria, and therefore archaea-stage dynamics are a topic for future research[242,243].

We investigated methanogen variants, and found the majority of variation occurred in periods when other archaea were dominating (preweaning and growth adult; Ch3_SNV_results.xlsx). We performed single-nucleotide variant (SNV) analysis on our two archaea-MAGs that showed continuous detection throughout the host lifetime (Cluster C MAGs: Ar-7 and Ar-8; Figure 3B). We attributed the majority of variances to the unweaned host, while fewer variances were identified in the growth adult. Interestingly, the variants were highest during times where other archaea-MAGs were predominantly identified (Figure 3B). We hypothesized that the variation found in the growth adult host could indicate a competitive microbial environment, while fewer

variants as compared to the earlier growth stages could be due to a largely already developed gut microbiome. In a competitive gut microbiome system, it is beneficial to have genetic diversity which translates to increased functional diversity[244]. A similar competitive environment and SNV diversity was demonstrated in the human gut bacterial community[244]. Comparatively, as the gut developed and microbes established with focused functions, the variation decreased when humans reached 2 years of age[244]. Human gut development is similar to the relatively faster development of the swine microbiome during preweaning and within 10 day days post weaning[245]. The conditions which encouraged the increased variation in the growth adult in our study could have been a change of diet, host stress, or other host-associated and environmental conditions[246].

While we demonstrated differing archaea and SNV association with age, we were primarily interested in methanogen function. We hypothesized methanogenic function influenced our resolved methanogen MAGs' ability to establish in the microbiome at different host stages through energy acquisition via host diet. Phylogenetic similarity in archaea across geography and hosts prompted an investigation into whether our archaea-MAGs were identified in other hosts of similar developmental ages, and therefore similar archaeal functions.

Methanogens span host species, millennia, and geographic distance

We wanted to further demonstrate not only global and host distribution, but also temporal identification of our methanogens beyond genetic similarity, as illustrated in our phylogenetic analyses. We mapped metagenomic sequencing reads from young and aged hosts to our archaea-MAGs from the following hosts: swine (n=16)[204], humans (n=429)[205,206], mice (n=60)[207], chicken (n=71)[208], and cattle (n=34)[209] (Figure 4; Ch3_metagenome_mapping_metadata.xlsx and Ch3_metagenome_mapping_detection.xlsx). Our archaea-MAGs were identified in older humans

and varying aged swine metagenomes, but not in the chicken, mice or cattle metagenomes. We also demonstrated evidence of our archaea-MAGs in the ancient human gut and global distribution. Altogether we determined within a host species, archaeal age-association appeared to be similar, but some archaea span multiple host species, and for millennia[215]. We hypothesized differential archaeal function may be essential to the gut microbiome of many modern and ancient monogastric hosts.

We determined our swine-associated methanogens were not present in poultry, mice and ruminant metagenomes (Ch3_metagenome_mapping_detection.xlsx). Given the drastic differences in the ruminant digestive system compared to the monogastric gut, we were not surprised that our swine archaea-MAGs were not found in cattle from the United States (US) State of Pennsylvania. Although not identified consistently in all cattle, *Methanobrevibacter smithii*[247–250] and *Methanobrevibacter gottschalkii*[251–254] have been associated with the cow digestive tract. Similarly, *UBA71* has been identified in adult chickens previously[255]. Given that similar taxonomic methanogens are present in cattle and chickens, we hypothesized the methanogens of these hosts were genetically distinct from the methanogens we identified in swine. Additionally, since the methanogens we identified were not consistently detected across our aging hosts, it was very probable that other metagenomes from these host populations could contain our methanogens. Future research is necessary to evaluate how distinct methanogen members function individually and collectively within the microbiome system to influence gut health in different host species.

Interestingly, we could only find a singular example of archaea attributed to the mouse gut: *Methanomassiliicoccaceae DTU008*[256]. Remaining attempts, encompassing more than 1,000 metagenomes, proved unsuccessful in identifying mice gut archaea[257–260]. In fact, an investigation

of murine gut composition across 17 rodent species demonstrated, beyond the instance of mouse *DTU008*, only North American porcupine (*Erethizon dorsatum*), capybara (*Hydrochoerus hydrochaeris*), and guinea pig (*Cavia porcellus*) contained archaea[256]. Although murine hosts have a monogastric digestive system, there appears to be a lack of understanding if and when archaea are present in mouse gut[260].

Although from a different continent, Chinese swine demonstrated the closest age-associated detection to our US swine methanogens. Even though the Chinese preweaning swine were not weaned, the methanogen presence appeared to more closely resemble the US swine weaned, nursery gut. The exception to the nursery resemblance being Ar-5, which more closely resembled our US swine preweaning gut. Many factors, including breed, weaning age (China at 42 days; US at 18-20 days), and housing, are known to influence microbiome development, and therefore could have resulted in the different archaeal age-establishment patterns[89,261,262]. In terms of our earlier detection clusters, Cluster A still appeared more prevalent in the growth adult host, and Cluster C was similarly prevalent throughout both the preweaning and growth adult stages. Although the detections of Ar-5 and Ar-6 (Cluster B) were not shared between the Chinese and US swine, as Ar-6 demonstrated relatively low detection in the preweaning stage. Given that the Chinese swine dataset was from a single day in preweaning and growth adult, future research should investigate longitudinal distribution of methanogens from monogastric swine according to various characteristics, such as country of origin, breed, diet, housing environment, etc. This would further develop our understanding of global methanogen distribution according to associated variables.

Overall, we demonstrated genetic support for the same, or very closely related, methanogens circulating in both US and Chinese swine with similar age-associated detection. This further

demonstrates the ubiquity of archaea to the monogastric swine host which we hypothesized are distributed with host age according to archaeal function.

Our resolved archaea-MAGs not only appeared in Chinese swine, but we also provided evidence of these archaea-MAGs in adult humans from modern age (Mexico and Sweden) and ancient time (modern day US and Mexico). In contrast to the swine gut, we identified merely two methanogens in the human gut: *M. smithii* and *MX-02 sp006954405*. With the exception of one Swedish 12 month sample demonstrating Ar-5 presence, the infant data illustrated comparatively minimal to no archaeal presence of our methanogens. Given that many publications demonstrate identification of multiple *Methanobacteriales* and *Methanomassiliicoccales* in the human gut[160,162,263,264], it is possible that there were genetically distinct methanogens present in these human samples beyond our *M. smithii* and *MX-02 sp006954405*. The modern adult human samples, both the Mexican and Swedish datasets, only demonstrated *M. smithii* presence. Multiple publications have identified increasing *M. smithii* in the human gut with age[264,265]. Interestingly, we identified *MX-02 sp006954405* and *M. smithii* in the palaeofaeces from the US and Mexico, suspected to be between 1,000-2,000 years old[205]. While many paleobiology studies have investigated ancient methanogens of water sediments[266–274], we identified merely two human-related paleobiology methanogen studies pertaining to: the archaic human gut[205] and neanderthal dental plaques[275]. *M. smithii* has been previously identified in ancient humans[205], but the identification of *MX-02 sp006954405* appeared to be the first evidence of human-associated ancient *Methanomassiliicoccales*. The methanogen *MX-02* has been identified in the human gut previously[174], but we illustrated novel evidence for *MX-02 sp006954405* in the ancient human gut, suggesting that *MX-02 sp006954405*, or close relatives, were likely present in the modernized human gut, but we did not identify genetic resemblance in the 122 modern human metagenomes we evaluated. Future research is necessary

to provide further insights into the gut-associated archaea to elucidate genetic phylogeny, evolution of archaeal functions, and association with ancient humans.

Given our findings indicating our US-swine associated archaea-MAGs were present in Chinese swine, US humans, and Mexican humans, we wanted to further understand the role of these methanogens in the monogastric gut.

Critical methane metabolism functions were conserved across methanogens

Our primary goal was to profile the expressed genomic potential that contributed to the methanogenesis of the swine gut. In our study, we constructed and demonstrated the first swine complete methanogenic pathways, which is crucial for understanding the role of archaea within the microbiome system and to host health. We analyzed methanogenesis pathways of *Methanobacteriales* and *Methanomassiliicoccales* (Figure 5, Ch3_gene_annotations.xlsx). We identified 44 genes in methane metabolism[276].

The shared genes represented crucial functions in the final methanogenesis steps of energy and methane production. Nine genes were shared across the 8 archaea-MAGs: 3 heterodisulfide reductase (*hdrA*, *hdrB*, and *hdrC*), 3 methylviologen-dependent Ni,Fe hydrogenase (*mvhA*, *mvhG*, and *mvhD*), and 3 methyl-coenzyme M reductase (*mcrA*, *mcrB*, and *mcrG*). HdrABC, MvhAGD, and McrABG are critical to the final steps of methanogenesis. HdrABC and MvhAGD form an electron-bifurcating complex to regenerate coenzyme M (HS-CoM) and coenzyme B (HS-CoB) from heterodisulfide CoM-S-S-CoB[277]. An intermediate step, not shared by all archaea-MAGs but discussed in the following section, generates methylated cozyme M (CH_3-S-CoM)[183]. McrABG then catalyzes the final methanogenesis step where the methyl group of CoM-CH_3 is reduced to

methane, with HS-CoB utilized as an electron donor[183]. This formation of methane generates CoM-S-S-CoB for reduction again by HdrABC/MvhAGD[278]. The final step in producing methane is crucial for methanogens, and our archaea-MAGs supported the roles of HdrABC, MvhAGD, and McrABC in these final methanogenesis reactions.

Differential Methanobacteriales and Methanomassiliicoccales methane metabolic pathways may relate to age-associated detection

While we supported the clear ubiquity of *hdr*, *mvh* and *mcr* to methanogens, we were primarily interested in how our archaea differed in methanogenic potential since they exhibited differential colonization through the swine growth stages. We noticed our taxonomically distinct archaea-MAGs harbored different genetic components for divergent methane metabolic pathways (Figure 5). We identified the first genetic support of an acetoclastic *Methanomassiliicoccales*, and first acetoclastic methanogen in a monogastric host. Moreover, *Methanomassiliicoccales* also contained genes for the methylotrophic methanogenic pathway, indicating the potential to utilize various substrates from acetate to methylated compounds. Alternatively, *Methanobacteriales* contained genes for the hydrogenotrophic pathway with CO_2 as the substrate input. We surmised that these alternate pathways played a role in energy acquisition according to differential nutrients available during the host lifetime, and therefore growth stage associated diet.

The hydrogenotrophic methanogenic pathway was the key methanogenic pathway identified in all of our *Methanobacteriales* MAGs. During hydrogenotrophic methanogenesis, CO_2 is reduced to methane (CH_4) with four molecules of H_2[183,279]. Our *Methanobacteriales* archaea-MAGs contained all genes crucial to the hydrogenotrophic methanogenic pathway: formylmethanofuran dehydrogenase (*fwd*; A-H), formylmethanofuran-tetrahydromethanopterin N-formyltransferase

(*ftr*), methenyltetrahydromethanopterin cyclohydrolase (*mch*), coenzyme F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase (*mtd*), methenyltetrahydromethanopterin hydrogenase (*hmd*), methylenetetrahydromethanopterin reductase (*mer*), and methyltetrahydromethanopterin-coenzyme M methyltransferase (*mtr*; A-H)[217,280].

We identified both *mtd* and *hmd*, indicating the methanogens can potentially utilize H_2 with or without F_{420} to reduce methenyl-H_4MPT, as Mtd requires F_{420}[281]. As mentioned previously, we believe the CH_3-CoM in our *Methanobacteriales* archaea-MAGs was generated via MtrABCDEFGH. This *mtr* complex has been demonstrated to transfer the methyl group from tetrahydromethanopterin (H_4MPT) to CoM, therefore coupling the hydrogenotrophic pathway to the final methane production steps[170,282,283]. *Methanobacteriales* have been associated with the hydrogenotrophic pathway previously[183].

Our *Methanobacteriales* archaea-MAGs contained all of the aforementioned genes, with one exception: Ar-5 lacked *mch*. We attribute this to the incompleteness of the metagenome assembled genome, as Ar-5 exhibited the lowest completion of our methanogen MAGs at ~72% (Table 1, Ch3_anvio_results.xlsx). Interestingly, only two hydrogenotrophic pathway genes (*fwdD* and *fwdH*) were identified in two *Methanomassiliicoccales* archaea-MAGs. The lack of a hydrogenotrophic pathway clearly indicated the *Methanomassiliicoccales* utilized a distinct methane pathway.

All *Methanomassiliicoccales* archaea-MAGs, and our *M. smithii* archaea-MAGs, indicated varying ability to metabolize methanol, mono-, di- and trimethylamine through the methylotrophic methanogenesis pathway. Genes we identified in our archaea-MAGs included:

methanol:coenzyme M methyltransferase (*mtaB* and *mtaC*), monomethylamine (MMA) methyltransferase (*mtmB* and *mtmC*), dimethylamine (DMA) methyltransferase (*mtbA*, *mtbB*, and *mtbC*), and trimethylamine (TMA) methyltransferase (*mttB* and *mttC*)[217]. *Methanomassiliicoccales* is known to perform methylotrophic methanogenesis with all of the previously discussed substrates[284]. Conversely, *M. smithii* was thought to be a hydrogenotrophic population[285]. Given we only identified methylotrophic genes in one *Methanobacteriales* population, future research is necessary to support this genetic potential.

Although all *Methanomassiliicoccales* methanogens and the *M. smithii* population contained the *mtaBC* genes for methanol metabolism, we did not identify *mtaA* in the *Methanomassiliicoccales* genetic content. The MtaABC complex transfers the methyl group from methanol to coenzyme M, generating CH_3-S-CoM for McrABG reduction[286]. We identified a candidate *mtaA* homolog through a literature review: uroporphyrinogen III decarboxylase (*hemE*)[287] (designated *mta-like* in Figure 5 and Ch3_gene_annotations.xlsx). This is the first publication identifying the homolog in methanogens. As such, future research is critical to analyze how HemE might interact with MtaBC, and how the enzyme performs in the methylotrophic pathway.

Only three (Ar-2, Ar-3, and Ar-4) out of the five *Methanomassiliicoccales* archaea-MAGs contained a complete genetic pathway associated with monomethylamine (*mtmBC*) and dimethylamine (*mtbBC*) utilization[217,288]. Moreover, only two *Methanomassiliicoccales* populations (Ar-2 and Ar-4) contained *mttBC,* associated with trimethylamine (TMA) utilization[217,288]. TMA has been associated with increased cardiovascular disease, so TMA metabolism is beneficial for the host[160,161]. Further research is necessary to evaluate if other methylated compounds may play roles in cardiovascular disease, and therefore the host aided by

archaeal metabolism. Although the majority of our archaea-MAGs appeared to be able to utilize methanol in the methylotrophic methanogenesis pathway, fewer were able to use mono-, di- and trimethylamine. As noted previously, this might be due to the incompleteness of the archaea-MAGs, especially since our *Methanomassiliicoccales* archaea-MAGs completeness ranged from ~80-99% (Table 1, Ch3_anvio_results.xlsx). Still, there is a possibility that these *Methanomassiliicoccales* archaea-MAGs might have different abilities in utilizing methylated sources due to contrasting biological necessity and evolutionary selection[289].

To the best of our knowledge, we identified the first complete acetoclastic methanogenic pathway in *Methanomassiliicoccales*[290,291]. Three *Methanomassiliicoccales* (Ar-1, Ar-2, and Ar-7) archaea-MAGs contained genetic support, with two complete pathways (Ar-1 and Ar-7), for the acetoclastic, or also called aceticlastic, pathway. Acetate is reduced to acetyl-CoA and then H_4MPT via acetyl-CoA synthetase (*acs*) and carbon monoxide dehydrogenase (*acsC* and *acsD*)[217,288,292]. Two of our *Methanomassiliicoccales* archaea-MAGs (Ar-3 and Ar-4) did not have acetoclastic genes identified, while *acsD* was not found in Ar-2.

Acetoclastic methanogenesis is typically performed by aquatic methanogens[293–295]. The only prior identification of acetoclastic archaea in gastrointestinal tracts was in *Methanosarcinales* of a ruminant (cow)[160,296]. Therefore, we identified the first evidence for acetoclastic methanogenesis in monogastrics. Moreover, only *Methanosarcinales* and *Methanococcales* were known to perform acetoclastic methanogenesis[290,296,297]. The ability of our *Methanomassiliicoccales* to perform both methylotrophic and acetoclastic methanogenesis parallels *Methanosarcinales*, since many *Methanosarcinales* also are able to perform both of these pathways[291]. Conversely, acetoclastic-able *Methanococcales* are also able to perform hydrogenotrophic methanogenesis[296]. Acetoclastic

methanogenesis requires an ATP input to convert acetate to acetyl-CoA, which impairs the energy efficiency of this methanogenic pathway[298]. The diminished energy return of acetoclastic methanogenesis likely plays a role in the dual acetoclastic-methylotrophic metabolic potential of our *Methanococcales* archaea-MAGs. The ability to utilize different substrates via varying methanogenic pathways is beneficial. The methanogen can potentially still metabolize energy as their substrate source changes. Changing substrates is common in the gastrointestinal system, as the host changes diets and the associated microbiome changes and produces different metabolites[242].

Although the *Methanomassiliicoccales* archaea-MAGs contained genes for both methylotrophic and acetoclastic methanogenesis, this dynamic substrate capability did not appear to allow *Methanomassiliicoccales* archaea-MAGs to prevail more than their counterparts: *Methanobacteriales*. In fact, the *Methanobacteriales* populations in general were detected at more times during the host life than *Methanomassiliicoccales* (Figure 3A). This may indicate CO_2 is more available in the monogastric system during the host lifetime. The exception to this being the during the growth stage where *Methanomassiliicoccales* appear abundantly, therefore mylated compounds or acetate could have transitioned to being the dominantly available substrate, suggesting that the stage-associated characteristics, such as dietary composition had a high influence on the methanogens dynamics. Although diet influences substrate availability, we cannot rule out other factors, including host age and other gastrointestinal organisms (including bacteria and protist), which alter the gut microbiome system[160,242,296,299].

Collectively, with the novel acetoclastic *Methanomassiliicoccales* and methylotrophic *Methanobacteriales M. smithii* archaea-MAGs, there is a knowledge gap surrounding the

functional potential of methanogens. Taken together with the phylogenetic analysis, we are lacking a holistic understanding from global and host distribution of methanogens to their methanogenic actions. Other publications have also discussed the lack of overall methanogen knowledge, especially knowledge surrounding archaeal functions[300–302]. Archaea are a member of the gut microbiome alongside bacteria, fungi and viruses, and without understanding their distribution and functions, we will not understand how archaea influence the gut microbiome system and host health.

Conclusions

We performed a longitudinal study of the monogastric microbiome where we produced 1,130 MAGs, with 8 methanogen archaea-MAGs. The novel archaea-MAGs clustered with geographically diverse methanogens from various animal and human hosts, indicating global distribution of closely related archaea. We also determined that our archaea-MAGs were detected in swine and humans from distinct continents and time. Given the stark distinctions in detection and distribution, we wanted to evaluate if energy acquisition associated with methanogenesis could be related to these factors, especially age-distribution. Our *Methanobacteriales* archaea-MAGs contained genes for hydrogenotrophic methanogenesis, indicating the ability to metabolize CO_2. Alternatively, *Methanomassiliicoccales* archaea-MAGs appeared to have the capability to utilize a range of substrates from methylated compounds, including methanol and methylamine, and acetate, through the methylotrophic and acetoclastic pathways, respectively. We identified the first acetoclastic *Methanomassiliicoccales*, and also the first acetoclastic methanogens of monogastrics. Moreover, we identified a *Methanobacteriales* population with methylotrophic genes. Previously, *Methanobacteriales* was thought to only perform hydrogenotrophic methanogenesis. We hypothesized the distinct diets, given according to age, provided different substrates which

influenced archaeal establishment and therefore detection patterns. Still, we know there are multiple other growth stage-associated and microbiome dynamics which likely play a role in archaeal growth.

In order to continue developing our understanding of archaea, we must continue to evaluate their global prevalence across diverse hosts and ecosystems. Moreover, we should evaluate the significance of acetoclastic methanogens to monogastrics, including how these methanogens influence other microorganisms and host health. Future studies should also investigate how growth stage-associated factors influence methanogenic potential and therefore archaeal abundance. In pursuing this archaea research, we can better determine how methanogens provide beneficial or detrimental consequences to host health, and how we might utilize or deter methanogens in animals and humans alike.

Declarations

Ethics approval and consent to participate

Pigs were managed according to the Kansas State University Institutional Animal Care and Use Committee (IACUC) approved protocol #4036.

Availability of data and material

We uploaded our metagenome raw sequencing data to the SRA under NCBI BioProject PRJNA798835. All other analyzed data, in the form of databases and fasta files, and bioinformatic scripts are accessible at figshare 10.6084/m9.figshare.20431713.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

B.F., R.G. and S.T.M.L. designed the study. Sample collection was performed by B.F. B.F., V.D., K.R., and K.W. completed DNA extraction and Nanodrop and Qubit quality analysis. B.F. and Q.R. performed anvi'o and PATRIC bioinformatic analyses. B.F. and S.T.M.L. attributed biological relevance, wrote the manuscript, prepared figures, and supplemental files. B.F. and S.T.M.L. performed major manuscript and figure refinement while remaining authors contributed to lighter refinement. All authors read, contributed to manuscript revision, and approved the submitted version.

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Figures

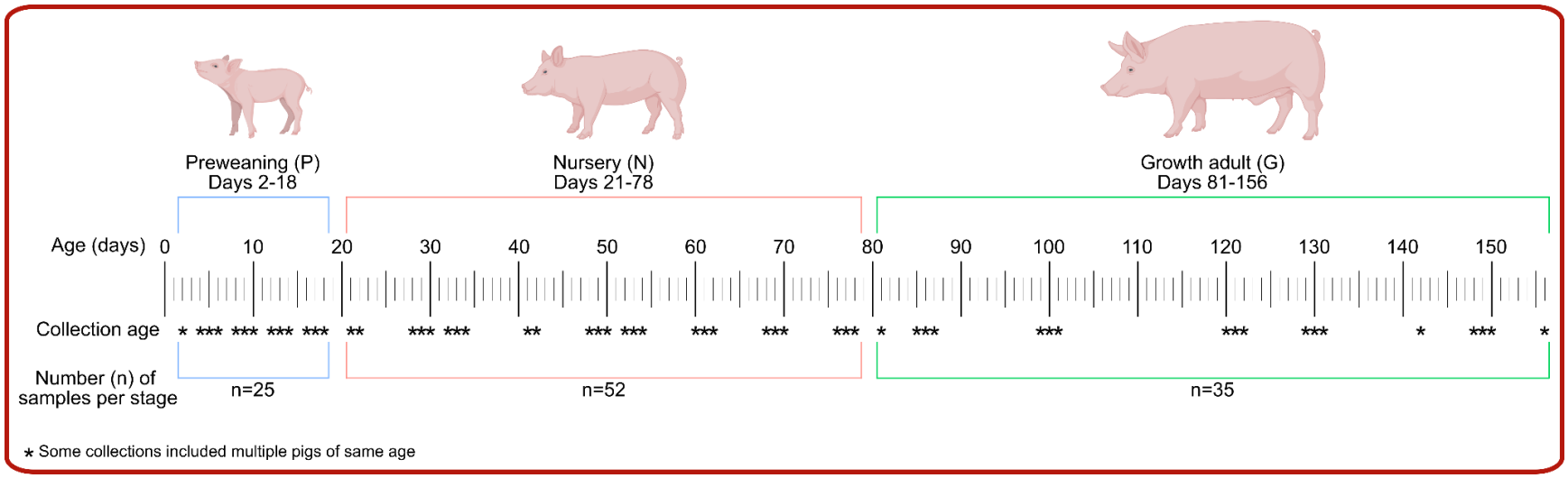

Figure 3.1: Study schematics of 7 swine hosts including fecal sampling ages and developmental stages.

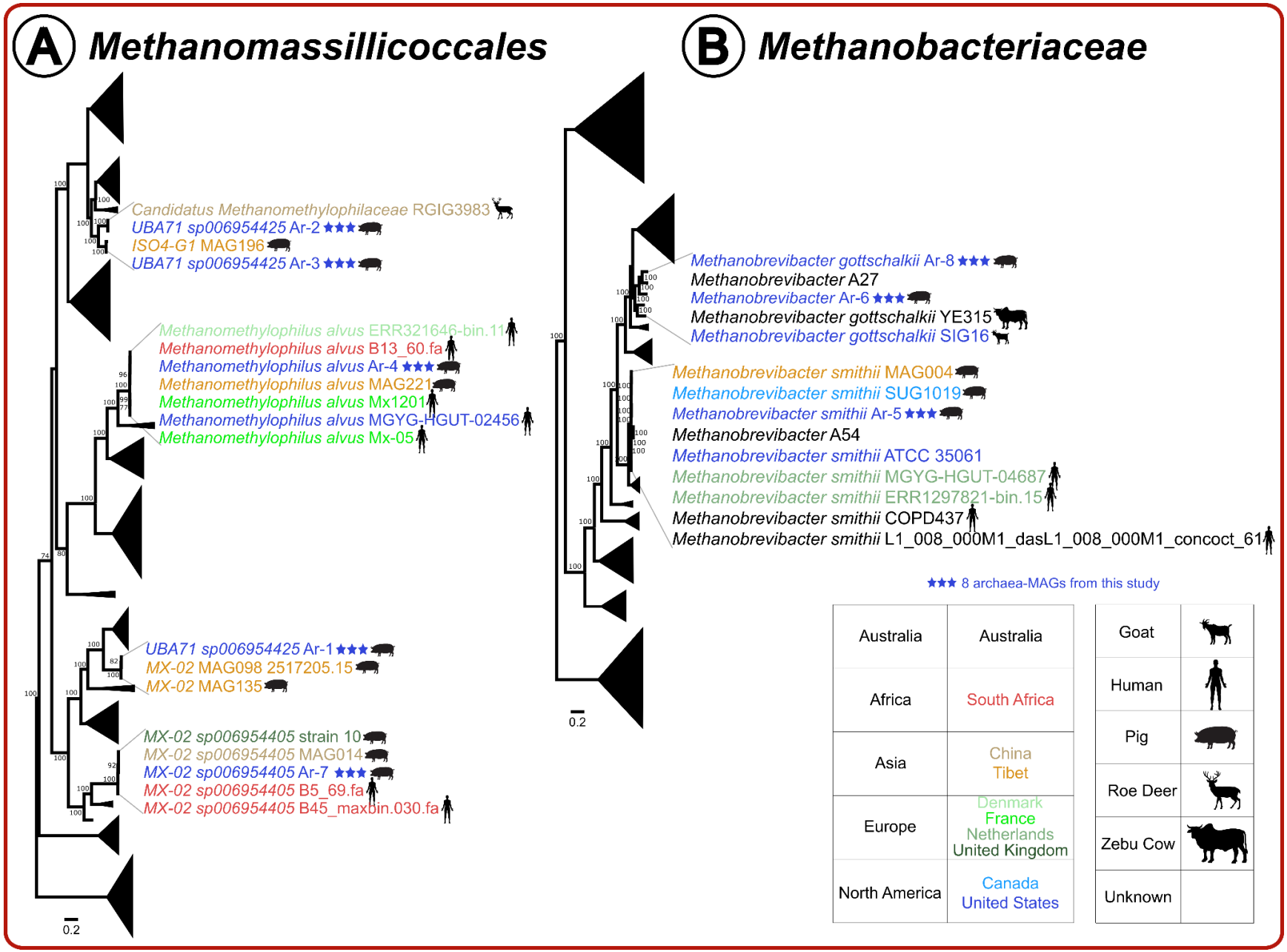
A Methanomassillicoccales
B Methanobacteriaceae
Candidatus Methanomethylophilaceae RGIG3983
UBA71 sp006954425 Ar-2 ★★★
ISO4-G1 MAG196
UBA71 sp006954425 Ar-3 ★★★
Methanomethylophilus alvus ERR321646-bin.11
Methanomethylophilus alvus B13_60.fa
Methanomethylophilus alvus Ar-4 ★★★
Methanomethylophilus alvus MAG221
Methanomethylophilus alvus Mx1201
Methanomethylophilus alvus MGYG-HGUT-02456
Methanomethylophilus alvus Mx-05
UBA71 sp006954425 Ar-1 ★★★
MX-02 MAG098 2517205.15
MX-02 MAG135
MX-02 sp006954405 strain 10
MX-02 sp006954405 MAG014
MX-02 sp006954405 Ar-7 ★★★
MX-02 sp006954405 B5_69.fa
MX-02 sp006954405 B45_maxbin.030.fa
0.2
Methanobrevibacter gottschalkii Ar-8 ★★★
Methanobrevibacter A27
Methanobrevibacter Ar-6 ★★★
Methanobrevibacter gottschalkii YE315
Methanobrevibacter gottschalkii SIG16
Methanobrevibacter smithii MAG004
Methanobrevibacter smithii SUG1019
Methanobrevibacter smithii Ar-5 ★★★
Methanobrevibacter A54
Methanobrevibacter smithii ATCC 35061
Methanobrevibacter smithii MGYG-HGUT-04687
Methanobrevibacter smithii ERR1297821-bin.15
Methanobrevibacter smithii COPD437
Methanobrevibacter smithii L1_008_000M1_dasL1_008_000M1_concoct_61
0.2
★★★ 8 archaea-MAGs from this study
Australia
Australia
Africa
South Africa
Asia
China
Tibet
Europe
Denmark
France
Netherlands
United Kingdom
North America
Canada
United States
Goat
Human
Pig
Roe Deer
Zebu Cow
Unknown

Figure 3.2: Phylogenetic trees of (A) *Methanomassiliicoccales*[218–227] and (B) *Methanobacteriales*[221,227–230,303–308] with bootstrap values ≥ 70 indicated at nodes. Branches were collapsed for non-immediate phylogenetic relatives of our archaea-MAGs while branches containing these 8 MAGs were magnified for clarity. Original non-collapsed trees with statistics are in Ch3_original_phylogenetic_trees.pdf .

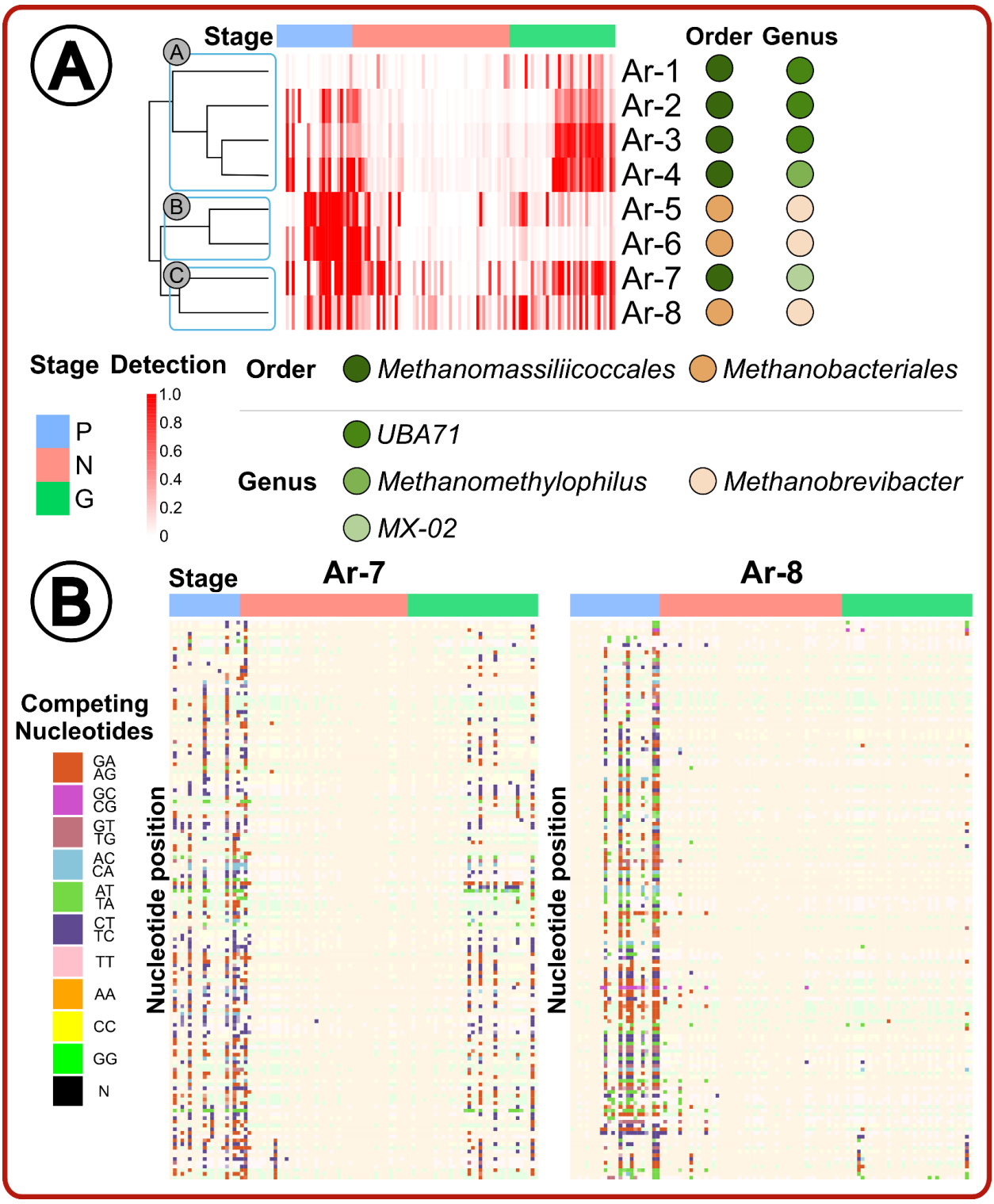

Figure 3.3: (A) Detection heatmap of archaea-MAGs (rows) across all individual sample metagenomes (columns) with MAG taxonomy and stage annotation (Preweaning [P]; nursery [N];

growth adult [G]). (B) Single-nucleotide variant (SNV) analysis of Ar-7 and Ar-8 where box colors indicate competing nucleotides and stage is indicated along the bottom.

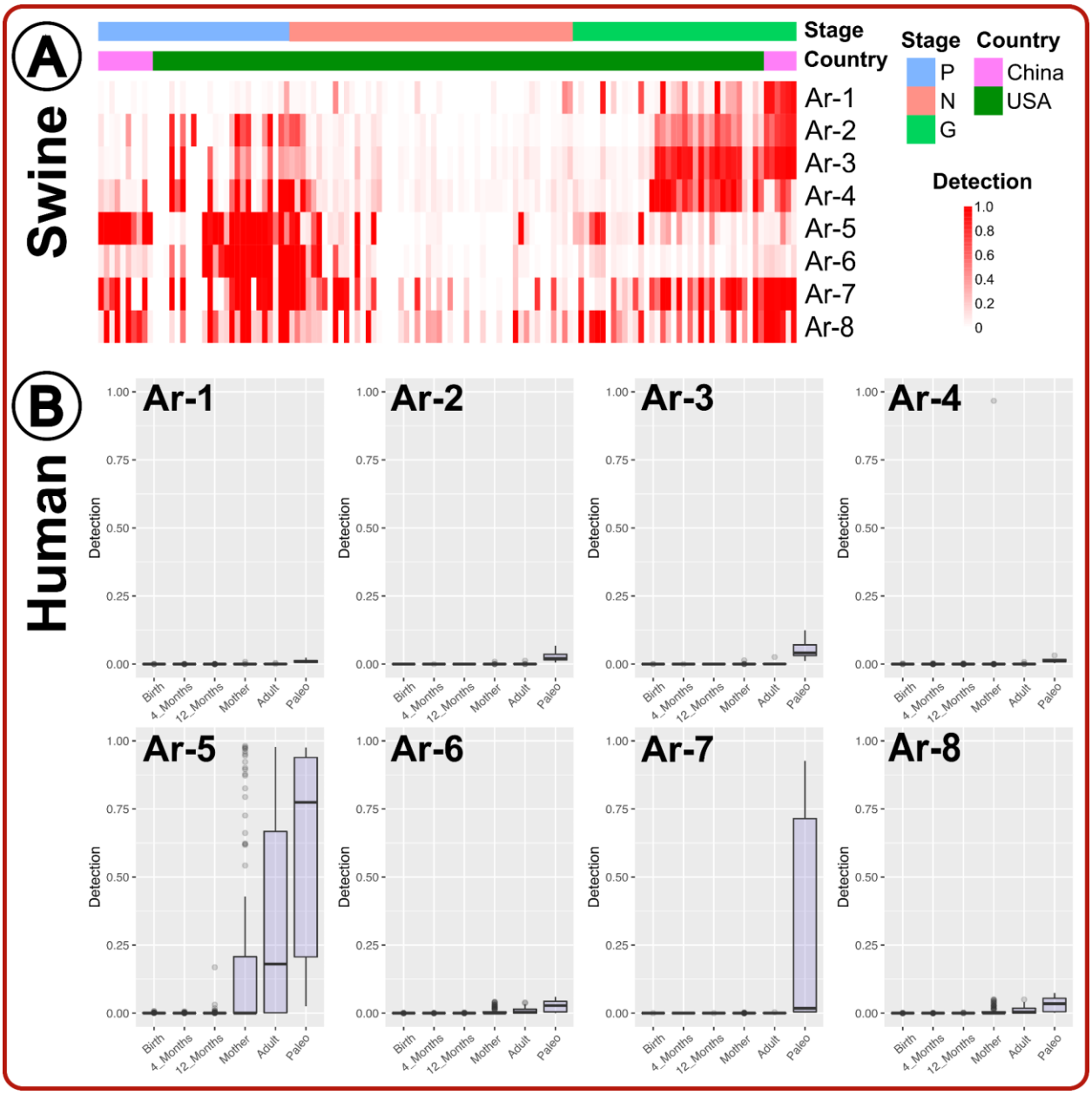

Figure 3.4: (A) Detection heatmap of previously published swine metagenomes[204] mapped to this publication's archaeal MAGs (Preweaning [P]; nursery [N]; growth adult [G]). (B) Detection box plots of previously published human metagenomes[205,206] mapped to our archaeal MAGs ("Adult" from Mexican humans; "Paleo" from present day US and Mexico ; all remaining groups from Sweden).

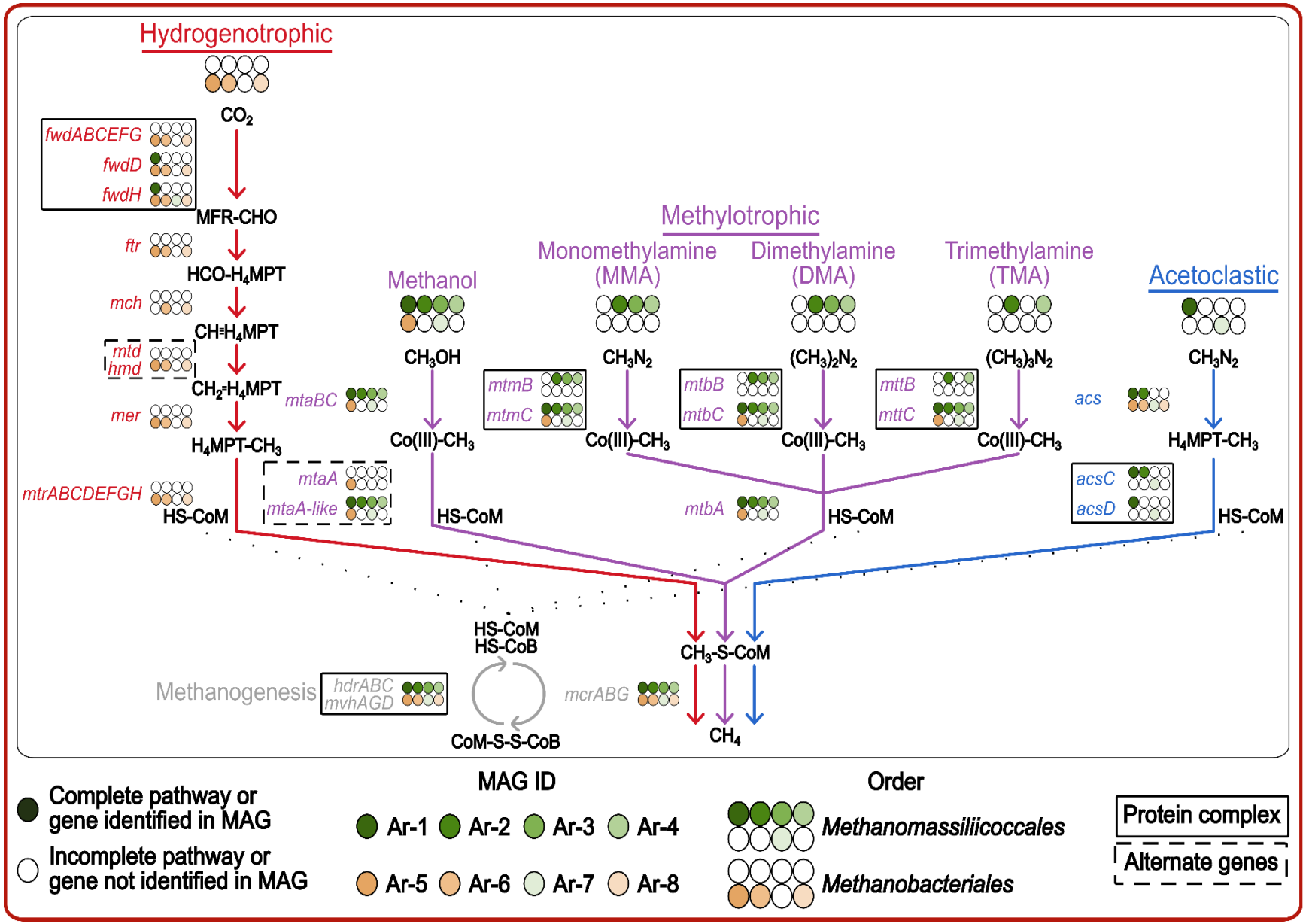
Hydrogenotrophic
CO2
fwdABCEFG
fwdD
fwdH
MFR-CHO
ftr
HCO-H4MPT
mch
CH≡H4MPT
mtd
hmd
CH2=H4MPT
mer
H4MPT-CH3
mtrABCDEFGH
HS-CoM
mtaABC
mtaA
mtaA-like
Methylotrophic
Methanol
Monomethylamine (MMA)
Dimethylamine (DMA)
Trimethylamine (TMA)
Acetoclastic
CH3OH
CH3N2
(CH3)2N2
(CH3)3N2
CH3N2
mtmB
mtmC
mtbB
mtbC
mttB
mttC
acs
Co(III)-CH3
Co(III)-CH3
Co(III)-CH3
Co(III)-CH3
H4MPT-CH3
HS-CoM
mtbA
HS-CoM
acsC
acsD
HS-CoM
HS-CoM
HS-CoB
CH3-S-CoM
Methanogenesis
hdrABC
mvhAGD
mcrABG
CoM-S-S-CoB
CH4
Complete pathway or gene identified in MAG
Incomplete pathway or gene not identified in MAG
MAG ID
Ar-1
Ar-2
Ar-3
Ar-4
Ar-5
Ar-6
Ar-7
Ar-8
Order
Methanomassiliicoccales
Methanobacteriales
Protein complex
Alternate genes

Figure 3.5: Methane metabolic pathway genes detected in our archaeal MAGs distinguished by pathway[217,288,309,310].

Table

Table 3.1: Anvi'o results, including taxonomic assignment, of 8 archaea-MAGs.

MAG ID	Total length (nucleo-tides)	Number of contigs	N50	GC content	Percent completion	Percent redun-dancy	Domain	Phylum	Class	Order	Family	Genus	Species
Ar-1	1,634,787	68	46,847	53%	97%	0%	Archaea	*Thermoplasmatota*	*Thermoplasmata*	*Methanomassiliicoccales*	*Methanomethylophilaceae*	*UBA71*	*UBA71 sp006954425*
Ar-2	2,118,112	250	15,626	54%	95%	1%	Archaea	*Thermoplasmatota*	*Thermoplasmata*	*Methanomassiliicoccales*	*Methanomethylophilaceae*	*UBA71*	*UBA71 sp006954425*
Ar-3	960,519	435	2,416	56%	80%	9%	Archaea	*Thermoplasmatota*	*Thermoplasmata*	*Methanomassiliicoccales*	*Methanomethylophilaceae*	*UBA71*	*UBA71 sp006954425*
Ar-4	1,269,388	112	15,439	56%	83%	4%	Archaea	*Thermoplasmatota*	*Thermoplasmata*	*Methanomassiliicoccales*	*Methanomethylophilaceae*	*Methanomethylophilus*	*Methanomethylophilus alvus*
Ar-5	957,172	18	59,251	32%	72%	0%	Archaea	*Methanobacteriota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobrevibacter*	*Methanobrevibacter smithii*
Ar-6	1,724,540	38	68,759	33%	100%	0%	Archaea	*Methanobacteriota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobrevibacter*	N/A
Ar-7	1,341,189	53	49,597	48%	99%	0%	Archaea	*Thermoplasmatota*	*Thermoplasmata*	*Methanomassiliicoccales*	*Methanomethylophilaceae*	*MX-02*	*MX-02 sp006954405*
Ar-8	1,513,577	47	51,497	31%	100%	1%	Archaea	*Methanobacteriota*	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobrevibacter*	*Methanobrevibacter gottschalkii*

Chapter 4 - High proportions of single-nucleotide variations associated with multidrug resistance in swine gut microbial populations

Brandi Feehan[1], Qinghong Ran[1], Kourtney Monk[1], T. G. Nagaraja[3], M. D. Tokach[4], Raghavendra Amachawadi*[2] , and Sonny T M Lee*[1]

*Corresponding authors

[1]Division of Biology, College of Arts and Sciences, Kansas State University, Manhattan, Kansas, 66506, United States of America.

[2]Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506, United States of America.

[3]Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506, United States of America.

[4]Department of Animal Sciences & Industry, College of Agriculture, Kansas State University, Manhattan, Kansas 66506, United States of America.

Abstract

Background: Antimicrobial resistance (AMR) is a significant global public health concern associated with millions of deaths annually. Agriculture has been attributed as a leading factor in AMR and multidrug resistance (MDR) associated with swine production is estimated as one of the largest agricultural consumers of antibiotics. AMR research has received increased attention in recent years. However, we are still building our understanding of genetic variation within a complex gut microbiome system that impacts AMR and MDR. In order to evaluate the gut resistome, we evaluated genetic variation before, during, and after antibiotic treatments. We studied three treatment groups: non-antibiotic controls (C), chlortetracycline (CTC) treated, and tiamulin (TMY) treated. We collected fecal samples from each group and performed metagenomic sequencing for a longitudinal analysis of genetic variation and functions. **Results**: We generated 772,688,506 reads and 81 metagenome assembled genomes (MAGs). Interestingly, we identified a subset of 11 MAGs with sustained detection and high sustained entropy (SDHSE). Entropy described genetic variation throughout the MAG. Our SDHSE MAGs were considered MDR as they were identified prior to, throughout, and after CTC and TMU treatments as well as in the C piglets. SDHSE MAGs were especially concerning as they harbored relatively high variation. Consistently high variation indicated that these microbial populations may contain hypermutable elements which has been associated with increased chance of AMR and MDR acquisition. Altogether, our study provides comprehensive genetic support of MDR populations within the gut microbiome of swine.

Introduction

Antimicrobial resistance (AMR) is a ubiquitous threat around the world, estimating to be the third cause of global human deaths[311]. Antibiotic resistance was estimated to be associated with 4.95 million deaths globally in 2019[311] and 2.8 million illnesses annually in the US alone[312]. AMR has also burdened medical systems and economies, and scientists see this as a sustained trend expecting $100-210 global losses due to AMR by 2050[313–315]. The burden and repercussions of AMR are a one world health concern as antibiotics are utilized for animals in addition to humans[316]. AMR describes bacteria containing genetic components which allow microbes to survive through antimicrobial treatment. Particular concern arises when microbes exhibit resistance to multiple drugs[37,317]. These multidrug resistant (MDR) organisms (MDRO) can persist, at times, beyond all medicinally utilized antibiotics[37,317,318]. Moreover, multidrug resistant microbes can spread through individuals, and between humans and animals, increasing the prevalence of AMR[319]. With the global burden of AMR, we need an enhanced understanding of AMR to combat infections caused by MDRO.

Animal agriculture has been identified as the largest antibiotic consumer[320]. Antibiotics have been used in agricultural animals, much like humans, to treat bacterial infections, but antibiotics are also for growth promotion in agriculture[321]. Swine production was estimated to be the current largest agricultural animal antibiotic-use sector in 2017[320,322]. Moreover, antibiotic resistance rates are rising in the swine industry as the proportion of antibiotics, with resistance higher than 50%, increased in the swine industry from 0.13 to 0.34 from 2000 to 2018[323]. Global surveys[324–328] and smaller-scale studies[329–335] have in-large identified high consumption of tetracycline antibiotics in the past two decades in animal agriculture and swine. Tetracycline was estimated to account for 43% of antibiotic usage in agricultural animals from 2015 to 2017[328]. Unfortunately tetracycline

antibiotics are not exclusively utilized in animals. For example, chlortetracycline is used in both swine and humans[336,337]. With continued use of antimicrobial drugs, especially when utilizing the same treatments in humans and animals, and increasing resistance to antibiotics, AMR is a global concern to agriculture and humanity alike.

Across monogastric organisms, including pigs and humans, the gut microbiota has been identified as an AMR reservoir[338,339]. The gut microbiome has been recognized as a diverse environment in terms of antibiotic resistant genes[340,341]. With oral antibiotic use, the gut has been demonstrated to increase in resistant microbes[342]. Antibiotic treatments decrease the abundance of susceptible microbes which allows resistant microbes more resources, such as nutrients and space, to increase in abundance[343]. While the work in AMR is accumulating at a fast pace, we still have limited understanding on how genetic variations among the microbial populations contribute to the resistome.

Antibiotic use has been associated with microbes containing increased genetic variation and so-called hypermutable bacteria[35,344]. Antibiotic usage selects for microbes with genetic variation, or those with relatively high mutation rates termed hypermutable bacteria[35]. As microbes develop variation, this leads to an increased chance of developing resistance[35,344]. Therefore with subsequent antimicrobial treatments, we are continually selecting for hypermutable populations harboring increased variation in turn having more opportunities for further AMR acquisition and MDR[35,344]. This can lead to MDR bacteria with high mutation rates to evade future antimicrobial treatments. However, studies related to the understanding of cumulative genetic variation across AMR genes in MDR microbes in response to antibiotic supplementation (in vivo) among piglets

are lacking.. In studying microbial variation in these circumstances, we can further evaluate the risk of and potential treatments for MDR microbes.

Clearly, we need a deeper understanding of antibiotic resistance and MDR to enhance our approach to AMR. Here, we studied gut microbiota through two distinct antibiotic treatments (in-feed chlortetracycline [CTC] and in-feed tiamulin [TMU]) in addition to a non-antibiotic control (C). We utilized swine, with tetracycline and pleuromutilin class antibiotics, to provide an *in vivo* evaluation of a comparatively high and low utilized antibiotic classes[320,322], in the global swine industry[324–335]. As mentioned previously, tetracycline antibiotics accounted for 43% of antibiotic usage in animal agriculture during 2015-2017 whereas pleuromutilin only accounted for 3%[328]. For our study, we performed metagenomic sequencing to obtain genes for functional analysis. Following our subsequent genome assembly and manual genome refining, we identified a subset of 11 metagenome-assembled genomes (MAGs) with high genetic variation prior to and throughout both antibiotic treatments and in control swine. We also confirmed consistent detection of the 11 MAGs and termed these MAGs: sustained detection and high sustained entropy (SDHSE) MAGs. Our SDHSE MAGs are of concern as they contained genetic variation and demonstrated MDR to both CTC and TMU. Moreover, we identified 22 distinct AMR genes in our SDHSE MAGs. Altogether, we provide evidence of MDR bacteria present in swine with concerningly high levels of genetic variation in 11 distinct microbial populations. Our research transcends global health with insights into antibiotic resistance, and especially MDR, from a major contributor to global AMR.

Materials and Methods

Experimental design

The swine study was performed as previously described (Figure 1A-C)[345–347]. Swine (genetic line L337×1050, PIC, Hendersonville, TN) were housed in a commercial research nursery facility. Diets were fed with formulations as previously described[348]. All pigs were housed in one room with an enclosed, environmentally controlled, and mechanical ventilation system. Pens contained slatted floors with deep manure pits. Feed and water were provided *ad libitum* per pen with a six-hole stainless steel self-feeder (refilled via a robotic system) and pan waterer. This study utilized 648 pigs randomly distributed into 24 pens (27 pigs per pen), while working to minimize differences in average pen weight during distribution. Three treatments were administered, according to average pen weight, 7 days after weaning at 21 days of age, for a total of 14 days, each across 8 pens: control (no antibiotic; C), in-feed chlortetracycline (CTC; 22 mg/kg body weight; CTC-hydrochloride, Elanco Animal Health, Indianapolis, IN), and in-feed tiamulin (TMU; 5 mg/kg body weight; Denagard®, Elanco, Animal Health, Indianapolis, IN).

All swine were managed according to protocol #4033 with Kansas State University Institutional Animal Care and Use Committee (IACUC). Furthermore, methods are reported according to ARRIVE guidelines. The authors also confirmed that all methods were performed in accordance with relevant guidelines and regulations, and we affirmed that all methods were approved by Kansas State University.

Sample Collection

For this study, we considered each pig pen as an experimental unit, and there were eight replicate pens per antibiotic treatment (Figure 1A-C). Fecal collection occurred every seven days, starting

on the day of introduction to the pens. Fecal samples were collected via gentle rectal massage from five randomly selected pigs per two random pens per treatment, and each fecal sample was stored in individual sterile plastic bags (Whirl-Pak® bags, Nasco, Ft. Atkinson, WI) and kept on ice during transportation. Processing occurred within 24 hours of receipt, with intermittent storage at 4°C, at the Pre-harvest Food Safety laboratory, College of Veterinary Medicine, Kansas State University. To reduce research bias, laboratory personnel were blinded to the treatments.

DNA extraction

We stored fecal samples at -80°C until DNA extraction. For each pen and time-point, the five fecal samples were pooled for DNA extraction (Figure 1A-C). Total genomic DNA from fecal samples was extracted utilizing the DNeasy PowerSoil Pro Kits (QIAGEN Inc.; Valencia, CA), following the manufacturer protocols. We then quantified the extracted genomic DNA with a Nanodrop and Qubit™ (dsDNA BR Assay Kit [Thermo Fisher; Waltham, MA]) for DNA quality and concentration. Final storage of extracted DNA was at -80°C until library preparation and sequencing.

Metagenomic sequencing and 'omics workflow

Library preparation was performed on 30 samples with Nextera DNA Flex (Illumina, Inc.; San Diego, CA) (Figure 1B; Ch4_sample_metadata.xlsx). A Tapestation 4200 (Agilent; Santa Clara, CA) was employed to visualize libraries followed by size-selected via a BluePippin (Sage Science; Beverly, MA). The final library pool of 30 samples was quantified with the Kapa Biosystems (Roche Sequencing; Pleasanton, CA) qPCR protocol, and sequenced on an Illumina NovaSeq S1 chip (Illumina, Inc.; San Diego, CA) with a 2 x 150 bp paired-end sequencing strategy.

We performed a bioinformatics workflow using anvi'o v.7.1 (https://anvio.org/install/; 'anvi-run-workflow' program)[187,188]. The workflow utilized Snakemake[349] to perform multiple tasks: short-read quality filtering, assembly, gene calling, functional annotation, hidden Markov model search, metagenomic read-recruitment and binning[189]. Briefly, we processed sequencing reads using anvi'o's 'iu-filer-quality-minoche' program removing low-quality reads following criteria described in Minoche *et al.*[190]. We termed the resulting quality-control reads "metagenome" per sample. We co-assembled quality-control short reads from metagenomes into longer contiguous sequences (contigs) according to no-treatment (prior to treatment/after) and treatment groups (C, CTC, TMU). We utilized MEGAHIT v1.2.9[187,191] for co-assembly. The following anvi'o methods were then performed to further process contigs: (1) 'anvi-gen-contigs-database' to compute *k*-mer frequencies and identify open reading frames (ORFs) using Prodigal v2.6.3[187,192]; (2) 'anvi-run-hmms' to annotate bacterial and archaeal single-copy, core genes using HMMER v.3.2.1[187,193]; (3) 'anvi-run-ncbi-cogs' to annotate ORFs with NCBI's Clusters of Orthologous Groups (COGs; https://www.ncbi.nlm.nih.gov/research/cog)[194]; and (4) 'anvi-run-kegg-kofams' to annotate ORFs from KOfam HMM databases of KEGG orthologs (https://www.genome.jp/kegg/)[195].

We mapped all metagenomes' short reads to contigs with Bowtie2 v2.3.5[196]. We converted mappings with samtools v1.9[187,197,198] into BAM files. We profiled BAM mapping files ('anvi-profile')[187] with a minimum length of 1,000 bp. We then combined profiles with 'anvi-merge' into a single anvi'o profile. Next, we used CONCOCT v1.1.0[199] to group contigs into bins. We manually refined bins with 'anvi-refine' using bin tetranucleotide frequency and coverage across sample metagenomes[187,200,201]. After manual refining, we labeled bins that had ≥70% completion and <10% redundancy (both based on single-copy core gene annotation[350]) as metagenome-assembled genomes (MAGs). We analyzed MAG occurrences according to the "detection" metric.

We determined single-nucleotide variants (SNVs) on all MAGs after read mapping with 'anvi-gen-variability-profile' and '--quince-mode'[187]. We used anvi'o's DESMAN v2.1.1 to analyze SNVs to determine the number and distribution of subpopulations in the MAGs[203]. We accounted for non-specific mapping by removing any MAG subpopulations that made up less than 1% of the entire population and were explained by a singular MAG.

Data analyses

We used RStudio v1.3.1093[102] to visualize MAGs detection and entropy patterns in RStudio (https://www.rstudio.com/products/rstudio/) using: pheatmap (pretty heatmaps) v1.0.12[105], ggplot2 v3.3.5 (https://ggplot2.tidyverse.org/)[210], forcats v0.5.1 (https://forcats.tidyverse.org/)[211], dplyr v1.0.8 (https://dplyr.tidyverse.org/)[212], and ggpubr v0.4.0 (https://CRAN.R-project.org/package=ggpubr)[101]. We generated SNVs counts according to individual sample with anvi'o, anvi-summarize, and MAG entropies[351] were generated with anvi'o's anvi-gen-variability-profile[187,201]. Individual MAG entropy files and individual MAG statistical analysis files were combined respectively in RStudio with: tidyverse 1.3.1 (https://cran.r-project.org/web/packages/tidyverse/citation.html)[98] and 1.4.0 (https://stringr.tidyverse.org/)[352]. We performed Welch two sample T-test[353] statistical analysis on detection and entropy according to pre-treatment versus post-treatment and treatment groups. We used anvi'o COG annotations, as described above, for metabolic function analyses[187]. Our final figures were edited in Inkscape v1.2.1[157].

Results and Discussion

Antimicrobial resistance (AMR), and especially multidrug resistance (MDR), are a global concern. Agriculture has been identified as the top consumer of antibiotics with the swine industry

consuming the most of any agricultural sector[320,322]. In order to better understand AMR and MDR dynamics of the swine gut microbiome, we collected samples prior to, during and after antibiotic treatment. We utilized three distinct treatment groups: chlortetracycline, tiamulin, and non-antibiotic control. These antibiotics were utilized to allow analysis of distinctly utilized microbial classes across swine. Tetracyclines, the class chlortetracycline falls within, accounted for 43% of antibiotic usage in animal agriculture during 2015-2017 whereas pleuromutilin, including tiamulin, accounted for merely 3%[328]. Interestingly, we identified 11 distinct bacterial populations with similar detection levels pre- and post-treatment and between treatments. These microbes were especially of concern as they harbored high genetic variation. These 11 microbial populations, assembled from our metagenomic data, were termed sustained detection and high sustained entropy (SDHSE) metagenome-assembled genomes (MAGs). Already exhibiting MDR, high variation in our resolved SDHSE MAGs could result in enhanced multidrug resistance. We further identified 22 unique AMR genes with varying detection in SDHSE MAGs. Altogether, we detailed AMR of swine microbiota with genetic support of existing MDR prior to antibiotic treatments and sustained variation throughout treatments. Our study advances AMR and MDR research by providing analysis of a top consumer of antibiotics globally: swine.

Resolved identify of gut metagenome-assembled genomes

We assembled and analyzed high resolution metagenome-assembled genomes (MAGs) to postulate functional distinctions between gut microbiota before and after antibiotic treatment. Each MAG represents a "microbial population." We described a microbial population as an assemblage of coexisting microbial genomes in an environment that are similar enough to map metagenomic reads to the same reference genomes[354]. Metagenomic sequencing on an Illumina NovaSeq produced 772,688,506 paired-end reads from 30 fecal samples (Figure 1B;

Ch4_read_and_assembly_results.xlsx). After quality filtering, 741,143,268 paired reads (96%) were utilized in contig co-assembly. We generated 330,769 contigs from assembly which described 1,018,536,193 nucleotides and 1,270,711 genes. We performed contig binning to create 369 bins, and after automatic and manual refinement we resolved 205 MAGs (Ch4_anvi'o_results.xlsx). To ensure high quality MAGs in our analysis, we performed downstream analysis with MAGs greater than 2M nucleotides (n=81), as these would more accurately represent bacterial genomes[355]. Of these 81 MAGs, each MAG, contained 360 ± 232 contigs and an N50 value of 18,345 ± 16,569 nucleotides. MAG GC contents ranged from 26% to 62%. Moreover, the average MAG size was 2,424,923 nucleotides. Our MAGs described 6 bacterial phyla (*Actinobacteriota*, n=3; *Bacteroidota*, n=37; *Firmicutes*, n=38; *Planctomycetota*, n=1; *Proteobacteria*, n=1; and *Verrucomicrobiota*, n=1) with 96% of the MAGs resolved to 48 distinct genera.

We mapped each sample's metagenomic reads (i.e. metagenome) to the 81 MAGs to determine detection throughout the study (Figure 2 and Ch4_MAG_detections_sample_SNVs.xlsx). We confirmed detection of all 81 MAGs and determined general differential detection patterns according to detection clustering. The top branches broadly depict MAGs detected in the pre-treatment hosts. Comparatively, the middle clusters were sparsely detected. Finally, the bottom clusters were, in general, detected relatively high, compared to previous clusters, throughout the experiment regardless of pre- or post-treatment or treatment group. Altogether, our detection analysis suggested that association of microbial populations with our swine hosts was far more complex than just what microbes were affected by the use of antibiotics.

Previous studies suggested environmental pressures, such as antibiotic administration, increased genetic variation in microorganisms[356,357]. The genetic variation in bacteria results from single nucleotide polymorphisms (SNPs), and could lead to generation of novel bacterial strains[356]. Studies further demonstrated that bacteria often used mutations as a mechanism for stress response, which is termed as stress-induced mutagenesis[358]. Since one of the mechanisms for the diversification and adaptation of the genomes operates at the single nucleotide level, we proceeded to resolve a more complete understanding of the environmental forces that affect adaptive strategies of our resolved MAGs to survive in the environment they resided in. Therefore, while our MAGs were detected throughout the study, we were particularly interested in how MAG variants were changing according to treatment. Our bioinformatic analysis generated single nucleotide variants (SNVs) according to sample (Figure 2). We noticed relatively more SNVs associated with the post-treatment samples which suggested that our resolved MAGs might respond to the antibiotic induced environmental pressure leading to generation of new strains[356]. In light of this discovery, we proceeded to evaluate which MAGs were consistently high variation while maintaining detection even with different antibiotic treatments. These MAGs could potentially evade antimicrobial treatment with a multitude of variants, as demonstrated through sustained detection. Therefore, we next evaluated entropy throughout all 81 MAGs.

MAGs harboring high genetic variation persisted through antimicrobial treatment

We performed single nucleotide variant (SNV) analysis to calculate entropy on our 81 MAGs to investigate genetic variation due antibiotic induced environmental pressure (Ch4_entropy_results.xlsx). Entropy describes nucleotide ratios for a given position, and entropy is measured from 0 (no variation; A=0, T=0, G=0, C=1) to 1 (complete variation; A=0.25, T=0.25, G=0.25, C=0.25)[359]. We performed statistical analysis to determine which MAGs held high

sustained variation in the form of entropy and sustained detection (Ch4_detection_and_entropy_stats.xlsx). We discovered 31 MAGs with no statistical difference in entropy and detection (Ch4_detection_and_entropy_stats.xlsx). These MAGs represented microbial populations that were detected consistently regardless of antibiotic treatment. We further narrowed our selection to 11 MAGs with the relatively highest (33%) variation (Ch4_detection_and_entropy_stats.xlsx) because we were interested in MAGs harboring high variation, with potential multidrug evasion. Previous publications demonstrated the use of relative entropy analysis versus discrete entropy thresholds[360–362]. These 11 MAGs were termed sustained detection and high sustained entropy (SDHSE) MAGs (Figure 3; Table 1). Of these SDHSE MAGs, 5 (45%) were assigned to the gram negative *Bacteroidota* phylum, while 6 (55%) were annotated to gram positive *Firmicutes*. While members of both phyla have been associated with resistance to CTC and TMU, we identified only 2 (*Prevotella*[363–365] and *Ruminococcus*[366–368]) of 9 genera associated with CTC resistance and 0 with TMU. Of our 11 SDHSE MAGs, 8 (73%) MAGs were annotated to 8 distinct species. Akin to the genus level, we provided novel associations of bacterial species, within the SDHSE MAG populations, exhibiting MDR. The finding indicated there are likely additional genera and species, with CTC and TMU resistance, than are currently known. Still, we wanted to investigate how the genetic variation of our SDHSE MAGs was related to AMR and MDR.

Our SDHSE MAGs satisfied three important criteria - 1) consistent detention; 2) consistent high coverage of MAGs in the metagenomes; 3) consistent high variation of the MAGs in the metagenomes. Consistent detection demonstrated MDR, at least encompassing resistance to CTC and TMU, of the microbial populations. Consistent high coverage of the MAGs removed biases of identifying false variations among metagenomes due to coverage differences. Finally, previous

publications have described how microbes harboring variation are a concern for antimicrobial resistance[35,369,370]. When a microbial population contains a relatively high number of SNVs or contains a highly variable genetic background, the population contains genetic variation which may allow the microbe to persist even with antibiotic treatments. Here we demonstrated that our 11 SDHSE MAGs showed similar detection prior to and after distinct antibiotic treatments (CTC and TMU). The specific variants harbored in these MAGs are of particular interest to antimicrobial resistance (AMR) studies, thus, we surmised that the broad variation within these SDHSE MAGs likely contributed to the microbes adaptive ability to survive antibiotic induced environments. Moreover, harboring continued high variation even after antibiotic treatment suggested many variants were able to persist during and after CTC and TMU treatment[35,369,370]. Previous studies highlight the role of antibiotic selection for populations with higher mutations, called hypermutable bacteria, which leads to high genetic variation in subsequent generations[35,344]. Hypermutable bacteria, including our SDHSE MAGs harboring numerous variants, are a concern to AMR with their MDR potential.

We hypothesized that out 11 SDHSE MAGs likely contained AMR genes contributing to their continued detection. Therefore we next evaluated these MAGs for AMR genes within our functional potential annotations.

Abundance of antimicrobial resistance (AMR) genes associated with sustained detection and high sustained entropy (SDHSE) MAGs

We hypothesized genetic components associated with antimicrobial resistance (AMR) supported the ability for SDHSE MAGs to prevail regardless of CTC and TMU use. We used COG annotations to investigate genetic functions for our 11 SDHSE MAGs, and obtained a total of

21,025 COG annotations (average 1,911 per MAG). We observed numerous AMR genes within the high entropy contigs among the SDHSE MAGs (Ch4_annotations.xlsx). Within the COG annotations, we identified 19 unique gene annotations that coded for 18 distinct proteins or protein complexes related to AMR with an additional three genes (two complexes: YadH/YadG and RhaT) for drug efflux (Figure 4, Table 2, and Ch4_AMR_genes_resistance.xlsx)[371–408]. We identified genes associated with six different drug efflux pump superfamilies (ATP-binding cassette [ABC], multidrug and toxic compound extrusion [MATE], drug/metabolite transporter [DMT], major facilitator [MFS], resistance-nodulation-division [RND], and small multidrug resistance [SMR]) alongside genes coding for: antimicrobial peptides (AMP), **β**-lactamases, **β**-lactamase regulators, and penicillin binding protein (PBP) relatives. Interestingly, of the 11 SDHSE MAGs, the gram negative MAGs (n=5) were, on average, annotated with 13 (57%) of the 22 genes, whereas the gram positive MAGs (n=6) were annotated on average with 12 (52%) genes. This agrees with previous literature indicating AMR is more often associated with gram negative bacteria relative to gram positive bacteria[409]. Still, both gram negative and gram positive bacteria cause significant illnesses and mortalities globally[409–411]. Given the risk MDR bacteria, including our SDHSE MAGs, pose to society, we further investigated individual resistome genes and proteins to build the knowledge surrounding AMR and MDR.

We noticed all SDHSE MAGs contained a variety of drug efflux pump and other (non-efflux pump) genes. Looking further into suspected resistance to antibiotics, based on AMR gene annotations, we discovered all SDHSE MAGs harbored AMR genes associated with 5 distinct antibiotic classes (Table 2)[371–397]. Tetracycline resistance, including resistance to CTC, is suspected across all SDHSE MAGs due to shared presence of *ftsI* and *mepA*, alongside *mdlB*, *norB*, *tetA*, *acrA-acrB-tolC*, *emrE*, and *tetD*[371–397]. The shared presence of multiple AMR genes

could explain the consistent identification of these MAGs, regardless of antibiotic use. These microbes could have repressed effects of the antibiotics as a result of these, and likely other, AMR genes. As expected, our gram negative MAGs contained, on average, a broader antibiotic class resistance (n=8.8) compared to gram positive MAGs (n=7.0)[409]. The physical membrane distinctions between gram positive and gram negative bacteria have resulted in greater antimicrobial resistance in gram negative bacteria[409]. Overall, we showed that all SDHSE MAGs demonstrated multidrug resistance potential which likely contributed to their continual presence even after antibiotic treatment.

We identified tripartite efflux pumps solely in gram negative MAGs. We identified the RND tripartite AcrAB-TolC complex genes in nearly all of our SDHSE gram negative genomes; only SDHSE-03 lacked AcrAB-TolC identification. RND pumps facilitate efflux across the outer membrane[406]. Gram positive bacteria lack this outer membrane which coincided with the absence of *acrA-acrB-tolC* annotation in our gram positive MAGs[406]. Moreover, since the majority of efflux pumps only facilitate efflux across the first membrane, RND pumps such as *acrA-acrB-tolC* have been described as broader and supports association of *acrA-acrB-tolC* with CTC and TMU resistance (Ch4_AMR_genes_resistance.xlsx)[371,391,406]. A similar tripartite structure and efflux action have been described in the MFS efflux pump EmrAB-TolC, again in gram negative bacteria[406]. Although, EmrAB-TolC, to the best of our knowledge, has not been associated with CTC or TMU resistance. In our study, the identification of EmrAB-TolC in the CTC and TMU treatments suggested that EmrAB-TolC could have roles in CTC and TMU resistance. Further research is crucial to discover the range and action of tripartite efflux pumps in resistance and especially MDR.

We identified six of seven distinct efflux superfamilies, but we did not identify any proteobacterial antimicrobial compound efflux (PACE) annotations[412]. Although PACE has been demonstrated as prevalent in gram negative bacteria, previous literature has lacked identification in gram negative *Bacteroidota*, while PACE has been identified in gram positive *Firmicutes*[413]. PACE, the newest antibiotic class, was first described in 2015, with the second newest antibiotic class having been found in 2000[414]. Therefore, the breadth of knowledge surrounding PACE is growing and our MAGs could contain PACE efflux pump genes which have not been annotated within the COG database to date.

Beyond drug efflux pumps, we also identified genes coding for additional AMR proteins. As expected, we did not associate CTC or TMU resistance with **β**-lactamase or penicillin binding protein (PBP) related genes (Ch4_AMR_genes_resistance.xlsx). **β**-lactamase inactivates **β**-lactam antibiotics, including penicillins, carbapenems, and cephalosporins[415]. We surmised that genes coding for these AMR proteins identified in our SDHSE MAGs have not previously been associated with CTC and TMU resistance due to their biochemical action, limiting their range of resistance[415].

We did not identify any genes, outside of drug efflux genes, with suspected pleuromutilin (TMU) resistance. The only gene with suspected TMU resistance was the RND pump AcrAB-TolC[395], which has also been associated with resistance of 5 other antibiotic classes. Only 36% of SDHSE MAGs contained genes with resistance to pleuromutilin antibiotics like TMU[391]. There are likely additional genes beyond the COG annotations we evaluated, and perhaps additional resistance which has yet to be discovered[416]. Additionally, we found that the majority of proteins (56%) associated with our genes had no previous support for resistance to CTC or TMU. Together, these

results indicated a sizable knowledge gap in understanding the implications of strain level genetic variations among bacterial populations. Our SDHSE MAGs are likely harboring MDR to not only CTC and TMU, but other drugs, with genetic variations hindering targeted therapeutics[417]. While there has been a call for shifting our antibiotic usage to narrow or even strain-specific antibiotics to limit further AMR with application of broad antibiotics[418], bacterial populations with high genetic variation could minimize the success of such therapies[35]. Clearly bacterial populations, such as SDHSE bacteria, with high genetic variation are concerning as they demonstrate increased AMR and threaten further AMR through targeted antimicrobials[35]. Future research needs to investigate similar SDHSE populations to determine their prevalence and risk they pose to global health.

Conclusions

In order to investigate genetic variation pertaining to multidrug resistance (MDR) and antimicrobial resistance (AMR), we evaluate the gut microbiome for population dynamics before, during and after antimicrobial treatment. Our research is critical to understanding the implications of AMR on global health as we evaluated resistance in a sector dominating antibiotic use: agricultural swine[320,322]. We demonstrated evidence of MDR bacterial populations present prior to antibiotic administration through 11 distinct bacterial populations we termed sustained detection and high sustained entropy (SDHSE) MAGs. Within these MAGs, we indicated novel CTC and TMU resistance association with their taxonomic classifications at the genus and species levels. As work continues to discover gut-associated microbes, we should evaluate their AMR characteristics to combat further resistance. Further highlighting the need for heightened AMR research, we found that approximately a third of our SDHSE MAGs contained annotated genes associated with TMU resistance. Although given the consistent identification of these MAGs

during TMU treatment, there must be TMU resistance genes within the SDHSE genomes resulting in TMU resistance. While the number of antibiotic resistance studies published has increased substantially since 2010[419], the scientific community still has numerous topics to evaluate to better target AMR and MDR, all while under the pressure of rising antimicrobial resistance concerns[420].

Declarations

Ethics approval and consent to participate

Pigs were managed according to the Kansas State University Institutional Animal Care and Use Committee (IACUC) approved protocol #4033.

Availability of data and material

We uploaded our metagenome raw sequencing data to the SRA under NCBI BioProject PRJNA899060. All other analyzed data, in the form of databases and fasta files, and bioinformatic scripts are accessible at figshare (https://doi.org/10.6084/m9.figshare.21548445.v1).

Competing Interests

The authors declare no competing interests.

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Author Contributions

R.A designed the study. Sample collection and DNA extraction was completed by R.A. B.F and K.R. fulfilled Nanodrop and Qubit quality analysis. B.F. and Q.R., S.T.M.L. performed anvi'o bioinformatic analyses. B.F. and S.T.M.L. attributed biological relevance, wrote the manuscript, prepared figures, and supplemental files. B.F. and S.T.M.L. performed major manuscript and figure refinement while remaining authors contributed to lighter refinement. All authors read, contributed to manuscript revision, and approved the submitted version.

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Figures

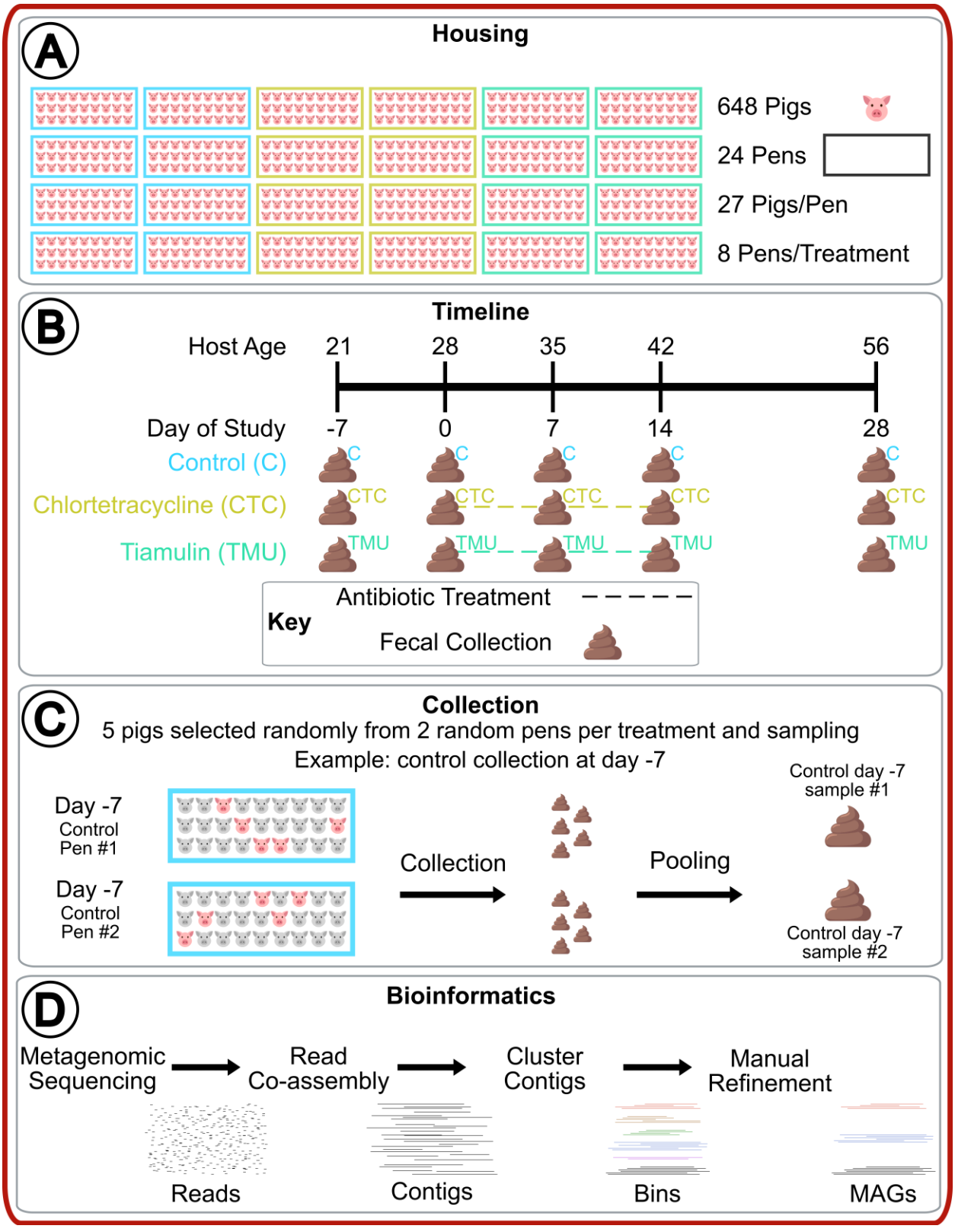
A
Housing
648 Pigs
24 Pens
27 Pigs/Pen
8 Pens/Treatment
B
Timeline
Host Age
21
28
35
42
56
Day of Study
-7
0
7
14
28
Control (C)
Chlortetracycline (CTC)
Tiamulin (TMU)
Key
Antibiotic Treatment
Fecal Collection
C
Collection
5 pigs selected randomly from 2 random pens per treatment and sampling
Example: control collection at day -7
Day -7
Control
Pen #1
Day -7
Control
Pen #2
Collection
Pooling
Control day -7
sample #1
Control day -7
sample #2
D
Bioinformatics
Metagenomic
Sequencing
Read
Co-assembly
Cluster
Contigs
Manual
Refinement
Reads
Contigs
Bins
MAGs

Figure 4.1: A) Pig and pen housing* allocation to treatments. B) Timeline of study. C) Fecal sample collection and pooling. D) Bioinformatics from sequencing reads to refined MAGs.

*Image denotes pen treatments in same location for simplification. Note that pens were not all located in one region of room, instead pens were dispersed to control for adjoining pen interactions[345–347].

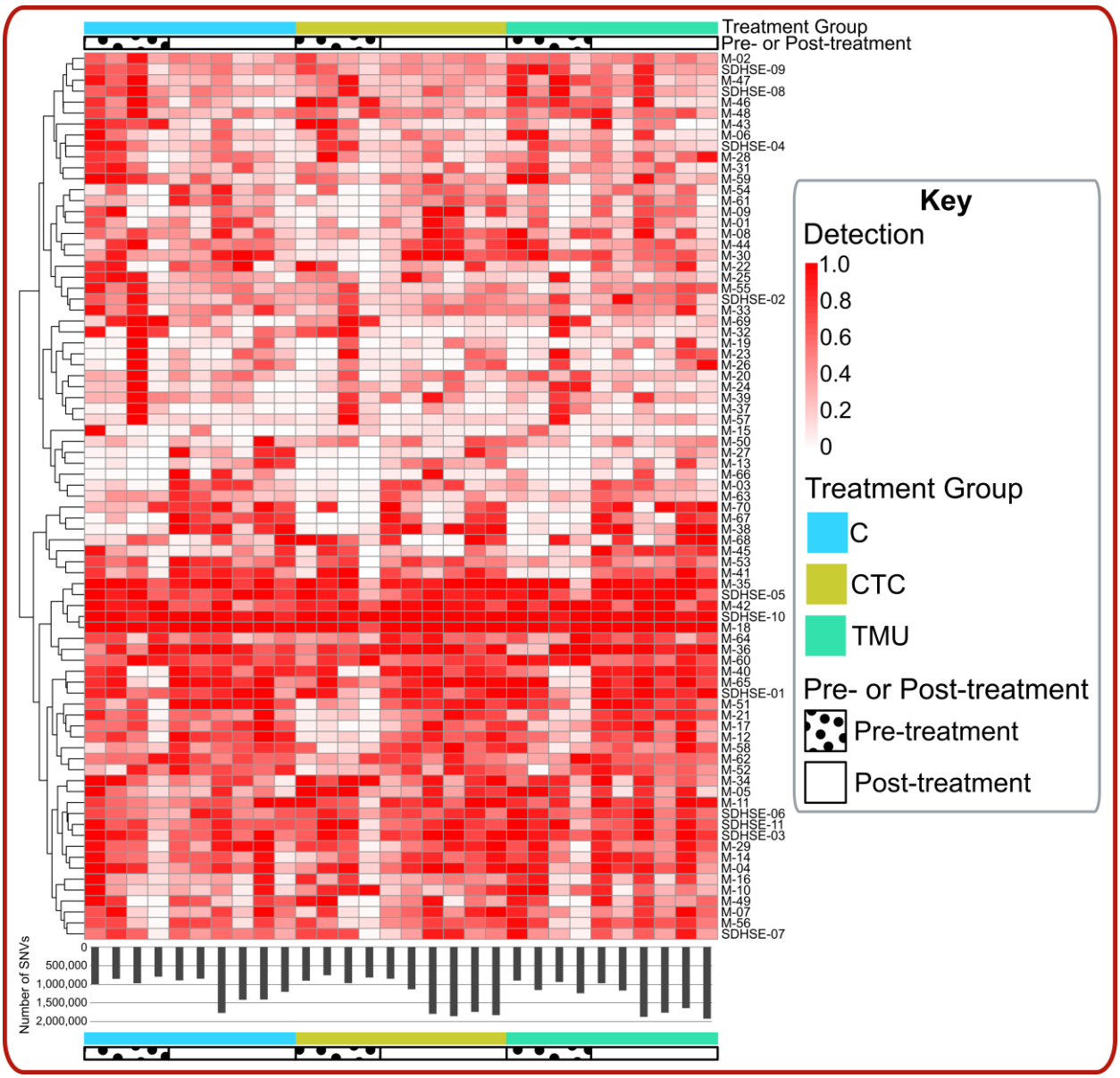

Figure 4.2: MAG detection heatmap (top) and single nucleotide variants (SNVs; bottom) according to treatment group and pre-/post-treatment (from left to right is earliest sampling [day -7] to last sampling [day 28] per treatment group) per sample.

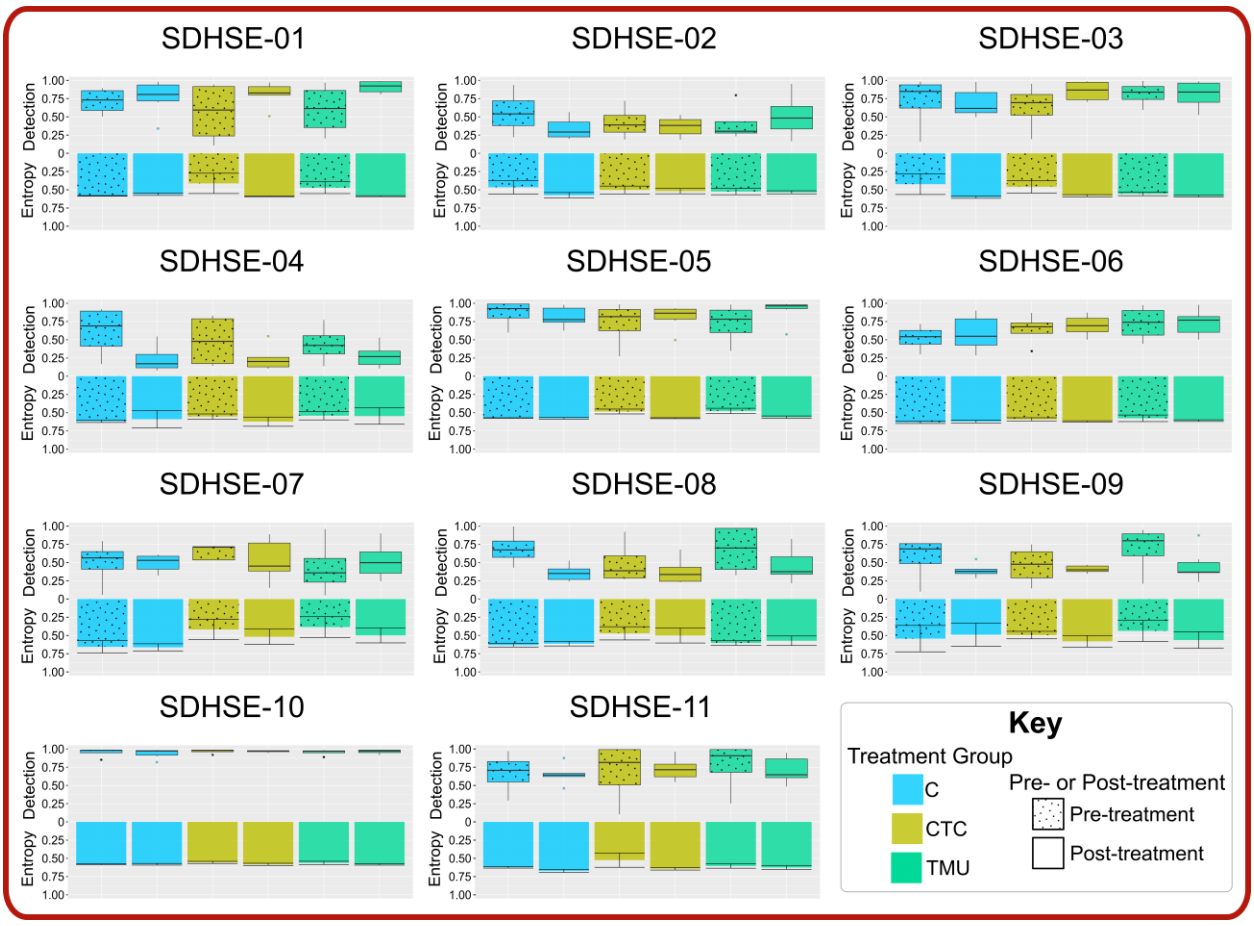

Figure 4.3: Detection boxplot and entropy bar graphs of our 11 sustained detection and high sustained entropy (SDHSE) MAGs.

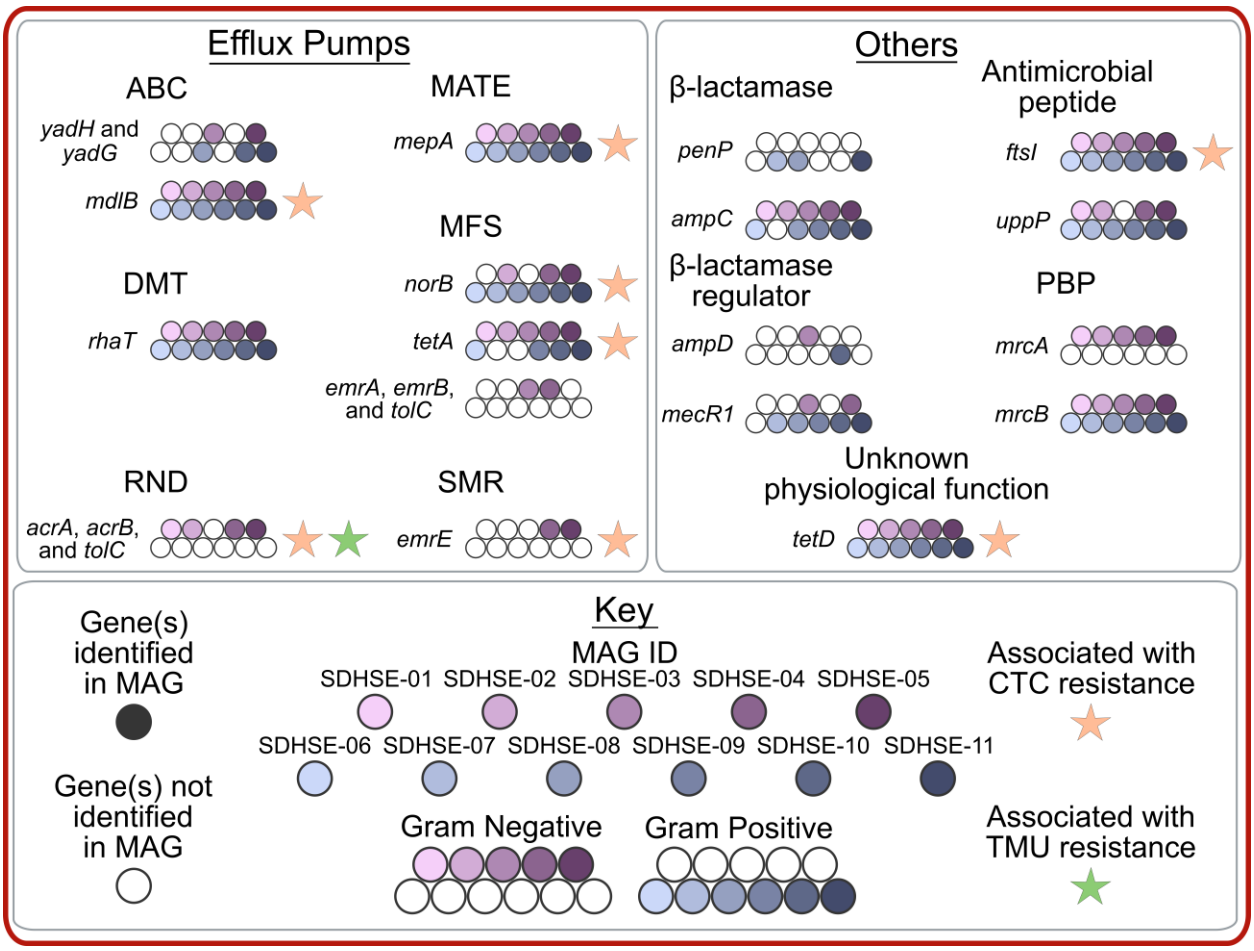

Figure 4.4: AMR genes detected in SDHSE MAGs annotated according to presence in our gram negative/positive MAGs and according to association with CTC (or tetracycline) or TMU (or pleuromutilin) previously[371–408].

Tables

Table 4.1: Anvi'o results, including taxonomic assignment, of sustained detection and high sustained entropy (SDHSE) MAGs.

MAG ID	Total length (nucleo-tides)	Number of contigs	N50	GC cont-ent	Percent comple-tion	Percent redun-dancy	Domain	Phylum	Class	Order	Family	Genus	Species
SDHSE-03	2,629,111	365	15,755	44%	79%	7%	*Bacteria*	*Bacteroidota*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Prevotella*	N/A
SDHSE-04	2,458,376	727	4,266	48%	93%	8%	*Bacteria*	*Bacteroidota*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*UBA6382*	*UBA6382 sp002439755*
SDHSE-01	2,343,180	332	9,899	49%	96%	8%	*Bacteria*	*Bacteroidota*	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*C941*	*C941 sp004557565*
SDHSE-02	2,593,352	391	11,592	51%	86%	8%	*Bacteria*	*Bacteroidota*	*Bacteroidia*	*Bacteroidales*	*UBA932*	*RC9*	*RC9 sp000431015*
SDHSE-05	2,812,877	455	10,884	52%	87%	3%	*Bacteria*	*Bacteroidota*	*Bacteroidia*	*Bacteroidales*	*UBA932*	*RC9*	*RC9 sp000433355*
SDHSE-08	2,753,895	203	29,028	41%	90%	6%	*Bacteria*	*Firmicutes*	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Blautia*	N/A
SDHSE-10	2,095,569	581	4,928	42%	94%	7%	*Bacteria*	*Firmicutes*	*Clostridia*	*Oscillospirales*	*Acutalibacteraceae*	*Ruminococcus*	N/A
SDHSE-06	2,196,155	223	21,375	52%	96%	1%	*Bacteria*	*Firmicutes*	*Clostridia*	*Oscillospirales*	*Acutalibacteraceae*	*Ruminococcus*	*Ruminococcus sp003531055*
SDHSE-07	2,289,369	499	7,653	56%	80%	4%	*Bacteria*	*Firmicutes*	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	*CAG-170*	*CAG-170 sp003516765*
SDHSE-11	2,034,772	145	22,117	61%	87%	1%	*Bacteria*	*Firmicutes*	*Clostridia*	*Oscillospirales*	*Ruminococcaceae*	*Gemmiger*	*Gemmiger sp004561545*
SDHSE-09	2,432,201	438	8,763	54%	96%	8%	*Bacteria*	*Firmicutes*	*Clostridia*	*Oscillospirales*	*Ruminococcaceae*	*Ruthenibacterium*	*Ruthenibacterium sp002315015*

Table 4.2: Antibiotic class resistance, based on previous publications and our annotated AMR and drug efflux genes, for our SDHSE MAGs[371–408]; green filled boxes indicate resistance associated with gene(s) whereas white demonstrates no AMR association.

Antibiotic Class		β-lactam	Fluoroquinolone	Glycylcycline	Sulfonamide	Tetracycline	Bacitracin	Quinolone	Chloramphenicol	Pleuromutilin	Aminoglycoside	Nitroquinoline	Sum Antibiotic Resistant Classes	Average	Standard Deviation
Gram Negative	SDHSE-01	■	■	■	■	■	■		■	■			8	8.8	1.7
	SDHSE-02	■	■	■	■	■	■	■	■	■			9		
	SDHSE-03	■	■	■	■	■						■	6		
	SDHSE-04	■	■	■	■	■	■	■	■	■	■	■	11		
	SDHSE-05	■	■	■	■	■	■	■	■	■	■		10		
Gram Positive	SDHSE-06	■	■	■	■	■	■	■					7	7	0.0
	SDHSE-07	■	■	■	■	■	■	■					7		
	SDHSE-08	■	■	■	■	■	■	■					7		
	SDHSE-09	■	■	■	■	■	■	■					7		
	SDHSE-10	■	■	■	■	■	■	■					7		
	SDHSE-11	■	■	■	■	■	■	■					7		
MAGs with Resistance Genes	Count	11	11	11	11	11	10	9	4	4	2	2			
	Percent	100%	100%	100%	100%	100%	91%	82%	36%	36%	18%	18%			

Chapter 5 - Conclusions

Brandi Feehan and Sonny T M Lee

Division of Biology, College of Arts and Sciences, Kansas State University, Manhattan, Kansas, 66506, United States of America.

The monogastric gut microbiome plays critical roles in maintaining host health, but it is also known to associate with host disease[1–3]. In order to elucidate the complexities underlying these host outcomes, we need to understand microbial membership, functions, and genetic variation in the gut under different environmental influences. There are numerous gut environmental variables that can impact the host associated microbiome, including age-associated factors and antibiotic treatments. To develop our knowledge on effects of environmental factors on gut microbial dynamics, I performed three monogastric microbiome studies.

In my second chapter, I described the dynamics and correlations of the bacteriome and the dominant swine fungus, *Kazachstania slooffiae*[82,83], through the host developmental stages. The stages of swine development are associated with environmental changes to the gut[89]. I noticed distinct differential patterns between bacteria and *K. slooffiae*. The bacteriome developed primarily during the preweaning stage, whereas *K. slooffiae* underwent an expansion and fall during the nursery stage. The implications of *K. slooffiae* dynamics in the nursery and growth adult host are not known and therefore are a topic for future research. I found 63 novel correlations between bacteria and *K. slooffiae*[87,88]. My correlations demonstrated the monogastric gut bacteria were competing with *K. slooffiae* at all developmental stages. The interactions between bacteria and fungi are known to impact host health[421]. Further studies can build upon my bacteria and *Kazachstania slooffiae* development study to enhance our understanding of gut health throughout the host lifetime. While my first study provided insights into microbiome development and interactions, I next wanted to determine microbial functional potential during host development.

I focused on methanogenic archaea, known as methanogens, which are under-studied in the developing monogastric gut within my third chapter. Following shotgun metagenomic sequencing

and bioinformatic applications, I found 8 distinct archaea metagenome assembled genomes (MAGs), with the first genetic support of 2 swine methanogens (identified as *Methanobrevibacter UBA71 sp006954425* and *Methanobrevibacter gottschalkii*)[215]. Furthermore, I described similar MAGs in paleontological humans and present-day monogastric hosts from around the globe[218–230,303–308]. Although, the novelty to my third chapter was my reconstruction of methanogenic pathways, central to ATP production[173,177–179]. I found novel genes for acetoclastic methanogenesis which indicated a previously unknown capability for acetate utilization in methanogenesis for monogastric methanogens[290,291]. Further studies are crucial to confirm these novel functions of methanogens for additional methanogens and monogastric hosts. This study provided a comprehensive evaluation of archaea methanogenic functions and how similar monogastric methanogens could be performing these functions, from past to present, worldwide.

In my final study found in my fourth chapter, I looked for microbiome dynamics associated with different antibiotic treatments. Swine were either given one of two different antibiotic treatments (chlortetracycline [CTC] or tiamulin [TMU]), or no antibiotics in the control group. I found 11 MAGs with no statistical difference in detection and statistically consistently high variation in the form of genetic entropy (SDHSE [sustained detection and high sustained entropy] MAGs). These MAGs were concerning given their detection prior to treatments and throughout antimicrobial administration which suggested not only antimicrobial resistance (AMR), but also multidrug resistance (MDR). Although I identified 22 AMR genes, less than a third of SDHSE MAGs contained genes associated with TMU resistance[391]. Given the resilience of SDHSE MAGs to TMU treatment, there are likely genetic functions contributing to TMU resistance which are not currently understood. With the threat of AMR and MDR[37,311,317,318], this study provided critical

insights into monogastric microbiomes harboring MDR organisms supported with gene functions and variation.

My studies contributed critical information about the gut microbiome during the host life and antibiotic treatments. With this knowledge, we know how microbial membership, functions, and genetic variation change alongside the local gut environment. Further research should build upon these findings to determine implications on host health. Moreover, these studies can aid clinical research in targeting gut diseases since the need for antibiotic alternatives and gut therapies are increasing[1–3,422]. As we strive to understand gut health, we must move beyond microbial membership and continue to evaluate microbial function and interaction studies. We need these functional approaches as we begin to deconstruct the numerous interactions within the complex gut microbiome to reconstruct its implications on host health.

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