Ecological drivers of gut microbiome and antimicrobial resistance in swine

by

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B.V.Sc., Purbanchal University, Nepal, 2011
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AN ABSTRACT OF A DISSERTATION

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Department of Diagnostic Medicine/Pathobiology
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Abstract

The pig gastrointestinal tract hosts a large and diverse microbial community, representing a complex and dynamic ecosystem. The microbial communities are not uniformly distributed and differ across the locations of the gastrointestinal tract. The microbial community composition influences the prevalence and distribution of antimicrobial resistance (AMR) in the gut. The microbial taxonomic composition, richness, and diversity are influenced by age of the pig, dietary composition, and antimicrobial drug use. The association between antimicrobial use and AMR development is of interest because of public health implications. Several studies in swine and cattle have reported a decrease in AMR in fecal bacteria with animal age; however, exact dynamics and contributing factors are largely unknown. Investigations on the AMR dynamics in gut microbiome during the production phase of food animals could aid in the design of a framework to address the problem of AMR in the food chain in a sustainable manner.

The primary hypothesis of our study was that the dynamics of gut microbiome and AMR in swine are largely a function of age and dietary composition. Therefore, the objectives of the studies were: 1. Perform a scoping review of the literature on the age-dependence of AMR of fecal bacteria in food animals. 2. Conduct longitudinal studies to evaluate the dynamics of fecal bacteriome and mycobiome taxonomic compositions and AMR prevalence between cohorts of production pigs (n=12) from birth to harvest and breeding sows (two cohorts, n=6 and n=12) from 3 weeks through first farrowing and weaning, to test the hypothesis that the dynamics are a function of age, rather than the production system. 3. Investigate interactions between the age-related dynamics and effects of diet (levels and sources of fiber) and antimicrobial treatments (injectable ceftiofur or penicillin G) in influencing the fecal microbiome taxonomic composition and AMR in finisher pigs. 4. Conduct a study to describe bacterial community composition

associated with luminal contents and mucosal epithelium from different segments of the gut of piglets. Culture-based and metagenomic analyses coupled with statistical modeling were utilized to monitor microbiome changes and estimate and infer AMR occurrence in gut bacterial communities in relation to age and diet.

The scoping review of published data suggested that the animal-level prevalence and within-animal abundance of AMR in enteric or fecal bacteria decreased with age during the production life-span in pigs, in beef and dairy cattle. The age-dependent dynamics of fecal bacteriome and mycobiome taxonomic compositions and associated animal-level prevalence and within-animal abundance of AMR were similar in a cohort of production pigs and two cohorts of breeding sows. The highest AMR prevalence and abundance occurred at the youngest age-points and decreased with age and stabilized around 5 to 6 months of age. The data suggested a strong age-dependence and additional independent diet effects on the fecal microbiome composition and AMR. Data also showed that the concentrations of ceftiofur metabolites in swine feces were lower on day 3 compared to day 1 of the 3-day ceftiofur treatment, irrespective of the animal diet or gender. In a study conducted in piglets (6-7 weeks old: n=3), luminal contents and mucosa were collected from the stomach, duodenum, ileum (at two locations), cecum, spiral colon, and the rectum. The bacterial community composition and AMR genes were determined, and the study showed that the bacterial taxonomic composition and AMR gene repertoire changed throughout the gastrointestinal tract of piglets. Genes encoding bacterial resistance or reduced susceptibility to tetracyclines, β-lactams, aminoglycosides, and glycopeptides were most abundant AMR genes in the samples. In summary, age and diet, in addition to the use of antimicrobials, play an important role in the establishment and maintenance of gut microbial diversity and AMR in pigs.

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Chapter 1 - Introduction

Background

The links between antimicrobial resistance (AMR) in humans, environment, and food animals are not fully understood. Several cross-sectional and longitudinal observational studies have suggested that AMR in enteric bacteria decreases with the age of the human or animal host. For example, the relative abundance of AMR in enteric populations of the fecal indicator bacterium *Escherichia coli* appeared to decrease during early life in humans (Kalter et al., 2010a; Literak et al., 2011b), cattle (Hoyle et al., 2004a; Hoyle et al., 2004b; Khachatryan et al., 2004; Berge et al., 2010; Edrington et al., 2012a; Mainda et al., 2015a), and pigs (Agga et al., 2015b). It is unclear whether the bacterial genes conferring AMR are present, introduced, or disseminated at or before the birth of the host or at some other point during early life. Antimicrobial resistance genes (ARGs) can be transferred between indigenous native and transient gut bacteria picked up from the food, water or the environment (Sommer et al., 2010; Hu et al., 2014).

The taxonomic composition, richness, and diversity of the pig gut microbiome change with the age of the pig (Slifierz et al., 2015b; Chen et al., 2017; Han et al., 2018). Similar observations have been made in humans (Claesson et al., 2012; Conlon and Bird, 2014), most noticeably regarding the bacterial genus *Prevotella* and associated genera (O'Toole and Jeffery, 2015). Mariat et al. (2009) found that the ratio of phyla *Firmicutes* to *Bacteroidetes* in the human gut was 0.4, 10.9, and 0.6 in infants (3 weeks to 10 months), adults (25–45 years), and elderly individuals (70–90 years), respectively, indicating that the relative abundances of major components of the human microbiota change with the age of the host. Similarly, in pigs, the taxonomic diversity of the gut microbiota increased as the age of the host increased from 25 days

to 240 days, with *Fusobacterium* as the dominant genus during the preweaning stage and *Firmicutes* and *Bacterioidetes* as the dominant phyla later on (Ke et al., 2019). Similarly, in another study, age-dependent shifts in the gut microbiome were reported in healthy pigs (*n*=32) born to five different sows, with *Bacteroides*, *Lactobacillus*, and *Prevotella* dominating at 10, 21, and 63 days after birth, and *Clostridium*, *Prevotella*, and an unclassified genus of family *S24-7* dominating at 93 and 147 days after birth (Han et al., 2018). Because the physiology of the human gut is similar to that of the pig gut, *Firmicutes* and *Bacteroidetes* were the dominant phyla in the enteric microbiomes of both species; although, their relative abundances varied among individuals (Tilocca et al., 2017).

The mechanisms of the fecal microbiome changes with animal age are not fully understood. Furthermore, age-related microbiome dynamics may be influenced by dietary and environmental factors (De Filippo et al., 2010; Yatsunenko et al., 2012; O'Toole and Jeffery, 2015). For example, in humans, a decrease in dietary fiber leads to a decrease in microbiome diversity (Flint et al., 2012), whereas increased dietary fiber leads to an increased diversity with enrichment of *Bacteroidetes* and depletion of *Firmicutes* (De Filippo et al., 2010). Similarly, dietary factors, especially the intake of crude fiber from corn, had a significant impact on the composition of the pig gut microbiome (Wang et al., 2019). Although the bacterial constituents of the gut microbial communities have been studied extensively, gut-associated fungi and their roles are poorly understood in humans (Seed, 2014) as well in animals (Lai et al., 2019).

Antimicrobial resistance is a natural phenomenon, and bacteria have been evolving resistance mechanisms to naturally occurring antibacterials produced by other bacteria and fungi (Blair et al., 2015). A longitudinal study of commercial production pigs with high levels of antimicrobial drug exposure showed that the AMR genes prevalence and abundance, microbiome

alpha diversity were not significantly influenced by the antimicrobial use; however, AMR abundance was higher in nursing piglets with low fecal microbiome diversity (Pollock et al., 2020).

In 2016, Xiao et al. (Xiao et al., 2016a) described the effects of antimicrobial use on the composition of the pig gut resistome. They reported that age, gender, and other host factors influenced the gut microbiome and resistome composition. Similarly, a study found that the presence of AMR genes in commercially raised pigs was not associated with the intensity of antimicrobial use; however, they did find a strong correlation between the pig gut resistome and the bacterial composition at the genus level (Munk et al., 2018b). Another study (Joyce et al., 2019) found a positive correlation between the total AMR gene abundance and the total microbial abundance in fecal samples from healthy pigs, indicating that the microbiome composition influences the resistome composition.

Study objectives

The primary hypothesis of our study was that the dynamics of gut microbiome and AMR in swine are largely a function of age and dietary composition. We hypothesized that the fecal AMR composition and abundance are driven by age-based dynamic changes in the taxonomic composition and interactions within the gut microbial community.

The main objectives were to:

1. Perform a scoping literature review to examine the extent, range, and nature of research activity and summarize the available data on the research question: “Does AMR in enteric/fecal bacteria shift in accordance with animal age?”
2. Compare the dynamics fecal microbiome and mycobiome taxonomic compositions and AMR between cohorts of production pigs and breeding sows.

3. Evaluate age-related dynamics and effects of dietary interventions with and without concurrent antimicrobial treatments (injectable ceftiofur or penicillin G) on the fecal microbiome taxonomic composition or AMR of finisher-stage production pigs.
4. Describe the bacterial taxonomic composition and AMR genes prevalence in the luminal contents and mucosal epithelium in different locations of the gastrointestinal (GI) tract of piglets.

Microbial culture-based and metagenomic analyses coupled with statistical analyses were used to estimate and infer AMR occurrence in fecal microbial communities in relation to age and diet.

Chapter 2 - Literature Review

Antimicrobial resistance in fecal bacteria of swine

Bacteria can be intrinsically resistant to certain antimicrobials, but resistance can arise in a susceptible bacteria population as a result of horizontal gene transfer or mutations in chromosomal genes (Blair et al., 2015). Different techniques have been applied to characterize AMR and ARGs in bacteria given samples, using culture-based and non-culture-based methods. Current efforts to monitor AMR are primarily based on the culturing of indicator bacteria followed by phenotypic AMR determination (Munk et al., 2018b), which likely overlooks a large portion of the resistome. The gut contains a collection of genes and genetic materials that exist within gut microbial ecosystem, conferring AMR to diverse the gut bacterial community (D'Costa et al., 2006).

Culture-based analysis

The culturing of samples on agar or selective medium has often been used to isolate fecal bacteria, which can subsequently be tested for antimicrobial susceptibility using phenotypic and genotypic methods. Such testing is a time-consuming process, and is often limited to culturable bacteria such as *Enterobacteriaceae* and *Enterococcaceae*, which means that many AMR phenotypes and ARGs may go undetected (van Schaik, 2015; Singh et al., 2019). In traditional phenotypic testing, bacteria are grown in the presence of different concentrations of various antimicrobials. Nowadays, however, detailed information of given bacteria can be obtained through new techniques called whole genome sequencing (WGS). With this method, entire genomic DNA or genes in bacteria can be evaluated, which allows us to understand AMR at a deeper level (Oniciuc et al., 2018). Further, bacteria that have similar AMR patterns caused by different mechanisms can be differentiated using WGS. In the US, the National Antimicrobial

Resistance Monitoring System (NAMRS) is currently monitoring AMR in foodborne culturable bacteria (mainly *E.coli*, *Salmonella* spp., *Campylobacter* spp.) incorporating the WGS method (McDermott et al., 2016). However, the culturable bacteria represent only a small portion of the enteric bacterial diversity, the majority of which cannot be cultured or evaluated in the laboratory. Similarly, bacteria may lose the whole plasmid or part of the plasmid (that encode the AMR genes), or all the resistance genes may not be fully expressed during culture. Further, single bacteria may not represent the source or target bacterial populations. Therefore, results from the WGS study may be biased if the organism does not truly represent microbial populations (Kanwar et al., 2014b).

Culture-independent approach to assess the epidemiology of AMR

Complex interactions among different bacterial species and culture media affect the growth of bacteria in culture. In general, it is believed that less than 20% of gut bacteria can be grown at all in defined growth media and that fast-growing microbes always dominate slow-growing microbes in heterogeneous cultures. Microbial populations and their constituent microorganisms evolve differently under different selection pressures in different ecological niches. Under the selective pressures of a controlled laboratory environment, which are likely very different from those of the natural environment, bacteria can lose whole plasmids or parts of plasmids that encode an ARG, and ARGs might not be adequately expressed to produce an AMR phenotype. Thus, a culture-based study might not capture the full picture of AMR in a bacterial community, and single bacterial species might not represent, in terms of ARGs, the source or target population in an epidemiological sense.

There are several culture-independent methods to identify ARGs and characterize the resistome of the gut microbiome using community DNA extracted directly from fecal samples

(van Schaik, 2015). For example, ARGs from gut microorganisms can be detected and quantified using quantitative polymerase chain reaction (PCR)/primer-based PCR (Kanwar et al., 2013); Gerzova et al. (2015b); (Birkegård et al., 2017) or by microarray hybridization (Card et al., 2014). Quantitative results and relative abundances of resistance genes or gene families can be determined using real-time PCR-based metagenomic methods. The results of targeted PCR methods tend to be skewed toward known ARGs and mechanisms; however, they can also be skewed by sequence heterogeneity in resistance genes of different species (Penders et al., 2013).

In the sequence-based metagenomic approach, community DNA is isolated from a sample (e.g., feces) and sequenced at a depth of 1.2 GB to 12.6 GB per sample (Raymond et al., 2019). The sequence data can be analyzed by mapping the sequence reads to a reference database or by assembling the reads into larger contiguous DNA fragments (van der Helm et al., 2017). AMR determinants are identified by aligning the sequence reads or assembled contigs to curated ARGs in one or multiple reference databases and identifying DNA fragments with 80% amino acid identity or 95% nucleotide identity with known ARGs (Ho et al., 2020).

Metagenomics allows the presence and dynamics of the resistome to be analyzed in the context of diverse microbial ecosystems. Metagenomic analysis has been used to explore the abundance and diversity of ARGs in various types of samples, such as fecal samples from pigs, cattle, and poultry (Ma et al., 2016; Munk et al., 2017). Recently, a metagenomic study found that the gut microbiome could serve as an ARG reservoir in which ARGs can be transferred between native and transient gut bacteria (Sommer et al., 2010; Hu et al., 2014). The metagenomic approach allows comparisons of the resistome and microbiome between samples and provides information about evolutionary shifts in AMR and the distributions of diverse ARGs among different ecological niches (Noyes et al., 2017). Although the metagenomic

approach has been expanded for use in AMR surveillance efforts, high-throughput metagenomics-based analysis of AMR still suffers from a lack of standard bioinformatic tools (Oulas et al., 2015). Also, the low sensitivity and specificity of current metagenomic tools and methods preclude the detection of minor microbial taxa and allelic variants, which are often present at levels below the detection limit but still exert an effect on the host phenotype (Lanza et al., 2018). Another challenge in metagenomics-based resistome analysis is the fact that the resistome comprises only a small portion of the total community DNA in given sample (Noyes et al., 2016a), and even relatively deep sequencing might not capture some portions of the resistome (Munck et al., 2015). The methods used to annotate metagenomic sequence data are still developing and improving; however, metagenomic sequencing data provide no information about the expression of ARGs.

Functional metagenomics is an approach in which a library of 5–40 kbp DNA fragments randomly isolated from a fecal sample is cloned into *E. coli* using a fosmid vector. The bacteria are then plated on antibiotic containing medium, resulting in the isolation of AMR clones (van Schaik, 2015). The functional metagenomics approach allows the identification of novel ARGs. For instance, Sommer et al. (Sommer et al., 2009a) characterized the resistance reservoir in fecal and saliva samples from healthy humans using functional screening of metagenomic DNA. They identified 95 unique functional ARGs that were evolutionarily distant from known AMR genes. Because of functional incompatibilities and the limited capacity of *E. coli* as a host for ARGs, not all ARGs can be expressed in functional metagenomics studies.

An alternative approach is to investigate how ARGs in gut microbial communities influence overall microbiome dynamics under certain environmental and clinical conditions. For example, one study found that the microbiomes of formula-fed infants are enriched with class D

β-lactamase genes, and with *Clostridium difficile* strains harboring those genes (Rahman et al., 2018).

Ecological drivers of the swine gut resistome

The age-dependent dynamics of the swine gut microbial community

The mammalian gut harbors a large, complex, and dynamic ecosystem that typically consists of diverse microbes, including bacteria, archaea, viruses, fungi, and protists (Ke et al., 2019). The majority of gut bacteria in the gut are yet to be discovered and are unculturable using current standard methods. The number of studies evaluating the bacterial communities of food-production animals has increased with the development of high-throughput sequencing technologies (Yeoman and White, 2014; Kim et al., 2017). Those microbial communities have important roles in host nutritional, immunological, and physiological processes (Carding et al., 2015). Recently, nearly 7.7 million unique genes representing more than 700 species were identified by deep metagenomic sequencing of fecal DNA from 287 pigs (Xiao et al., 2016a). Nearly 96% of the functional pathways in humans are also present in pigs, making pigs a preferred model species for biomedical research and investigations of gene function (Lunney, 2007; Xiao et al., 2016a). Several studies showed that age, host genetics, diet, and gender influence the microbiomes of both pigs and humans (Wagner et al., 2018; Ke et al., 2019). Therefore, given the high variability among individuals, longitudinal studies are needed to better understand the overall dynamics of gut microbial communities and outcomes of interest such as AMR.

Fecal microbiome composition

16s rRNA based microbiome study

The most commonly used method for gut microbiome analysis is the sequencing of the 16S rRNA gene, which is carried by bacteria and archaea and encodes a component of the 30S ribosomal subunit (Morgan and Huttenhower, 2012; Panek et al., 2018). The 16S rRNA gene contains roughly 1500 bp and includes both highly conserved regions, which can be targeted with PCR primers, and hypervariable sites (V1–V9), which are specific to each microbial species. V1–V3 and V4 are the sites most commonly targeted for the identification of different bacterial species (Goodrich et al., 2014).

Briefly, community DNA is isolated from samples (e.g., feces), and the bacterial taxa present in the community are identified by amplification and subsequent sequencing of the 16S rRNA gene. Then, highly similar sequences are clustered into OTUs. OTU clustering algorithms fall into three main categories: *de novo* (sequences clustered into OTUs without any external reference sequences/databases), closed (sequences are clustered on the basis of alignment to a reference database such as SILVA, GREEN GENES, or the ribosomal database project), and open –reference OTU (a two-step process comprising alignment to a reference database followed by *de novo* clustering of sequences that fail to match the reference database (Morgan and Huttenhower, 2012; Goodrich et al., 2014). OTUs can be defined at different taxonomic levels (phylum, class, order, family, genus, or species). One drawback of 16S RNA-based methods is that they are limited to bacterial species and ignore other members of the microbial community, such as viruses and fungi.

Shotgun metagenomics-based microbiome study

Metagenomics was first described by Handelsman and Rodon and has become an alternative method to identify taxa in community samples (Handelsman et al., 1998). Metagenomics provides a catalog of all the genes in a community by random sequencing of

DNA isolated from community samples, allowing improved taxonomic resolution (Wang et al., 2015). Functional metagenomics can be used to detect microbial community composition and diversity, novel functional genes, microbial pathways, ARGs, and interactions and co-evolution between microbial communities and hosts (Morgan and Huttenhower, 2012). Metagenomics are now widely used not only to study microbiomes but also to evaluate the ecological level of AMR in conjunction with microbiome analysis in humans and food-producing animals (Lepage et al., 2013; Xiao et al., 2016a; Noyes et al., 2017; Joyce et al., 2019).

Metagenomic approaches can help to provide a comprehensive understanding of the structure and function of microbial populations as a whole. There are still some limitations to metagenomic studies, however. For example, they cannot identify microbial gene expression; they require higher sequence coverage than 16S RNA analysis; they depend on complex bioinformatics analysis; and they involve substantial time and cost investments (Wang et al., 2015). Furthermore, millions of sequences are generated from each sample, and it is challenging to assign functions unambiguously on the basis of sequence similarity, which leads to misannotation (Schnoes et al., 2009).

The creation of standardized microbial DNA-isolation techniques, robust computation algorithms, a complete standardized reference database, and a standard for statistical analysis would improve metagenomics. With the rapid development of metatranscriptomics, metaproteomics, and metabolomics, now of studies tries to understand and identify the functional activities of microbial communities (Poretsky et al., 2009; Kolmeder et al., 2012; Heinken et al., 2014).

Fecal mycobiome composition

The study of fungal microbiota, known as the mycobiome, is a relatively new and rapidly progressing field. Microbial colonization of the GI tract begins at birth. Several studies have shown that the fungi present in the pig gut are ubiquitous members of the rare biosphere of microorganisms (Huffnagle and Noverr, 2013; Summers et al., 2019). Although the bacterial constituents of the gut microbiome have been studied intensely, gut-associated fungi and their functions are poorly understood and remain largely unexplored in humans (Seed, 2014) and animals (Lai et al., 2019). There is currently no accurate estimate of the gut mycobiome, but it is assumed to represent ≤0.1 of the total gut microbiome (Qin et al., 2010). Despite that, the gut mycobiome is thought to be more diverse than the bacterial microbiome (Dethlefsen et al., 2008) and essential for the maintenance of microbial community structure, immune response, gut homeostasis, and host physiology in humans (Huffnagle and Noverr, 2013; Lai et al., 2019), pigs, and other animals (Zlotowski et al., 2006; Erb Downward et al., 2013). In addition, studies suggest that commensal fungal communities might enhance immune tolerance of commensal bacteria (Li et al., 2019). The phenomena in which fungi interact with non-fungal communities and their role in AMR is still unexplored.

Transitions between different facilities (weaning, nursery, and finisher) are stressful events in a pig's life and can lead to increased susceptibility to diarrhea and other production-related diseases. The abrupt shift from a milk-based diet to a solid-based diet during the weaning transition can lead to a significant change in the gut microbiota of pigs (Dou et al., 2017; Guevarra et al., 2018). Several authors have investigated the effects of housing, age, and diet on the pig gut microbiome diversity and composition (Frese et al., 2015b; Guevarra et al., 2018; Wang et al., 2019), but the effects of the mycobiome remain poorly understood.

Ingestion of feed contaminated with mycotoxins led to immunosuppression in piglets and susceptibility to infectious diseases (Arfken et al., 2019). Similarly to humans, pigs harbor Candida species (Van Uden et al., 1958), which are known to be opportunistic pathogens under stressful conditions. Recently, (Summers et al., 2019) assessed the temporal dynamics of the mycobiome and microbiome compositions using internal transcribed spacer (ITS) and 16S rRNA sequencing of the feces of swine from birth to weaning age (35 days after birth). Overall, microbiome diversity and abundance increased over the study period. The dominant fungi were Saccharomycetaceae, *Dipodascaceae*, Cladosporiaceae, Aspergillaceae, Malasseziaceae, and Nectriaceae. The mycobiome also showed a shift in the relative abundance throughout the weaning transition. From day 1 through day 21, the mycobiome composition was highly variable but showed a predominance of *Cladosporiaceae*. After weaning, *Dipodascaceae* and *Aspergillaceae*, began to appear. In another study, (Arfken et al., 2019) investigated the microbiome and mycobiome sampled from the GI tract and feces of pigs (n = 23) from birth through day 35 after birth using the V4 and ITS2 regions of the bacterial 16S rRNA and fungal ITS genes, respectively. The piglets were nursed with their mother until 21 days of age (weaned on day 21) and then received nursery diet 1 (days 21–28) followed by nursery diet 2 (days 29–35). These piglets were not treated with antimicrobials or antifungal at any time during the study period. The most dominant bacterial phyla in the GI tract and feces were *Bacteroidetes*, *Firmicutes*, and *Epsilonbacteraeota*, and the dominant fungal phyla were *Ascomycota* (90%) and *Basidiomycota* (9%).

The mycobiome composition of newborns is poorly understood. (LaTuga et al., 2011) performed a fecal microbiome and mycobiome analysis of preterm infant babies (n = 7) and found that the most abundant fungal order was *Saccharomycetales*, which was represented by

several Candida species. Members of the *Malasseziales*, *Eurotiales*, *Botryosphaeriales*, and *Filobasidiales* were also observed in fecal samples. Similarly, Hoffmann et al. (2014) conducted deep sequencing of ITS1 regions to characterize the fecal mycobiome of healthy humans (*n* = 98) and identified a total of 184 species, including 66 fungal genera along with 13 additional unidentified fungal taxa. Among the known taxa, *Saccharomyces* were the most abundant (89%), followed by Candida (57%) and *Cladosporium* (42%). In the same study, *Candida* and *Saccharomyces* were positively correlated with the archaea *Methanobrevibacter* and the bacteria *Prevotella* and were most abundant in individuals with high-carbohydrate diets.

The Human Microbiome Project investigated the mycobiome diversity and abundance in 317 fecal samples using the ITS2 region, the 18S rRNA gene, and a shotgun metagenomics approach (Nash et al., 2017). The fungal diversity was lower than the bacterial diversity, with *Saccharomyces cerevisiae*, *Malassezia restricta*, and *Candida albicans* being the most abundant species, appearing in 97%, 88%, and 81% of the samples, respectively. Th

Identification of fungi in the metagenome samples

Two approaches can be used to identify the fungal taxonomic composition of samples. Briefly, DNA extracted from samples (e.g., pig feces) and preparation of libraries via either-a) shotgun metagenomic approach (DNA fragmented, adapter and multiplexing barcode ligation and sequencing) - b) targeted-amplicon approach (amplification of fungal marker, ITS1, or ITS 2 region between 18s, 5.8s and 28S rRNA gene and multiplexing barcodes and sequencing). The bioinformatics analysis of the raw reads generated from sequencing for each method differs significantly (Forbes et al., 2018), and both methods have their own disadvantages.

The molecular identification of fungal species in community samples is mostly based on high-throughput, “next-generation” sequencing of rRNA regions. The 18S rRNA gene and the

ITS regions between the 18S, 5.8S, and 28S rRNA genes have been used in sequencing-based approaches to determine fungal abundance and diversity (White et al., 2013; Nilsson et al., 2019). The ITS regions are made up of space DNA situated between the small-subunit and large-subunit rRNA genes in the chromosome.

Only a fraction of the DNA of soil fungi (≥ 40%) is accounted for by ITS sequences (Carini et al., 2016). Hence, genetic markers, primers, and PCR amplification are critical for mycobiome studies. For ITS sequencing of fungal communities, ITS4ngs reverse primer can be used in combination with the gITS7ngs and ITS9MUNngs forward primers to target the ITS2 subregion and the full ITS region, respectively (Nilsson et al., 2019). Taxonomic resolution can be improved by using ITSx to remove flanking genes, USEARCH to eliminate chimeras, and VSEARCH for quality assessment (Nilsson et al., 2019). Most mycobiome studies cluster the final sequences into species-level OTUs using 97–98.5% ITS sequence-similarity thresholds.

Although new approaches that cluster free OTUs (e.g. DADA2) have been used for microbiome studies, however DADA2 is not recommended for fungal analysis, given that fungal genes are sometimes present multiple times in each genome (Lindner et al., 2013).

With the decreasing cost of sequencing, the research gradually shifts away from 16S rRNA amplicon sequencing toward shotgun metagenomic sequencing. Unlike 16S rRNA sequencing, shotgun metagenomic sequencing can read all genome DNA in a sample, rather than just one region of DNA. Thus, the advantage of shotgun metagenomics is that sequencing data can be used to identify a wide range of species (bacteria, fungi, viruses, etc.) in the given metagenomic sample. However, only a few studies applied to fungal identification in metagenomic samples. Recently, Soverini and his colleagues (Soverini et al., 2019) proposed the "HumanMycobiomeScan," a new bioinformatics tool, for the characterization of fungal

communities in metagenomic samples. Similarly, Donovan and his colleagues (Donovan et al., 2018) developed "FindFungi" to identify fungal sequencing in public metagenomic databases.

In any analysis based on high-throughput sequencing, biases should be evaluated and corrected for high-quality and reproducible results. Examples of such biases include those related to DNA/RNA extraction, markers, primers, PCR amplification, library preparation, sequencing, and bioinformatics/analyses. For instance, in the case of the shotgun metagenomic approach, fungal genome databases are not comprehensive; thus, the fungal taxonomic resolution could be low due to incomplete databases or low fungal reads generated in comparison to bacterial reads. Similarly, in the case of amplicon sequencing, there is a high possibility of primer and amplification bias, which requires adjusting the region (e.g., ITS1) specific analysis during the bioinformatic steps (Forbes et al., 2018). In addition, strong bacterial and fungal interactions or antagonisms have been inferred from ARGs, indicating that the bacterial and fungal biomes need to be considered together for a better understanding of the AMR phenomenon in complex systems (Lindner et al., 2013). Recently, metatranscriptomics has emerged as an approach that allows researchers to target expressed genes in community samples, providing information about functional aspects of the fungal community.

Microbiome-mycobiome interactions

Several studies have revealed antagonism between certain members of the mycobiome and mycobiome. For instance, mouse models demonstrated that immune suppression or disturbances of the mycobiome promote *Candida* colonization of the gut (Naglik et al., 2008). A study suggested that the bacterial microbiome produces inhibitory substances such as volatile fatty acids or secondary bile acids that can reduce *C. albicans* adhesion to the gut epithelium (Yamaguchi et al., 2005). Furthermore, a reduction of *Lactobacilli* in the gut of mice that were

fed a purified diet contributed to long-term gastric candidiasis (Yamaguchi et al., 2005). Other studies showed that *Lactobacillus-Candida* antagonism could be a two-way process in which the presence of Candida inhibits the regrowth of *Lactobacillus* after antimicrobial therapy (Mason et al., 2012). (Arfken et al., 2019) observed potential interactions between bacterial and fungal genera in the porcine gut, with positive correlations between the fungus *Kazachstania* and several bacterial species, including *Lactobacillus*. Conversely, *Aspergillus* demonstrated negative relationships with the short-chain fatty acid-producing bacteria *Butyricoccus, Subdoligranulum,* and *Fusicatenibacter*. The results of another study indicated that differences in microbiome structure and diversity coupled with antibiotic use at different time points could manifest as a microbiome-mediated physiologic process leading to a fungal expansion in the gut (Huffnagle and Noverr, 2013).

Operational taxonomic units (OTUs) versus amplicon sequence variants (ASVs) for microbiome and mycobiome analysis

Analyses of targeted microbiome sequencing (16S ribosomal RNA (rRNA) gene amplicon sequencing) data commonly use bioinformatics pipelines such as Quantitative Insights into Microbial Ecology (QIIME), MOTHER, and USEARCH as well as tools such as DADA2 and Qimme2-Deblur. The Qimme-uclust, MOTHUR, and USEARCH-UPARSE pipelines usually group sequences that share 97% identity (or clusters of reads that differ by fixed threshold, typically 3%) into clusters called OTUs, whereas other pipelines (e.g., Qiime2-Deblur, DADA2, and USEARCh-UNOISE3) attempt to generate the exact biological sequences that are present in the metagenomic sample, also known as ASVs (Callahan et al., 2017; Prodan et al., 2020). The DADA2 pipeline resolved ASVs better than other methods, and the USEARCH-UPARSE and MOTHUR pipelines performed well, generating OTUs, but with lower specificity

than the ASV pipelines (Prodan et al., 2020). Several studies showed that ASV methods had better sensitivity and specificity and discriminate ecological patterns better than OTU methods (Tikhonov et al., 2015; Callahan et al., 2016; Caruso et al., 2019). Furthermore, the methods to define OTUs can be problematic. De novo OTUs (*i.e.* sequence reads are grouped into OTUs based on sequence similarity) are invalid outside of a given data set, whereas closed reference OTUs (*i.e.* reads are mapped to a sequence in a reference database) cannot capture real variation outside of the reference database. By contrast, the ASV method captures the biological variation in the data and also allows independent comparisons among different studies or different samples (Callahan et al., 2017). Furthermore, the ASV method can distinguish sequence variations as small as 1 nucleotide within a gene region. Among the methods for ASV determination, DADA2 has been shown to be the most sensitive to low-abundance sequences. Overall, the use of the ASV approach is increasing in studies of the swine microbiome (Raman et al., 2019; Bergamaschi et al., 2020) and the human microbiome (Bodkhe et al., 2019; Martinson et al., 2019). Some researchers argue, however, that existing sequencing methods is not sufficient to resolve exact sequences accurately, and that overall biological trends can be obscured by the ASV method and are easier to identify using the OTU method.

Microbial composition, diversity, and AMR across the GI tract

The mammalian GI tract is comprised of a complex ecosystem that harbors a diverse microbial community. The microbial community differs along the GI tract of pigs and is the most abundant in the lower part of the intestine, cecum, and colon (Simpson et al., 1999). Similarly, mucosa-associated bacterial communities differ from those recovered from feces and intestinal contents (Zoetendal et al., 2002). At birth, the piglet GI tract is sterile, but after fetal membrane rupture, the piglet is exposed to diverse microbes via contact with the vagina, feces, and skin of

the sow, so that within a few days, the piglet microbiome changes and becomes unique for each individual (Katouli et al., 1997). A meta-analysis to define the core microbiome of the swine GI tract found that *Proteobacteria* were common to all GI samples and that *Firmicutes* and *Bacteroidetes* accounted for nearly 85% of the total 16S rRNA sequences across all GI locations (Holman et al., 2017a). Another study showed that a moderate change in dietary protein level did not affect the fecal microbiome composition as dramatically as it affected the small-intestinal microbiome (Fan et al., 2017). In that study, the ileal bacterial richness declined from 16% to 10% in finishing pigs when the crude protein of the diet was descreased from 16% to 10%. When the pigs were fed a high-protein diet, *Clostridium sensu stricto* 1 was the dominant genus in the ileum. When the dietary protein was reduced, the proportion of *Clostridium sensu stricto* 1 increased significantly in the colon.

The mammalian GI tract is a diverse ecosystem with a unique, stratified environment. Hence, it contains a variety of distinct microbial communities along its length spanning the small intestine, cecum, and large intestine (Donaldson et al., 2016). The variation in the bacterial community across the GI tract might be due to physiological, chemical and nutrients gradients and differences in the host immune activity at different locations. It has been demonstrated that the cecum and colon have a denser and more diverse microbial community than other locations in the GI tract. One study showed a heterogeneous distribution of bacterial species along the large intestine and further suggested that intestinal bacteria are distributed along two axes corresponding to the distal axis and the radial axis (from the lumen to the mucosa) (Takahashi and Sakaguchi, 2006). A mouse study using laser-capture microdissection found a significant difference between the central lumen and the interfold region, with the *Firmicutes* families *Lachnospiraceae* and *Ruminococcacea*e enriched explicitly in the interfold region and the

Bacteroidetes families *Prevotellaceae*, *Bacteroidaceae*, and *Rikenellaceae* enriched in the digesta, suggesting that the inner lining of the intestinal tract (mucosa) is an important location for the microbial cluster (Nava et al., 2011). A different study found distinct microbial communities in the mucosa of humans and mice that differed from those in fecal samples (Zoetendal et al., 2006). Hill et al. (Hill et al., 2010) found that mucosa-associated microbial communities differ from those in the intestinal lumen and may vary across the different parts of the GI tract. Further phylogenetic analysis demonstrated significant temporal and spatial effects on the luminal and mucosal microbial communities that included a reduction in luminal *Firmicutes* and mucosa-associated *Lactobacilli* following antibiotic treatment, which led to a reduction of IFNγ and IL-17A production by mucosal CD4+ T lymphocytes (Hill et al., 2010).

Looft et al. (2014) performed a metagenomic study of the different sections of the GI tract (ileum, cecum, and mid-colon) as well as the gut contents and freshly voided feces from piglets ~3 months of age and found that the mucosa-associated ileal microbiota harbored greater bacterial diversity than the lumen, and the ileal contents (control and medicated) had reduced richness and abundance compared with other parts of the intestinal tract. These results further suggest that the mucosal bacterial community of the ileum might serve as a source for the large intestine. In the same study, an oral antibiotic led to a significant increase in the size of the *E. coli* population in the ileum (lumen and mucosa) relative to that in the feces and other parts of the GI tract. Similarly, an analysis of multiple colonic mucosal sites and feces from healthy humans indicated significant variability in microbial communities among individuals and also between feces and mucosa (Eckburg et al., 2005). That study also showed that the microbial concentration increases along the GI tract, with the lowest concentration in the stomach and the highest concentration in the colon. A different study showed that *Bacteroides* and *Firmicutes*

were the dominant mucosa-associated bacteria in the small intestine and colon (Sekirov et al., 2010). In the proximal gut, *Lactobacilli*, *Veillonella*, and *Helicobacter* were the most abundant taxa; *Bacilli*, *Streptococcaceae*, *Actinomycinaeae*, and *Corynebacteriaceae* were abundant in the duodenum, jejunum, and ileum; *Firmicutes* and *Bacteroidetes* were dominant in the colon.

Influence of diet on microbiome and mycobiome composition

Several studies have shown that diet and age contributed to the gut microbial community composition (Gill et al., 2006), and this may, in turn, influence the fecal resistome. A study has demonstrated age and diet-dependent microbial community succession process in piglets (Bian et al., 2016). Of these factors, the diet would be the easiest approach to modify for short- or long-term intervention. The study showed that the fecal microbial communities were clustered into major enterotypes - *Bacteroides* (protein and animal fat-based diet) and *Prevotella* (carbohydrate-based diet) based on the long-term dietary intervention. The microbial community changes were detected within 24 hours of introduction of high fat/low fiber or low fat/high fiber-based diet (Wu et al., 2011). Similarly, a bacterial community dominated by *Firmicutes* (65-75%) was reported during the first 3 days after the birth of the piglet. However, the introduction of solid feed and subsequent weaning was found to be the major event contributing to the gut microbial communities in the early life of pigs (Bian et al., 2016).

Similarly, Frese et al. (Frese et al., 2015a) reported that the diversity of bacterial taxa increased with dietary changes from sows milk to a plant-based diet, and the relative abundance of *Lactobacillaceae*, *Rumimococcaae*, *Veillonellaceae*, and *Prevotellaceae* increased in the weaned piglet. Recently, (Zhang et al., 2016) reported that moderately increased fiber (both soluble and insoluble) in the diet influenced the gut microbial composition in piglets fed with different levels of fiber-containing feed compared with a control diet from postnatal day 7 to day

22. A study also showed changes in the relative abundance of *Lactobacillus* spp. and *Streptococcus suis* in the stomach, jejunum, and ileum of piglets after weaning (Su et al., 2008). These studies show that the gut microbial community could be modulated by various dietary treatments such as dietary fiber, dietary protein level etc. for long term intervention.

Other studies showed that dietary changes coupled with other management factors such as in-feed antimicrobials, prebiotics, and probiotic administration, played an important role in shaping the gut microbial community in pigs (Bian et al., 2016; Guevarra et al., 2019). It is still unclear how quickly the gut microbial community responds to dietary modification.

Similarly, an earlier study showed that the first fungi detected in the infants gut are *Saccharomycetalean* yeasts, particularly *Candida* species. These Candida species are common fungi of the skin, colon, and vaginal mucosa (Bliss et al., 2008). These fungi are often influenced by the availability of the type and source of the diet. For instance, dietary-induced changes in the gut microbial communities depend on whether animal-based or plant-based diets. Overall microbiome composition was mostly driven by the type of diet (fiber vs. protein and fats), while mycobiome composition appeared to be affected by food colonization (David et al., 2014). They also found that an animal-based diet had a more significant impact on the gut microbiome than the plant-based diet. However, in the same study, the species diversity (Shannon index) did not significantly change during either the plant-based or animal-based diet.

Further, Hoffmann and his colleagues (Hoffmann et al., 2013) found that the *Candida* abundance was significantly associated with a carbohydrate diet, while *Bacteroides* (bacterial taxa) were more abundant in a diet with high protein. The study also suggests that the metabolism of the fungal cell wall (e.g., Beta-glucan) may influence the growth of *E. coli* and other bacteria in the gut; for example, dietary beta-glucan decreased the fecal *E. coli* counts and

benefited the growth performance in weaned pigs (Zhou et al., 2013). Further, the gut mycobiome of the healthy cohort from the Human Microbiome Project showed that the diet, environment, and host genetic factors played a significant role in influencing the human gut mycobiome composition (Nash et al., 2017).

Antimicrobial use and their effects on microbial communities and antimicrobial resistance

Antimicrobial use in swine production

Antimicrobials have been used in swine production for disease treatment, control, and prevention and to increase feed efficiency and growth performance since the early 1950s (Zeineldin et al., 2019). Swine are usually raised in confinement in farrow-finish or aggregated management systems with the purpose of controlling infectious diseases. Antimicrobials are given to all animals at low (subtherapeutic) concentrations to promote growth, whereas they are given at higher (therapeutic) concentrations to control the spread of infection (metaphylaxis) or to prevent infection (prophylaxis) (Aarestrup, 2005). Antimicrobial use for the treatment, control, or prevention of disease is considered therapeutic use, whereas antimicrobial use to promote growth is considered non-therapeutic use (Lekagul et al., 2019).

Antimicrobials for growth promotion or disease prevention are given in feed at low concentrations, especially after weaning (as starter feed), and are typically removed at the finisher stages of production to avoid drug residues in the final products (McEwen and Fedorka-Cray, 2002). It is suggested that 50% of all antimicrobials are used in food production animals, and nearly 33–62% of nursery units and 30–44% of grower-finisher units use antimicrobials for growth improvement (Holman and Chenier, 2015; Zeineldin et al., 2019). In addition, 46% of breeding sows regularly receive antimicrobials in their feed (Cromwell, 2002). Parenteral

therapy via intramuscular injection is generally used for acute and severe infections. The average duration of antimicrobial use for disease prevention typically ranges from 20 days to 40 days (Stone et al., 2009), whereas that for growth promotion can last up to 77 days (Dewey et al., 1997). Prolonged exposure to low antimicrobial concentrations might increase the risk of AMR development, which might subsequently be transferred to humans (Aarestrup et al., 2008).

Many classes of antimicrobials used in swine (or other food animals) are also used in humans, including critically important antimicrobials used in human medicine. One study suggested that the penicillin and tetracycline classes of antimicrobials, used mainly to treat age-specific, production-related diseases, were the most commonly used antimicrobials in many countries (Lekagul et al., 2019). Another study showed that the most common antimicrobials given to production pigs during the nursery stages for any reason were ceftiofur and penicillin G (APHIS, 2008). The common antimicrobial classes and agents used in swine production are presented in Table 2.1.

Table 2.1

The common antimicrobial classes used in swine production

Antimicrobial Class and Agent	Used in Animal Species	Used in Human Medicine
Aminocoumarins[d]		
Novobiocin	Beef and dairy cattle, poultry	No
Aminoglycosides[a]		
Dihydrostreptomycin	Beef and dairy cattle, swine	No
Gentamicin	Beef and dairy cattle, swine, poultry	Yes
Hygromycin B	Poultry, swine	No
Neomycin	Beef and dairy cattle, poultry, swine	Yes
Spectinomycin	Beef and dairy cattle, poultry, swine	Yes
Amphenicols[b]		
Florfenicol	Beef and dairy cattle, poultry	Closely related analogs
Cephalosporins[a]		
Ceftiofur	Beef and dairy cattle, poultry, swine	Analogs
Cephapirin	Beef and dairy cattle, poultry, swine	No
Diaminopyrimidines[d]		
Ormetoprim	Poultry	No
Fluoroquinolones[a]		

Danofloxacin	Dairy cattle	Analogs
Enrofloxacin	Beef cattle	Analogs
Glycolipids[d]		
Bambermycins	Beef and dairy cattle, poultry, swine	No
Ionophores[d]		
Laidlomycin	Cattle	No
Lasalocid	Cattle	No
Monensin	Poultry	No
Narasin	Poultry	No
Salinomycin	Poultry	No
Lincosamides[b]		
Lincomycin	Beef and dairy cattle, poultry, swine	Yes
Pirlimycin	Beef and dairy cattle	Analogs
Macrolides[a]		
Erythromycin	Beef and dairy cattle, poultry, swine	Yes
Gamithromycin	Nonlactating dairy cattle, beef cattle	No
Tildipirosin	Dairy and beef cattle	No
Tilmicosin	Poultry, swine	Analogs
Tulathromycin	Beef and dairy cattle, swine	No
Tylosin	Beef and dairy cattle, poultry, swine	Analogs
Tylvalosin	swine	No
Orthosomycins[d]		
Avilamycin	swine	No
Penicillins[a]		
Amoxicillin	Beef and dairy cattle, poultry, swine	Yes
Ampicillin	Beef and dairy cattle, poultry, swine	Yes
Cloxacillin	Beef and dairy cattle	Yes
Penicillin	Beef and dairy cattle, poultry, swine	Yes
Pleuromutilins[b]		
Tiamulin	Swine	No
Polymyxins[a]		
Polymyxin B	Beef and dairy cattle	Yes
Polypeptides[c]		
Bacitracin	Beef and dairy cattle, poultry, swine	Yes
Quinoxalines[d]		
Carbadox	Poultry, swine	No
Streptogramins[b]		
Virginiamycin	Poultry, swine	Analogs
Sulfonamides[b]		
Sulfadimethoxine	Beef and dairy cattle, poultry, swine	Analogs
Sulfamethazine	Beef and dairy cattle, poultry, swine	Analogs
Tetracyclines[b]		
Chlortetracycline	Beef and dairy cattle, poultry, swine	Analogs
Oxytetracycline	Beef and dairy cattle, poultry, swine	Yes
Tetracycline	Beef and dairy cattle, poultry, swine	Yes

[a] Critically important antimicrobials, [b] highly important antimicrobial, [c] important antimicrobials, [d] not medically important classified according to World Health Organization Human Medicine 5th revision Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) October 2016; adapted from (Mathew et al., 2007; Collignon et al., 2016; FDA, 2018; Zeineldin et al., 2019).

Bacterial resistance mechanisms

Bacteria can be intrinsically resistant to certain antimicrobials, but they can also acquire or develop resistance to antimicrobials via mutations in chromosomal genes or via horizontal gene transfer. Resistance to antimicrobials can be mediated by several different mechanisms, including those that minimize the intracellular concentration of the antimicrobial as a result of reduced cell-membrane permeability or increased efflux, those that modify the molecular target of the antimicrobial by genetic mutation or post-translational modification, and those that inactivate the antibiotic by enzymatic hydrolysis or modification (Blair et al., 2015).

Cephalosporins are members of the β-lactam class of antimicrobials and are widely used in human and veterinary medicine. They exhibit antimicrobial properties by binding to penicillin-binding proteins (PBPs) and disrupting the synthesis of the peptidoglycan layer of the bacterial cell wall (Fair and Tor, 2014). Since their introduction in 1964, cephalosporins have commonly been prescribed for human patients because of their clinical utility (FDA, 2012; Chaudhry et al., 2019). The World Health Organization declared third-generation and fourth-generation cephalosporins to be critically important for human health (Collignon et al., 2016).

There are two cephalosporins currently approved for use in food animals in the United States: ceftiofur and cephapirin (FDA, 2012). Ceftiofur is a semisynthetic, broad-spectrum, third-generation cephalosporin (NCBI, 2020) and is approved for intramuscular injection to treat bacterial respiratory diseases in beef and dairy cattle and swine. Ceftiofur is marketed in a short-acting formulation (ceftiofur sodium, Naxcel®), a more consistent formulation introduced in 1996 (ceftiofur hydrochloride, Excenel®), and a longer-acting formulation introduced in 2003 (ceftiofur crystalline free acid, Excede®) (Zoetis Animal Health, NJ, U.S.A.). Following intramuscular administration, ceftiofur is absorbed in its free-acid form and rapidly metabolized

into desfuroylceftiofur (DFC) and furoic acid (Beconi-Barker et al., 1996). DFC quickly forms conjugates in plasma and tissues or is further metabolized into disulfides (e.g., DFC–cysteine disulfide, DFC-dimer; (Beconi-Barker et al., 1995; Beyer et al., 2015). Free forms of DFC contain an intact beta-lactam ring and a major biologically active metabolite. The association between antimicrobial use and the development of AMR in swine production has been documented (Allen et al., 2011; Holman and Chenier, 2013). The use of antimicrobial agents in animals selects for resistant bacteria that in turn, can serve as a reservoir of resistance genes to (Wegener, 2003).

Cephalosporins bind to bacterial PBPs and are responsible for the hydrolysis of the cross-linkages of newly formed peptidoglycan. Resistance occurs when the PBPs (transpeptidases) are modified or protected by *β*-lactamases. The *β*-lactamases are produced by bacteria from chromosomal or plasmid DNA. The level of *β*-lactamase-mediated resistance depends on the amount of enzyme produced by the bacteria with or without induction and the kinetics of the enzymatic activity (Livermore, 1987).

β-lactamases are grouped into four classes according to the Amber structural classification based on sequence similarity: A, B (metallo-*β*-lactamases, B1, B2, and B3), C, and D (serine β-lactamases) (Silveira et al., 2018). Class A *β*-lactamases (TEM, SHV, CTX-M, and the carbapenemases KPC) are often associated with plasmids. Class B metallo-*β*-lactamases (NDM, IMP, and VIM) provide resistance to penicillin, cephalosporins, carbapenems, and other *β*-lactamase inhibitors. The genes encoding class B metallo-*β*-lactamases can be located on the bacterial chromosome, on plasmids, or on integrons. Class C enzymes (AmpC *β*-lactamases and CMY) are usually encoded by *bla* genes located on the bacterial chromosome, but they can also be encoded on plasmids. Class D *β*-lactamases confer resistance to penicillins, cephalosporins,

extended-spectrum cephalosporins (OXA-type extended-spectrum β-lactamases (ESBLs)), and carbapenems (OXA-type carbapenemases) (Bonomo, 2017). The most important β-lactamase enzymes for cephalosporin resistance are the ESBLs and AmpC cephalosporinases.

Impact of antimicrobial therapy on the gut microbial community

Antibiotics also have significant effects on the gut microbiome composition. It has been demonstrated that exposure to ciprofloxacin changes the fecal microbiome composition, particularly to a shift in the abundance of *Bacteroides*, *Faecalibacterium*, and *Ruminococcadeae* (Dethlefsen and Relman, 2011) but then stabilized by the end of the study period. Such effect could be manifested by both the direct effect of antibiotics and the indirect effect due to the microbial interaction community, which is often driven by age and diet. Further, the effect of antibiotics on the gut microbial community depends on the antibiotic class. For instance, an earlier study (Kanwar et al., 2014a) found a significant increase in ceftiofur resistance and decrease in tetracycline resistance among a treated group of steers (randomized control field trial with two treatment regimens-ceftiofur crystalline free acid with and without therapeutic doses of chlortetracycline in 176 steers) measured by changes in the target AMR gene ($bla_{\text{CMY-2}}$, $bla_{\text{CTX-M}}$, *tet*(A), *tet*(B) and 16s rRNA genes) copies. Similarly, the effect of macrolides on fecal bacterial composition persisted up to two years after treatment, while the effect of β-lactams lasted less than one year in two to seven-year-old children studied (Korpela et al., 2016). It is also possible that age can affect AMR in regard to the microbiome composition. For instance, a study also showed that AMR gene diversity in the human gut microbiome was age related (Lu et al., 2014b).

Longitudinal studies have attempted to identify the impact of antimicrobial interventions on the swine GI microbial community (Gerzova et al., 2015a; Holman et al., 2018; Zeineldin et

al., 2018). For instance, one study found that geographic location had a greater influence on AMR in the fecal microbiome than the method of husbandry (organic or conventional) (Gerzova et al., 2015a). Similarly, Kalmokoff et al. (Kalmokoff et al., 2011) reported that the addition of either tylosin or virginiamycin to pig feed over 15 weeks had no effect on the animals' fecal microbiome (16S rRNA). In another study, tylosin supplementation in pig feed resulted in an increase in the presence of tylosin-resistant anaerobes (from 11.8% to 89.6%) and also a ten-fold increase in the frequency of the macrolide-resistance gene *erm*(B) in fecal samples (Holman and Chenier, 2013). Another study showed that the frequency of ampicillin-resistant fecal coliform bacteria and the resistance patterns of fecal *E. coli* isolates were not different between the control and experimental weaned pigs, suggesting that antimicrobial administration in newborns has little influence on the development of a normal intestinal microbiome and the selection of ARGs (Yun et al., 2017). Similarly, Kim et al. (2012) found no difference in bacterial phyla between control pigs and tylosin-fed pigs (n = 10) (Kim et al., 2012).

A study showed that young pigs had higher ARG diversity than that of sows, but ARG abundance and prevalence were not influenced by antibiotic use (Pollock et al., 2020). In a different study, tetracycline-resistance genes and macrolide-resistance genes were frequently detected in pigs that were not exposed to antimicrobials (Holman and Chenier, 2013). A shotgun metagenomic approach was performed to evaluate the effect of therapeutic doses of oxytetracycline on the microbiome and resistome dynamics in the feces of weaned pigs (n = 16) over 21 days (Ghanbari et al., 2019). Antimicrobial use significantly increased the abundance of the resistome (mainly tetracycline, β-lactams, and MDR—multidrug resistance). In addition, there was a shift in bacterial taxa, mainly in the Escherichia (*Proteobacteria*) and *Prevotella*

(*Bacteroidetes*) populations, after seven days of antibiotic administration. Other study showed that antimicrobials exposure is not the single factor of ARG levels (Birkegard et al., 2017).

(Graesboll et al., 2019) evaluated the influence of antimicrobial use on resistome levels over time by following 1,167 pigs from five different farms in Denmark. Antimicrobial use resulted in increased abundances of the efflux pump-encoding *tet*(A) gene and the ribosomal protection proteins *tet*(O) and *tet*(W), although there were no significant differences between five different treatment strategies using oxytetracycline. Looft et al. (2012) investigated the effect of 3 weeks of in-feed administration of ASP250 (chlortetracycline, 100 mg/kg feed; sulfamethazine, 100 mg/kg feed; penicillin, 50 mg/kg feed) on the diversity and abundance of ARGs in post-weaned pigs. The ARG diversity increased in the treated pigs, with notable shifts in the gut microbiome (*Bacteroidetes* and *Proteobacteria*). Similarly, in a different study, the abundance of bla_{TEM} genes was increased in the feces of ampicillin-treated pigs, and the frequency of AMR in *Enterobacteriaceae* exceeded 50% in all treated pigs by days 4 and 7 (Bibbal et al., 2007). Agga et al. (2014) found that pigs that were fed with chlortetracycline after weaning had a higher abundance of the *tet*(A) gene in their feces than pigs that were not fed with chlortetracycline. Similarly, a study was conducted to determine the impact of parental antibiotics (ceftiofur crystalline free acid, ceftiofur hydrochloride, oxytetracycline, procaine penicillin G, and tulathromycin) administered according to the label dose and route on fecal microbiome composition of grower pigs (n = 20). Analysis of 16S rRNA showed an antimicrobial-dependent shift in microbiome composition over time from day 0 (before antimicrobial administration) and almost a return to the pretreatment composition by day 14 (Zeineldin et al., 2018).

The role of the gut microbiome in ARGs dissemination

The gut microbiome typically consists of diverse microbial communities, some of which possess antibiotic resistance determinants (ARDs) or serve as reservoirs for ARGs (Joyce et al., 2019; Ruppé et al., 2019; Singh et al., 2019). Bacteria in the gut can exchange genetic material through transformation, conjugation, and transduction, and they can increase level of their resistance by acquiring external ARDs present on mobile genetic elements such as plasmids, transposons, integrons, and phage (Huddleston, 2014). The gut microbiome is composed of diverse microbial flora, including bacterial pathogens such as *Enterobacteriaceae* and Enterococcus spp. that can transfer their mobile resistome to other members of the community. One study showed that mobile ARGs are mainly present in four bacterial phyla: the *Proteobacteria, Firmicutes, Actinobacteria,* and *Bacteroidetes* (Huddleston, 2014). The same study showed that *Streptococcus agalactiae*, *E. coli*, and *Streptococcus suis* are the three most abundant bacterial species that are shared between animal and human intestines and consistently harbor known ARGs. Recently, several studies suggested that the gut microbiota forms a large reservoir for diverse ARGs (van Schaik, 2015) that may play a significant role for the emergence of AMR in human pathogens (Sommer et al., 2009b).

Recently, a study used comparative modeling to compare the structures of known AMR proteins to those of proteins produced by the human gut bacterial microbiome (Ruppé et al., 2019). A total of 6,095 ARDs were predicted among a catalog of 3.9 million proteins from the human intestinal microbiome. The predicated ARDs shared ~30% amino acid identity with the known ARDS. The same study grouped 663 participants into six primary resistome clusters (resistotypes) based on resistome composition. The results showed that the different resistotypes were linked to specific enterotypes. Resistotypes 1 (rich in ANT- aminoglycoside

nucleotidyltransferase) and 3 (driven by tet(M) and class C *β*-lactamases) were associated with the *Clostridiales* enterotype. Similarly, resistotype 4 (enriched with tet(X) and class A β-lactamases) was associated with the *Bacteroides* enterotype, and resistotype 6 (class B1 β-lactamases and sul) was associated with the *Provotella* enterotype.

Deep metagenomic sequencing of fecal DNA from 287 pigs identified 7.7 million unique genes representing 719 species (Xiao et al., 2016a). The study further quantified the relative abundances of AMR in the samples and the genes conferring resistance to tetracycline, bacitracin, cephalosporin, macrolide, and streptogramin B in the fecal DNA of all of the pigs. In another study, a positive correlation between the total resistome size (257 unique ARGs) and the total number of OTUs in the microbiome was demonstrated in fecal samples from 16 sows, indicating that the microbiome composition might influence the resistome composition (Joyce et al., 2019). It may also be true that, conversely, microbial communities are shaped in part by the resistome. Antibiotics are used at different stages of pig growth to treat production-related diseases such as diarrhea- especially in weaning piglets. Thus, strong selective pressure is exerted on the gut microbial community. There is evidence that the diversity of ARGs in the gut microbiome is related to the age of the host, increasing and becoming more complex from an early age to adulthood (Lu et al., 2014a).

Statistical approaches to the analysis of microbiome, mycobiome, and AMR data

The data from microbiome studies can be used to build statistical models for hypothesis testing. Currently, most microbiome studies are performed to characterize the microbiome composition and understand the biotic and abiotic factors associated with the microbiome (Spor et al., 2011). Although statistical tests primarily focus on a core theme that explores the impacts

of external factors or interventions on the dynamics of microbiome composition, they can also be used to investigate microbial diversity (alpha and beta diversity), richness, evenness, dominance, or OTU abundance. Recently, a statistical association between AMR determinants in microbial communities and different agricultural or environmental conditions was reported (Noyes et al., 2016b; Yang et al., 2016b). Given the high dimensionality, excessive zero, non-normality, and hierarchical or phylogenetic nature of the data, an appropriate statistical approach is needed as microbiome research shifts from hypotheses about correlations to hypotheses about causal associations between different factors.

Comparisons of diversity across groups

One of the primary goals in studies of gut community ecology is to understand species compositional diversity and its relative influences. The term diversity usually refers to the number of different species that can be identified in a particular environment. Diversity indices applied to microbiome data consist of differing weights of two-component richness and evenness. For instance, the number and variety of taxa present in metagenomic samples can be described using several alpha diversity indices, such as the diversity of taxa (Shannon diversity index or Fisher's alpha diversity index), the evenness of taxa (Simpson's index), the richness of taxa (Chao1, Menhinick's richness index, or Margalef's richness index), and the dominance index of taxa (1-Simpson's index and Berger-Parker dominance index). Similarly, beta diversity refers to how different the microbial composition is between communities or samples. Beta diversity can be measured by several statistics, such as Bray-Curtis dissimilarity (abundance or count-based), Jaccard distance (does not depend on abundance information but is based on the presence or absence of species), and UniFract distance, which is based on the fraction of branch length shared between two communities within a phylogenetic tree of 16S rRNA sequences from

community DNA samples. The UniFrac distance can be further classified as Unweighted Unifrac (based on sequence distances with no abundance information) or Weighted UniFrac (branch lengths are weighted by the relative abundance).

An understanding of gut microbial diversity is important in investigations of how the composition, abundance, function, and dynamics of the gut microbial community are associated with host physiology (Kim et al., 2017). The diversity indices compare the diversity among and between samples (for example, treatments and controls); however, there are no standards for which microbial diversity index is the best to use. Taxonomic diversity explains how many different bacteria are found in terms of their taxonomic ranking as species, genera, orders, or phyla. Depending on how the index values are distributed, several tests such as the T-test and parametric and nonparametric analysis of variance (ANOVA) can be applied to test hypotheses (Rosner, 2011).

Classical univariate statistical analysis

Many traditional statistical tests, including the t-test and parametric and nonparametric ANOVA, can be used to test hypotheses on microbial taxa by comparing alpha and beta diversity metrics. For instance, a t-test can be used to compare alpha diversity metrics between two groups, and the two-sample t-test and its nonparametric analogs (Wilcoxon rank-sum test and Mann–Whitney test) are commonly used in microbiome studies to compare continuous variables between two groups (Bokulich et al., 2016; Chen et al., 2017; Xia and Sun, 2017). Depending on the normality of response variables, ANOVA or the nonparametric Kruskal−Wallis test can be used to compare more than two groups. For example, alpha diversity indices (richness and evenness, Simpson reciprocal index, and equitability indices) were compared between various locations in the GI tract of swine using two-way ANOVA (Holman et al., 2017b). The non-

parametric Kruskal−Wallis one-way ANOVA was used to compare the fungal and bacterial proportions of microbiome data within samples (Gorzelak et al., 2015). A Chi-square test can also be used to compare categorical microbiome data. A simple univariate correlation analysis was performed to determine if there was a linear relationship (Pearson's correlation) or a monotonic relationship (Spearman's correlation) between genes or taxa and metabolites (Chong and Xia, 2017). However, univariate methods do not have enough power to reduce the high rate of false-positive results that are inherent in metagenomic studies.

Negative binomial (NB), zero-inflated, and overdispersion models

The abundance of the gut microbial community is often characterized by overdispersion and zero-inflation, especially at low levels of taxonomic resolution. In addition, distributions can be skewed by the low sensitivity of diagnostic methods and, therefore, require transformation. Hence, the normality and homogeneity of variance assumptions are not typically relevant for the relative abundances of taxa. Such count analyses are usually handled using NB, zero-inflated, or hurdle models, which can be applied to fit microbiome count data by extending the Poisson distribution and allowing the variance to be different from the mean (McMurdie and Holmes, 2014; Xia and Sun, 2017; Calle, 2019). Several zero-inflated models, including zero-inflated Gaussian, lognormal, NB, and beta models, were used to analyze excess zero counts and over dispersed counts in microbiome data. For example, a zero-inflated Gaussian distribution mixed model (metagenomeSeq R package) was used to evaluate different microbiome features and resistome (Rovira et al., 2019). One study showed that hurdle and zero-inflated models are more accurate for parameter estimation with high power, strong goodness of fit, and well-controlled type I errors (Xu et al., 2015).

Longitudinal studies are commonly used in veterinary medicine to capture the inherent dynamic properties of microbiome composition. An appropriate statistical model is, therefore, required for time series as well as microbiome count data. It is important to accurately identify and understand these associations in order to make predictions about which taxa harbor AMR. A mixed-effects ANOVA model with the enterotype-cluster type as a fixed effect and the piglet nested within pen as a random effect was used to test for dependencies between animals within a given pen (Le Sciellour et al., 2019). Similarly, several studies used linear mixed models (LMMs) to account for time-dependent correlations in longitudinal microbiome studies by assuming a normal distribution of response variables (Benson et al., 2010).

Recently, (Zhang et al., 2018) proposed a negative binomial mixed model (R package NBZIMM) for longitudinal microbiome studies to account for time-dependent/changing trends and correlation structures among samples with various fixed and random effects. Similarly, Jian et al. (Jiang et al., 2019) proposed an integrative Bayesian zero-inflated NB regression model that can distinguish between differently abundant taxa with distinct phenotypes while estimating the covariate-taxa effect.

Multivariate analysis

Multivariate data imply that there are multiple numeric values for each data point. Given the high-dimensional, hierarchical, and sparsity characteristics of microbiome data, multivariate statistical analysis (distance-based or model-based) can be useful to address complex ecological questions (Ramette, 2007; Calle, 2019). Several tests such as Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA), principal coordinate analysis (PCoA), analysis of group similarities (ANOSIM), multi-response permutation procedure, and

Mantel's test (MANTEL) are commonly used to test differences between groups, time-points, or other variables in microbiome data (Rivera-Pinto et al., 2018).

PERMANOVA is a nonparametric, multivariate ANOVA test based on dissimilarities with permutations (Anderson, 2001). PERMANOVA can be performed with non-transformed data and is perhaps the most widely used distance-based method to test a null hypothesis of no difference in composition (e.g., OTU abundances) among groups. Distance measures such as unweighted similarity metrics (e.g., Jaccard or unweighted UniFrac) or weighted similarity metrics (e.g., Bray-Curtis or weighted UniFrac) can be used in a PERMANOVA test. The unweighted UniFrac takes into account the presence or absence of OTUs (community membership) or the number of taxa that are unique to either sample, whereas the weighted UniFrac accounts for the relative abundance of OTUs in the microbial community (Navas-Molina et al., 2013). Then, the variability within and between groups is tested using the ANOVA F test, but the partition of the sums of squares is applied to dissimilarities. Statistical significance is evaluated by a permutation to generate a distribution of pseudo-F-tests under the null hypothesis (Calle, 2019). Like PERMANOVA, ANOSIM was used to determine whether there were differences in microbial composition between nursing and weaned pigs based on weighted as well as unweighted UniFrac distance metrics (Guevarra et al., 2018). Recently, MANTEL was used to test for an association between cecal microbial composition and the plasma metabolome in pigs (Wu et al., 2020).

The Dirichlet-multinomial distribution, a parametric multivariate analysis for count data, was found to be suitable for hypothesis testing across groups based on location (mean) as well as scale (variance) (La Rosa et al., 2012). The Dirichlet-multinomial distribution accounts for overdispersion and is used to estimate parameters describing microbiome properties. Several

authors proposed Dirichlet multinomial mixtures (DMM) for the probabilistic modeling of microbial community data to determine enterotypes (Holmes et al., 2012). For instance, probabilistic modeling using DMM was used to examine the microbial communities associated with different production stages of pigs (Slifierz et al., 2015b). Because a simple DMM model does not account for the strong positive and negative correlations that can exist between different species, (Ren et al., 2017) proposed a Bayesian approach using a joint model for the multinomial sampling of OTUs in multiple samples that allows for correlations between OTUs and thus gives more accurate estimates of the microbial distributions.

Machine learning methods are commonly used to identify patterns in highly complex microbiome data and can be used to characterize differences between microbiomes in different types of communities, and also to build models that accurately classify unlabeled data (Knights et al., 2011). Several classifier tools such as random forests (RFs), nearest shrunken centroids, the elastic net, and support vector machines have been applied to microbiome classification. Recently, RF models were used to classify the resistome of formula-fed babies and breast milk-fed babies, and feature importance scores were used to select ARGs (Rahman et al., 2018) and to investigate the association between avian taxonomic orders and functional profiles (Marcelino et al., 2019).

Ordinations

Given the high dimensionality of microbiome data, dimensionality reduction techniques (i.e., ordinations) such as principle component analysis, PCoA/multidimensional metric scaling, non-metric multidimensional scaling, correspondence analysis, and Procrustes analysis have been used to visualize microbiome data (Vázquez-Baeza et al., 2017). The main purpose of dimension reduction is to optimally represent (dis)similarities between samples so that samples

that are similarly represented are depicted close together in a two—or three—dimensional plot, while the main trends of the data and the distances among samples are preserved as much as possible. Both unconstrained ordination (variability in data) and constrained ordination (exploration of the role of sample-specific variables in shaping microbial communities) have been used to visualize high-dimension data (Hawinkel et al., 2019).

Correlation/interaction-based microbial community networks

The reduction of the dimensionality of the microbial community structure might obscure intra-microbiome interactions. The relationships among microbial species can be represented as a network (correlation/interaction-based) where taxa or genes are presented as nodes, and their interactions are presented as edges connecting the nodes (Chong and Xia, 2017). Such networks provide information about links within the microbial community. Faust et al. (2012) analyzed microbial co-occurrence and co-exclusion patterns among 18 body sites in a cohort of 239 healthy humans (from the Human Microbiome Project) using an ensemble method based on multiple similarity measures in a combination of generalized boosted linear models to develop a single global microbial association network for taxa in the healthy commensal microbiome. Recently, (Ke et al., 2019) developed phylogenetic co-occurrence networks of the pig fecal microbiome at different host-ages, with nodes showing the abundances of OTUs and edges representing correlations between OTUs. Similarly, network analysis using the SparCC algorithm was used to indicate growth stage-associated interactions between bacterial features in pigs (Wang et al., 2019). Recently, associations between sexual habits, menstrual hygiene, demographics, and the vaginal microbiome were evaluated using bayesian network analysis (Noyes et al., 2018).

Summary of literature review

Several studies have used phenotypic or genotypic assays to evaluate antimicrobial use and levels of resistance in food animals. AMR in the commensal bacteria of food animals is often complex. The effects on AMR development due to antibiotic use for disease treatment or growth enhancement in livestock is still not clear. A study of commercial production pigs (with high antimicrobial usage) showed that the prevalence and abundance of ARGs were not influenced by antibiotic use. Other studies found that age, diet, and environmental/management factors could affect the level of AMR in gut microbial communities. Recently, studies demonstrated that the gut microbiome could serve as a reservoir from which ARGs can be transferred to native or transient gut bacteria. The gut microbiome (and mycobiome) composition, richness, and diversity change significantly with the age of the pig. The diet, especially the crude fiber content, was found to be an important factor shaping the pig gut microbiome. Although the bacterial constituents of the gut microbiome have been intensely studied, the gut-associated fungal diversity, abundance, and functions are poorly understood in food animals. Recent studies have shown potential interactions between bacterial and fungal genera in the porcine gut.

There has been extensive research to understand AMR in single microbial species of interest, but results in single species do not truly represent whole microbial communities and cannot fully explain AMR in a natural host system that consists of diverse microbial communities. It is evident that microbial communities (e.g., the gut microbiome) are highly dynamic and often depend on dietary and environmental factors. Therefore, diet and the timing of sampling need to be considered because they influence the diversity and composition of the microbial community. It is important to understand the factors that drive the evolution,

enrichment, and persistence of AMR in complex microbial communities to develop sustainable solutions. Various tools have been applied to characterize AMR in gut microbial communities, including culture-based and metagenomics-based approaches. Even in an era of molecular techniques, bioinformatic tools and increasingly advanced statistical approaches, it is still a complicated matter to address core hypotheses.

This study summarizes the literature published since the early 1970s on the age-dependency of AMR in fecal bacteria from animals raised in different geographical locations. Our study examines AMR in fecal microbial communities in swine using culture-dependent and metagenomic methods coupled with statistical analyses in order to estimate and make inferences about the relationships between AMR and the age and diet of the host animals. It investigates the potential linkage between AMR and the taxonomic compositions of the bacterial and fungal communities. It also examines differences in the bacterial community and ARG frequencies among the different parts of the GI tract of piglets. An understanding of the relationships among host factors, the microbiome/mycobiome, and AMR in pigs will help scientists and other stakeholders to design a systematic framework to address the problem of AMR in a sustainable way.

Chapter 3 - Age Dependence of Antimicrobial Resistance Among Fecal Bacteria in Animals: A Scoping Review

Abstract

A phenomenon of decreasing antimicrobial resistance (AMR) among fecal bacteria as food animals age has been noted in multiple field studies. We conducted a scoping review to summarize the extent, range, and nature of research activity and the data for the following question: "Does AMR among enteric/fecal bacteria predictably shift as animals get older?". This review followed a scoping review methodology framework. Pertinent literature published up until November 2018 for all animals (except humans) was retrieved using keyword searches in two online databases: namely, PubMed® and the Web of Science™ Core Collection, without filtering publication date, geographical location, or language. Data were extracted from the included studies, summarized, and plotted. Study quality was also assessed using GRADE guidelines for all included papers. The identified papers with detailed relevant data (*n*=62) were conducted in food animals, poultry, and dogs. These included longitudinal studies (*n*=32), cross-sectional studies of different age groups within one food animal production system or small-animal catchment area (*n*=16), and experimental or diet trials (*n*=14). A decline in host-level prevalence and/or within-host abundance of AMR among fecal bacteria in production beef, dairy cattle, and swine was reported in nearly two-thirds (65%) of the identified studies in different geographic locations from the 1970s to 2018. Mixed results, with AMR abundance among fecal bacteria either increasing or decreasing with age, have been reported in poultry (broiler chicken, layer, and grow-out turkey) and dogs. Quantitative synthesis of the data suggests that the age-

dependent AMR phenomenon in cattle and swine is observed irrespective of geographic location and specific production practices. It is unclear whether the phenomenon predates or is related to antimicrobial drug use. However, a majority of the identified studies predate recent changes in antimicrobial drug use policy and regulations in food animals in the United States and elsewhere.

Introduction

Epidemiological studies in food animals have suggested that enteric antimicrobial resistance (AMR) changes as the host ages. For example, studies in beef and dairy cattle production systems in the Pacific Northwestern and Southwestern U.S. (Khachatryan et al., 2004; Berge et al., 2010; Edrington et al., 2012b), Great Britain (Hoyle et al., 2004a; Hoyle et al., 2004b), and Zambia (Mainda et al., 2015b) have shown that AMR gene copy abundance decreases in the fecal indicator bacterium *Escherichia coli* (*E. coli*) in cattle as animals age during early life. One of the studies conducted in the U.S. suggested that the decline in the abundance of multidrug-resistant (MDR) fecal *E. coli* in cattle during the first months of life may be independent of the transition from milk to solid diet (Edrington et al., 2012b). Other studies in pig production systems in the Midwestern U.S. showed that AMR gene copy abundance decreases in fecal *E. coli* in pigs during early life; in fact, the effect of age surpasses in relative magnitude that of concurrent feeding with antimicrobial drugs (Agga et al., 2015b). Others have reported that this decline in pigs continues beyond the first two weeks in the nursery, when the enteric microbiome changes due to the transition from milk to solid diet (Kim et al., 2011; Frese et al., 2015a; Slifierz et al., 2015a). Hence, the decline does not seem to be solely driven by the dietary transition of weaning. Understanding the dynamics of AMR is essential because, first, the body mass and fecal output of food animals increase with age (ASAE, 2005). Thus, AMR among the fecal bacteria of larger older animals poses greater mass burden for AMR transmission via manure from production systems to the environment. Second, AMR among fecal bacteria at the age when the animal is harvested poses the greatest direct risk of AMR transmission to the consumer via carcass contamination at slaughter. Understanding the age-dependent AMR dynamics and their drivers could help lower both environmental and food safety risks.

Assessing the available research reports pertaining to age-related AMR in food animals enhances our understanding of how the prevalence of animals carrying antimicrobial-resistant bacteria or else the abundance of antimicrobial-resistant bacteria (or AMR genes) within those animals will change according to host age; in turn, this will help to formulate a risk-based AMR mitigation program to lower the environmental and food safety risk. However, it is unclear what kind of research information is available in the literature about age-dependent AMR in animals in different geographical regions of the world and across different time periods. Thus, the objectives of this review were to examine research reports and summarize the available data per the following question: "Does AMR among enteric/fecal bacteria predictably shift as animals get older?". A scoping review was chosen as the approach for the study, given the goal was to examine the nature, extent, and range of research activity on this broad question (Levac et al., 2010; Murphy et al., 2016) as well as to identify research gaps in the existing literature.

Here, we explore, summarize and present data on age-related AMR dynamics from the available literature that was systematically searched for all animals without filtering date, geographical location, or the language of the publication. Using the Grades of Recommendation, Assessment, Development, and Evaluation (GRADE) guidelines (Guyatt et al., 2011), we summarized the quality of the evidence for all those identified studies containing relevant data. Moreover, following the scoping review approach, data from all the identified studies were charted and compiled. The outputs from the review results were summarized to directly address the study question.

Materials and methods

We followed the scoping literature review methodology framework outlined by Arksey and O'Malley (Arksey and O'Malley, 2005). For rigor and reproducibility of the study, the review was implemented by adhering to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Liberati et al., 2009; Moher et al., 2009); more specifically, to the PRISMA extension for conducting scoping reviews (PRISMA-ScR) (Tricco et al., 2018) and for reporting purposes.

Study outcomes relevant to the study question

The following outcomes were defined for "AMR among enteric/fecal bacteria" in a sampled animal population: the abundance of AMR bacteria; the relative fraction of AMR bacteria; the quantities of total or specific genes encoding AMR in feces of the animals; and the proportion of animals carrying AMR bacteria or genes in their feces collected by individual fecal or rectal swabs or in a pooled sample from the pen/flock/barn. The study designs included any observational design employing a comparison group.

Keyword-based search strings were developed, refined, and implemented in two online databases: PubMed® maintained by the U.S. National Center for Biotechnology Information (NCBI) and Web of Science™ Core Collection maintained by the Thomson Reuters Corporation. The string for each database was refined until the search returned all publications known to the study team on the study question. The following final algorithms were used in PubMed®:

(antibiotic resistance OR antibiotic resistant OR drug resistance OR multiple drug resistance OR resistance genes OR antimicrobial resistance OR antimicrobial resistant OR bacterial resistance) AND (fecal OR feces OR faecal OR faeces OR stool OR intestinal OR

intestine OR enteric OR bacteria OR bacterial OR faecal coliforms OR fecal coliforms OR faecal coliform OR fecal coliform OR coliform bacteria OR faecal flora OR fecal flora OR feces collection OR faeces collection OR faecal examination OR fecal examination OR cecal OR caecal OR ceca OR caeca OR cecum OR caecum OR intestinal microorganism OR intestinal microorganisms OR Enterobacteriaceae OR Escherichia coli OR E. coli OR Salmonella OR Campylobacter OR Enterococcus OR Klebsiella OR Citrobacter OR microbial flora OR microbiome OR intestinal microorganisms) AND (age OR animals by age OR age groups OR age structure OR aging OR maturation OR cohort OR cohort studies OR longitudinal studies OR longitudinal distribution).

Similarly, the following final algorithms were used in the Web of Science™ Core Collection:

(antibiotic resistance OR antibiotic resistant OR drug resistance OR multiple drug resistance OR resistance genes OR antimicrobial resistance OR antimicrobial resistant OR bacterial resistance) AND (fecal OR feces OR faecal OR faeces OR stool OR intestinal OR intestine OR enteric OR bacteria OR bacterial OR faecal coliforms OR fecal coliforms OR faecal coliform OR fecal coliform OR coliform bacteria OR faecal flora OR fecal flora OR feces collection OR faeces collection OR faecal examination OR fecal examination OR cecal OR caecal OR ceca OR caeca OR cecum OR caecum OR intestinal micro-organism OR intestinal microorganisms OR Enterobacteriaceae OR Escherichia coli OR E. coli OR Salmonella OR Campylobacter OR Enterococcus OR Klebsiella OR Citrobacter OR microbial flora OR microbiome OR intestinal microorganisms) AND (age OR animals by age OR age groups OR age structure OR aging OR maturation OR cohort OR cohort studies OR longitudinal studies OR

longitudinal distribution) NOT (human OR man OR human disease OR human feces OR human stool OR children OR women OR infants).

Literature for all animals other than humans was searched (including farm, pet, or hobby animals and wildlife) without filtering the language, geographical location, or date of publication. The final searches were performed in November 2018. All citations identified by the searches in the two databases were imported, merged, and deduplicated in the web based RefWorks© v.2.0 platform (ProQuest, LLC, Ann Arbor, MI, USA). After deduplication, the citation list was imported into the web-based Rayyan platform for systematic reviews (Ouzzani et al., 2016).

Relevance screening and study selection criteria

The identified citations (*n*=8,073) were subjected to a title-based screening. A citation was excluded if the title indicated that the study was conducted in humans, was performed on the resistance to disease of multicellular organisms, was on infectious agents other than enteric/fecal bacteria, or was on bacterial resistance to drugs other than antimicrobial drugs. The citations retained after the title screening (*n*=383) were subjected to an abstract-based screening. A citation was excluded if the abstract met any of the title exclusion criteria. In addition, a citation was retained if the abstract identified that the study met these inclusion criteria: (*a*) performed in farm-, shelter-, or household-animals; (*b*) longitudinal study of >3 weeks or a cohort study or a cross-sectional study of multiple age groups in one production system or local population (*e.g.*, animals from multiple farms or owners); (*c*) enteric/fecal culturable bacteria or microbiome studied; and (*d*) AMR (phenotype or genes) measured in the bacteria or microbiome. The citations retained after the abstract screening (*n*=199) were subjected to full-text screening. The full text was excluded if any of the abstract exclusion criteria were met or if it was an *in vitro*

study or a cross-sectional study of animals at one age point or if the animal age was not specified. The full text was included if it met the abstract inclusion criteria if the publication contained age-specific data on enteric/fecal AMR in animals of a given species (and in a given production system for food animals) at more than one age point, and either the fecal/enteric bacterial species were isolated and antimicrobial drugs to which the isolate susceptibility was tested were specified or the fecal/enteric bacterial antimicrobial genes tested were specified. The screening was performed on the Rayyan platform. These steps yielded 62 studies. The references for all included studies are provided in Supplementary Table S 3.3.

Each title screening and abstract screening was performed independently by two reviewers. Citations on which the two disagreed were reviewed independently by a third reviewer whose judgment was the final decision. For refinement of the inclusion and exclusion criteria at the start of the title review, 50 titles were randomly selected from the citation list from PubMed® and independently reviewed following draft criteria by the first two reviewers. The three reviewers met to discuss the results and to clarify and refine the criteria. A similar criteria refinement procedure was performed at the start of the abstract review using 50 abstracts randomly selected from the citations (n=383) retained after the title screening. The scoping review diagram was created following the PRISMA guidelines (Liberati et al., 2009; Moher et al., 2009) using the PRISMA extension for scoping reviews (PRISMA-ScR) (Tricco et al., 2018). Given the difference in objectives from a systematic review, some PRISMA checklist items might not be relevant, while other important considerations may be missing; therefore, for transparent reporting purposes, we have followed a complete checklist relevant to PRISMA-ScR. In addition, an assessment of the quality of the final retained citations was made based on GRADE assessment and risk-of-bias approach (Guyatt et al., 2011).

Data extraction, characterization, and analyses

The characteristics of each full text, such as year of publication, geographical location of the study, study design (longitudinal, experimental trial of antimicrobial drug use or diet, and cross-sectional), sample size for the fecal sampling, animal species; animal production system (*e.g.*, dairy or beef), animal production category (*e.g.*, broiler breeder or chicken), age or age group of the sampled animals, individual or pooled fecal sampling (*e.g.*, from individual animals or pooled from multiple animals), sample type (*e.g.*, rectal swab, cloacal swab, fecal grab per rectum, grab sample from voided feces), and AMR assessment method (*e.g.*, testing phenotypic antimicrobial drug susceptibility of culturable bacterial species isolates, quantifying AMR genes in culturable bacterial species isolates, or AMR gene quantification via metagenomic sequencing of fecal samples) were recorded. For studies based on phenotypic AMR assessment, bacterial species isolated for testing the phenotypic susceptibility to antimicrobial drugs; number of isolates of each of the bacterial species; identity and class of each antimicrobial drug tested; percentage of the isolates of each bacterial species resistant to each individual antimicrobial drug; animal-level prevalence of AMR to each individual drug; percentage of the isolates of each bacterial species concluded to be MDR (≥3 drug classes); and animal-level prevalence of multidrug AMR were used in the study. For studies based on AMR gene quantification via metagenomics of fecal samples, the quantities of total and specific genes encoding bacterial AMR, identity and number of antimicrobial drug classes for which resistance is encoded by the detected genes, animal-level prevalence of the detected genes being carried in feces, the total number of isolates, and funding sources were extracted from the full-text citations. The above-described categories were not established *a priori* but were developed iteratively by reviewers and characteristics based on individual studies.

All the data captured above were recorded in Microsoft Excel 2010. To visualize the AMR data with the age of animals, data from individual studies were combined based on outcomes such as prevalence/proportion of animals carrying antimicrobial-resistant bacteria or AMR genes, abundance or proportion of phenotypically antimicrobial-resistant bacteria and quantities of total or specific AMR genes within the same enteric bacterial group (*e.g., E. coli, Enterococcus* spp., *Salmonella* spp., *Campylobacter* spp.). Some studies recorded the age of animals as the stage of production cycle, for instance, piglet, nursery, grower, and finisher in the age data for pigs. To make the data consistent, data from such studies were categorized based on production stages (*e.g*. for pigs, piglet 1-3 weeks, weaner 3-4 weeks, nursery 4-10 weeks, grower 10-14 weeks, and finisher 14-26 weeks). We used R software with the ggplot2 package (V.3.3.4) (Wickham, 2016) to summarize and visualize the data.

Results

A flow diagram of the process and the number of citations in the literature review is presented in Figure 3.1. The keyword searches in the NCBI PubMed database yielded 3,802 articles. The keyword search in the Web of Science™ Core Collection yielded 4,769 articles. The article sets were merged, and duplicate records were removed. This left 8,073 unique articles, the titles of which were screened by following the title exclusion criteria. After the removal of 7,690 titles, the abstracts of 383 records were reviewed and further screened based on inclusion and exclusion criteria, and finally, 199 full articles were reviewed. After further exclusion, a total of 62 studies met the inclusion criteria, providing relevant information based on the review question, and were included for qualitative analysis. A list of all the studies included in this review is presented in Supplementary Table S 3.5.

Based on the data gathered in this review, age dependency of AMR among fecal bacteria in animals has been reported since 1970. The absolute level of research activity peaked in 2010-2018 (*n*=33, 53%) (Figure 3.2). Of the *n*=62 studies, 48% (*n*=30) were conducted in Europe, 35% (*n*=22) in the U.S., 11% (*n*=7) in Asia and the remainder 5% (*n*=3) in Canada. The animal populations studied in the retained citations (*n*=62) were cattle (*n*=22, 35% including *n*=1 cattle and pig), pigs (*n*=24, 39% including *n*=1 pig and poultry), poultry (*n*=14, 23%) and dogs (*n*=2, 3%). The most common study type was observational (*n*=48, 77%) (Figure 3.3). The most frequently investigated enteric/fecal bacteria were *E. coli* (*n*=44, 71%), *Salmonella enterica* subsp. *enterica* (*n*=7, 11%) and *Enterococcus* spp. (*n*=6, 10%). All the studies were published as journal articles. The general characteristics of the retained articles are presented in Table 3.1, and details are presented in Supplementary Table S 3.3.

For the 22 cattle studies (including *n*=1 cattle and pig study), data outcomes were reported as: (*n*=9, 41%, the proportion of the animals carrying antimicrobial-resistant *E. coli*; *n*=8, 36%, abundance of antimicrobial-resistant *E. coli* or proportion of antimicrobial-resistant *E. coli*; *n*=1, 4%, proportion of antimicrobial-resistant *Salmonella enterica*; *n*=1, 4%, relative abundance of AMR in bacterial taxa; *n*=1, 4%, quantity of total or specific genes encoding AMR in feces of the animals; *n*=1, 4%, proportion of both the animals carrying both antimicrobial-resistant *E. coli* and abundance of antimicrobial-resistant *E. coli*; and *n*=1, 4%, abundance of both antimicrobial-resistant *E. coli* and AMR genes in feces of animals. Similarly, of the 24 pig studies (including one pig and poultry study), one study (cattle and pig) generated 25 combined data points, where the outcome was reported as *n*=2, 8%, the proportion of the animals carrying antimicrobial-resistant *E. coli*; *n*=12, 50%, abundance of antimicrobial-resistant *E. coli* or proportion of antimicrobial-resistant *E. coli*; *n*=4, 16%, proportion of antimicrobial-resistant

Salmonella spp.; *n*=1, 4%, proportion of antimicrobial-resistant *Campylobacter* spp.; *n*=1, 4%, proportion of antimicrobial-resistant *Enterococcus* spp.; *n*=3, 12%, quantities of total or specific genes encoding AMR in feces of the animals; and *n*=2, 8%, both abundance of antimicrobial-resistant *E. coli* or proportion of antimicrobial-resistant *E. coli* and quantities of AMR genes in feces of the animals.

Similarly, from a total of 14 poultry studies and one study from pigs and poultry, 15 data points were generated, where outcomes were reported as: *n*=7, 50%, abundance or proportion of antimicrobial-resistant *E. coli*; *n*=2, 14%, proportion of antimicrobial-resistant *Salmonella enterica* subsp. *enterica*; *n*=3, 21%, proportion of antimicrobial-resistant *Enterococc*us spp.; *n*=2, 14%, quantities of total or specific genes encoding AMR in feces of the animals; and *n*=1, 7%, abundance or proportion of both antimicrobial-resistant *E. coli* and *Enterococcus* spp.. We captured only two studies conducted in dogs, where outcomes were reported as: *n*=1, 50%, the proportion of antimicrobial-resistant *Enterococcus* spp.; and *n*=1, 50%, quantities of total or specific genes encoding AMR in feces.

Furthermore, we visualized the AMR dynamic data according to the age of the animals. Based on the combined data, we observed that the overall age-related AMR dynamics (both phenotypic and genotypic) were especially high at a young age and thereafter declined with the age of animals among fecal *E. coli* in cattle (Figure 3.4) and pigs (Figure 3.5). However, mixed age-related AMR dynamics were observed in fecal *E. coli*, *Enterococcus* spp. and *Salmonella* e*nterica subsp. enterica*. in poultry (including broiler chicken, layer, and meat-type turkey) (Figure 3.6) and fecal *Enterococcus* spp. in dogs**.** Furthermore, we also combined the age-related AMR data for other bacterial species to determine whether there were any discrepancies among bacterial species in terms of age-related AMR dynamics. In contrast, in an experimental study in

cattle where animals were treated with third-generation cephalosporin and chlortetracycline, the proportion of MDR *Salmonella enterica* subsp. *enterica* increased from day 4 through day 26 during the feeding period.

In addition to phenotypic AMR, we also evaluated the combined data for AMR genes from cattle, pigs, poultry, and dogs from the included studies. The combined data plot showed that the overall abundance of genes encoding AMR to different drug classes among fecal *E. coli* or among the total genes are similar to the phenotypic AMR data, with especially high values at earlier ages or sampling points in cattle and pigs. The age-related AMR genes among fecal bacteria were also observed in pigs (Figure S 3.7). Overall, two-thirds (65%, *n*=46) of the included studies from cattle and pigs showed a high prevalence and abundance of antimicrobial-resistant enteric/fecal bacteria early in life that subsequently declined with age. In contrast, half of the included studies in poultry showed an increase in the prevalence and abundance of AMR in fecal or cloacal bacteria with age. In dogs, mixed AMR dynamics with age were reported in the identified studies.

GRADE-based study evaluation to summarize the research activity (quality assessment)

To summarize the quality of research activity for the study question, an evaluation of the quality of evidence in the retained studies (*n*=62) was performed by adapting the GRADE assessment of study quality and the risk-of-bias approach (Guyatt et al., 2011). In summary, nearly all studies (*n*=60, 97%) clearly defined study objectives and sampling procedures (*e.g.*, description of participant/animal). However, the description of animal selection (inclusion/exclusion criteria for subject selection in the case of observational studies) was inadequate. In addition, very few studies (*n*=6, 10%) estimated the sample size. A majority of the

studies (*n*=13, 93% of experimental studies) clearly described the experimental group, but there were very few studies (*n*=6, 43%) that performed randomization for treatment allocation. Nearly all the studies *(n*=61, 98%) clearly defined the method to measure the outcomes. Finally, more than 50% of the studies described potential biases and/or confounders and adjusted or explained the results in the outcome and analysis section. Specific definitions used for quality assessment are provided in Table 3.2. The details of the quality assessment criteria are presented in Supplementary Table S 3.4. In addition to the GRADE assessment, we also found that most studies (*n*=51, 82%) reported the source of funding in their studies.

Discussion

This review assessed the extent, range, and nature of available research activity and systematically and transparently charted the main characteristics of AMR among enteric/fecal bacteria according to host age in food animals, poultry, and pet dogs. We identified research reports relating AMR prevalence or quantity among fecal bacteria with the age of the animal since the 1970s in different geographical regions of the world, with a surge of research (over 50% of papers) since 2010. Additionally, most of the studies were from Europe and North America. We believe that this distribution likely reflects the geographical location in which the phenomenon is frequently investigated.

The available evidence, including study findings, shows declining host-level prevalence and within-host abundance of AMR among fecal bacteria from early life in cattle and pigs to later production stages in the different production systems. Both observational and experimental field studies showed that the prevalence of AMR in fecal bacteria declined with the age of animals, and interestingly, this phenomenon has been observed over the decades across dispersed geographical locations and different production practices: from small-scale cattle farms in

Tajikistan in 1971 (Turushev, 1971) to housing of thousands of cattle in the Southwestern U.S. in the 2010s (Berge et al., 2010; Edrington et al., 2012b) to cattle farms in Zambia (Mainda et al., 2015b) and pig farms in Canada (Rosengren et al., 2008b) and the U.S. (Moro et al., 1998). This lends credibility to the notion that the phenomenon may not be driven solely by antimicrobial drug practices on farms. Similarly, healthy calves were reported to be rapidly colonized by antimicrobial-resistant *E. coli* shortly after birth and to shed MDR bacteria that are resistant to nine and ten antibiotics as early as one day of age (Donaldson et al., 2006). Furthermore, intestinal microbiomes are unique in younger calves compared to adult animals, favoring the survival of MDR bacteria (Khachatryan et al., 2004). Additionally, age-related dynamics are not limited to animals and have also been reported in humans (Kalter et al., 2010b; Literak et al., 2011a). For instance, age-specific AMR among *E. coli* was reported in a human in the UK, where the abundances of *E. coli* resistant to amoxicillin, co-amoxicillin/clavulanic acid, ciprofloxacin, cephalexin and extended spectrum beta-lactamase (ESBL)-producing *E. coli* were high at a young age and decreased with age, followed later by an increase (Robey et al., 2017b).

On the other hand, increased AMR in *E. coli* was reported in beef cattle (Catry et al., 2016) and in *Enterococcus* spp. in pigs (Butaye et al., 1999) for production systems in European countries that have dramatically altered antimicrobial drug use practices since the 1990s (Casewell et al., 2003; Hammerum et al., 2007). For example, in Belgium and other European countries, the use of avoparcin was banned in 1997. In Belgium, most surprisingly, the pigs in which the growth promoter was banned (sows) demonstrated the highest prevalence of vancomycin-resistant *Enterococci* (VRE) compared to piglets and finishers from later birth cohorts. Another finding from the same study was that the prevalence of VRE was higher in broilers compared to layers of the same age group; importantly, avoparcin had been used in the

past in broilers but never in layer chicks. In Denmark, higher tetracycline and sulfonamide resistance was observed in *S.* Typhimurium isolates from pigs as well from a human following ban on growth promoters went into effect (Casewell et al., 2003). Recently, analysis of the abundance and diversity of the fecal resistome in pigs and broilers in nine European countries found that resistome abundance and composition were very different in pigs and poultry; that is, with higher abundance in pigs but a more diverse resistome in poultry. However, estimated AMR genes were not associated with drug use, suggesting that some genes might be functional only in a specific host. Furthermore, the findings revealed that countries with similarity in antimicrobial use also exhibited the same general levels of AMR (Munk et al., 2018a).

Similarly, our review findings showed that the dynamics of AMR associated with age in poultry were different from those in cattle and pigs. Both increased AMR (Welton et al., 1998a; Santos et al., 2007; Ozaki et al., 2011) and decreased AMR (Hinton et al., 1982; Butaye et al., 1999) among enteric bacteria were reported in the poultry production cycles. These results might be due to blanket use of antimicrobials in feed or else due to direct and close contact between birds carrying antimicrobial-resistant strains. We did not capture a large number of citations for age dependency of AMR in dogs; however, we observed mixed dynamics of AMR in the included studies.

Based on the results, the following question arises: is this age-related phenomenon the result of previous or current antimicrobial exposure? For instance, a longitudinal study on calves demonstrated that there was no significant association between calves fed waste milk containing antibiotic residue or calves fed fresh milk in terms of the proportion of animals that shed CTX-M-positive *E. coli* during the 1- to 12-week age period (Brunton et al., 2014). Similarly, other studies have shown that resistome richness decreased significantly during the feeding period

(arrival and exit, ~ 32 weeks) of feedlots when they traced AMR in the feedlots; but at the same time, other resistome elements were detected against antimicrobials that are not approved for use in cattle production, suggesting that the relationship between antimicrobial use and AMR is not straightforward and that the use of antimicrobials alone cannot directly explain the presence of AMR (Noyes et al., 2016b). It has also been concluded that the elevated AMR in early life in cattle is not of maternal origin but likely acquired during the first weeks of life (due to factors other than antimicrobials added to fresh milk, if any) (Berge et al., 2005; Berge et al., 2010; Edrington et al., 2012b). More extensive research on the sources of AMR in the young has been performed in humans. Human newborns carry diverse AMR genes in their enteric microbiome before receiving antimicrobial treatments, and such individuals also have been born to mothers not treated with antimicrobial drugs during the last trimester of pregnancy (Zhang et al., 2011).

The purpose of this review was to extract essential information from the diverse body of work conducted to address the relevant research questions; scoping reviews typically do not assess the quality of the studies included in the review (Arksey and O'Malley, 2005). Quality assessment to control the biases in research analyses other than systematic reviews is rarely applied and poorly reported in veterinary science (Sargeant et al., 2006). For instance, some have reported quality assessments in scoping reviews (Murphy et al., 2016), whereas others did not report any quality assessment to identify the risk of biases (Greig et al., 2015; Rose et al., 2017; Travers et al., 2017; Baker et al., 2018; Gabriele-Rivet et al., 2019). A rigorous scoping review of scoping reviews was conducted by (Pham et al., 2014) to examine the approach and consistency. In these reviews, quality assessment of the included studies was infrequent, and only 22.4% of the 335 scoping reviews performed quality assessment checks. However, evaluation of quality or risk of bias in the included studies is recommended (O'Connor and

Sargeant, 2015). For this review, first, we adopted the PRISMA extension for scoping review (PRISMA-ScR) (Tricco et al., 2018) for the purposes of transparency and reproducibility; second, we implemented quality assessment of all retained citations by adapting GRADE assessment and a risk-of-bias approach (Guyatt et al., 2011) and summarized the data based on quality criteria (Table 3.2); however, we did not remove any articles from the assessment. In doing so, our review was able to provide a complete overview of all available research reports related to a topic, as per the objectives of the scoping review, and at the same time, we became aware of the quality of the evidence from the research analysis.

There are some limitations to our scoping review. Although nearly all (97%) the studies clearly mention the research objectives and outcome and are from journal articles, the data were from different types of study designs, and different interpretative criteria for antimicrobial susceptibility testing may have affected the findings. Any citations that were not listed in one of the search databases were not captured by this study. Therefore, this is something that needs to be taken into consideration when interpreting the data of this review.

Our scoping review has shown that the age-related dynamics in fecal/enteric AMR in food animals, including poultry and pet animal dogs, were consistent with other field study results. Although we do not fully understand the mechanism underlying the high AMR at early ages, it seems that the epidemiology of the age group of the animal population has been found to be an important factor in the quantification of AMR. We believe that this age-dependent AMR data finding helps to stratify further individual risk based on the study area, allowing potential targeting of surveillance (*i.e.*, threshold-based action-driven monitoring) or any other intervention in specific animal age groups in the population. However, this review specifically aimed to summarize the research reports related to the age-dependency of AMR, so many policy-

related questions remain unanswered. The higher AMR observed at early age is of concern, but there was no clear difference in animal age-specific trends between different antimicrobial agents, which could represent differences in antimicrobial use and, in turn, selection pressure. Well-researched areas of interest were identified in this review (for example, why higher AMR in fecal bacteria is observed at early ages in animals), and this finding could be extended to other food animals and to aquaculture, which will ultimately help with AMR risk categorization and planning for interventions to reduce environmental- and foodborne-risks to public health.

Conclusions

The decline in the prevalence and abundance of antimicrobial-resistant enteric/fecal bacteria with age in production pigs, beef, and dairy cattle has been reported since the 1970s in various geographical locations, and in two-thirds of the included studies in our scoping review. In broiler chickens and meat turkeys, mixed AMR dynamics associated with age have been reported. We captured very few studies in dogs, where mixed AMR dynamics with age also were reported. We found that the age of animals could be one of the factors affecting both phenotypic and genotypic AMR; however, other management factors may influence the overall findings. Hence, identifying such risk factors associated with resistance in the different production phases of food animals and poultry is crucial, and such findings could guide judicious antimicrobial use. The scientific evidence from the existing studies in these areas is limited. Therefore, further longitudinal research into related AMR phenomena should be undertaken to better guide the interpretation of our findings.

Table 3.1

Characteristics of included studies (*n*=62) from which the data were charted in this scoping review of literature on age dependency of antimicrobial resistance of fecal bacteria in animals.

Characteristic	Number of studies	Percentage of *n*=62 studies
Publication date		
1970-1979	1	2%
1980-1989	4	6%
1990-1999	5	8%
2000-2009	19	31%
2010-2018 (November)	33	53%
Study location		
UK, continental Western Europe, Eastern Europe, and USSR	30	48%
USA	22	35%
Canada	3	5%
Asia	7	11%
Study design		
Longitudinal	32	52%
Cross-sectional	16	26%
Experimental (antimicrobial drug treatment or diet trial)	14	23%
Animal species		
Cattle* (1 cattle and swine)	22	35%
Swine* (1 swine and poultry)	24	39%
Poultry (chicken, turkey)	14	23%
Dog	2	3%
Fecal sample		
From individual animals	51	82%
Pooled from multiple animals	11	18%
AMR identification		
Phenotypic AMR in culturable fecal bacteria	34	55%
Genes encoding AMR in fecal metagenome or culturable bacteria	1	2%
Both phenotypic AMR and AMR genes	27	44%
Fecal bacteria cultured		
E. coli	44	71%
Salmonella spp.	7	11%
Campylobacter spp.	1	2%
Enterococcus spp.	6	10%
E. coli and *Salmonella* spp.	1	2%
E. coli and *Enterococcus* spp.	1	2%
Staphylococcus spp.	1	2%

None, AMR genes in fecal microbiome analyzed	1	2%

Table 3.2

Descriptive summary of quality assessment of the retained studies (*n*=62) on age dependency of antimicrobial resistance of fecal bacteria in animals.

Individual quality criterion	Number of studies	Percentage of *n*=62 studies
Study objectives clearly defined (*n*=62)		
Yes	60	97%
Unclear	2	3%
Sampling method (animal selection) clearly described (*n*=62)		
Yes	59	95%
Unclear	3	5%
Sample size estimation included (*n*=62)		
Yes	6	10%
No	56	90%
Inclusion and exclusion criteria for the sampled animals stated (*n*=48 observational studies)		
Yes	15	31%
Partially	19	40%
No	14	29%
Experimental groups (treatment and control) clearly defined for an experimental study (*n*=14)		
Yes	13	93%
Unclear	1	7%
Sampling unit/animal randomly assigned to the treatment for an experimental study (*n*=14)		
Yes	6	43%
Unclear	8	57%
The methods for AMR analysis in the study clearly specified (*n*=62)		
Yes	61	98%
Unclear	1	2%
Potential biases or confounders listed and accounted for in the statistical analysis (*n*=62)		
Yes	36	58%
Unclear (not fully listed)	18	29%
Not reported	8	13%

Yes, quality criterion is met; No, quality criterion is not met; Partially, the criterion is not entirely met; Unclear, insufficient information to evaluate the quality criterion; N/A, not applicable

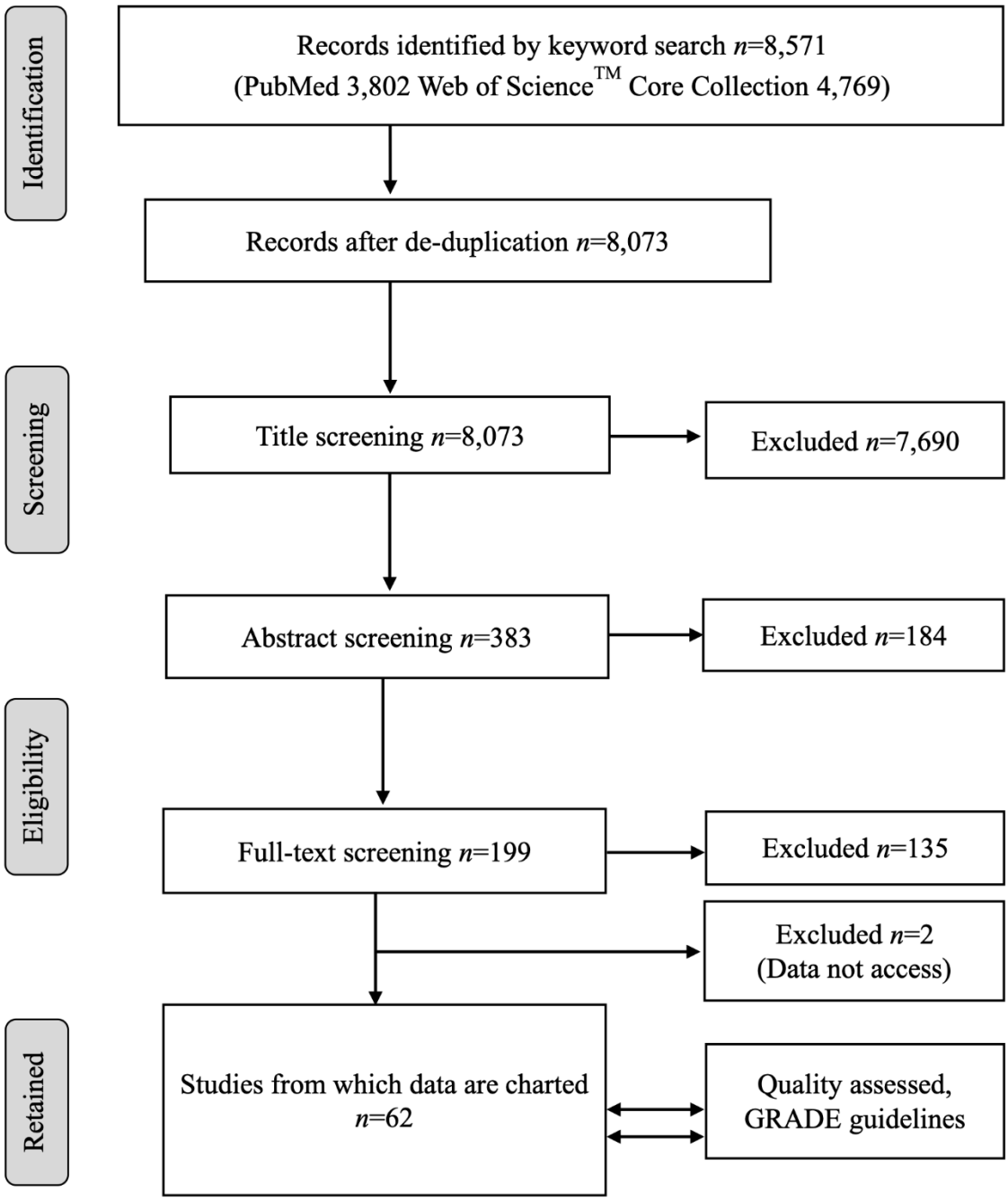

Figure 3.1. The PRISMA flow diagram of the scoping review of literature on the age-dependency of antimicrobial resistance of fecal bacteria in animals. *n*, number of studies.

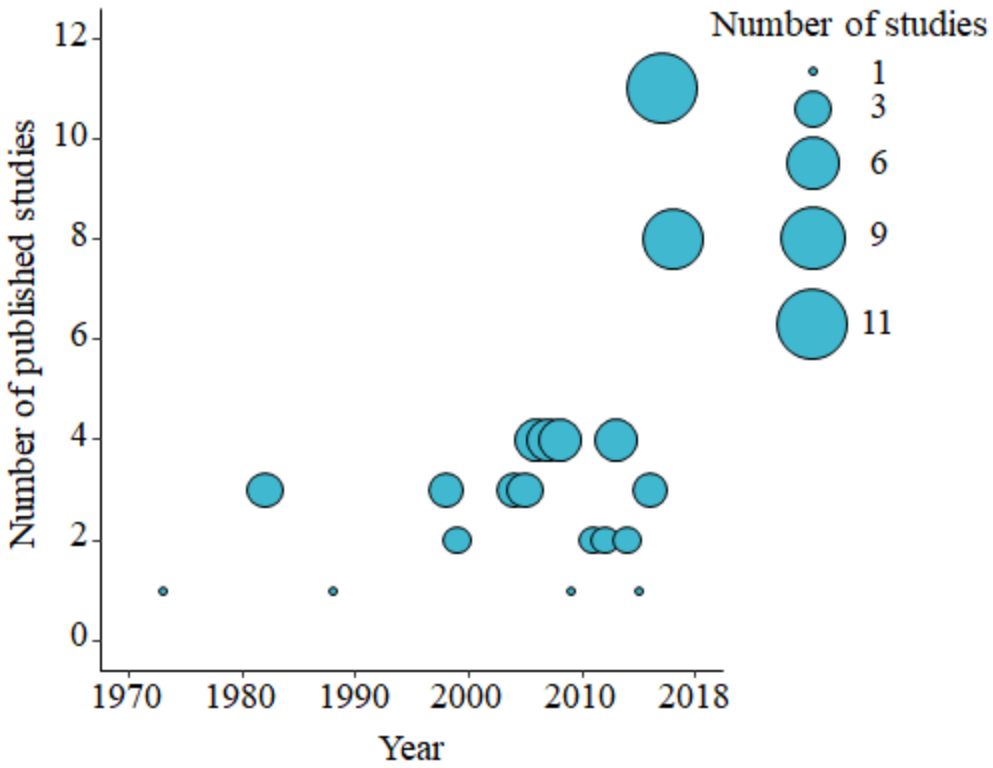

Figure 3.2. Bubble plot showing the number of published studies of age-dependent antimicrobial resistance in food animals by decade from the 1970s to 2010s (2000-November 2018). The bubble size is proportional to the number of studies in that decade. A total of 62 studies were identified and included in the analysis.

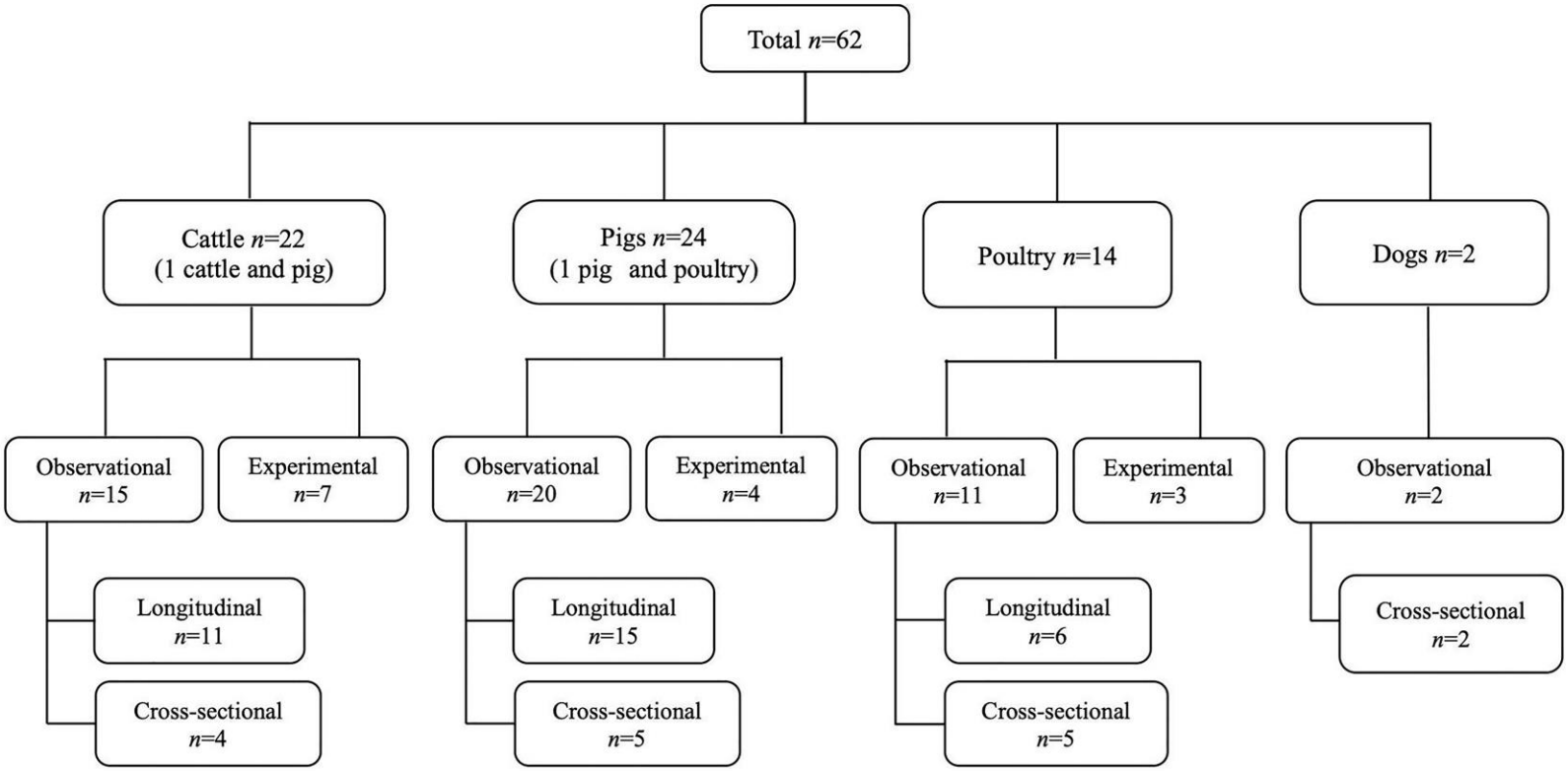

Figure 3.3. Distribution of the study design and animal species in the studies from which the data on the age-dependency of antimicrobial resistance of fecal bacteria in animals were extracted in this review. *n*, number of studies.

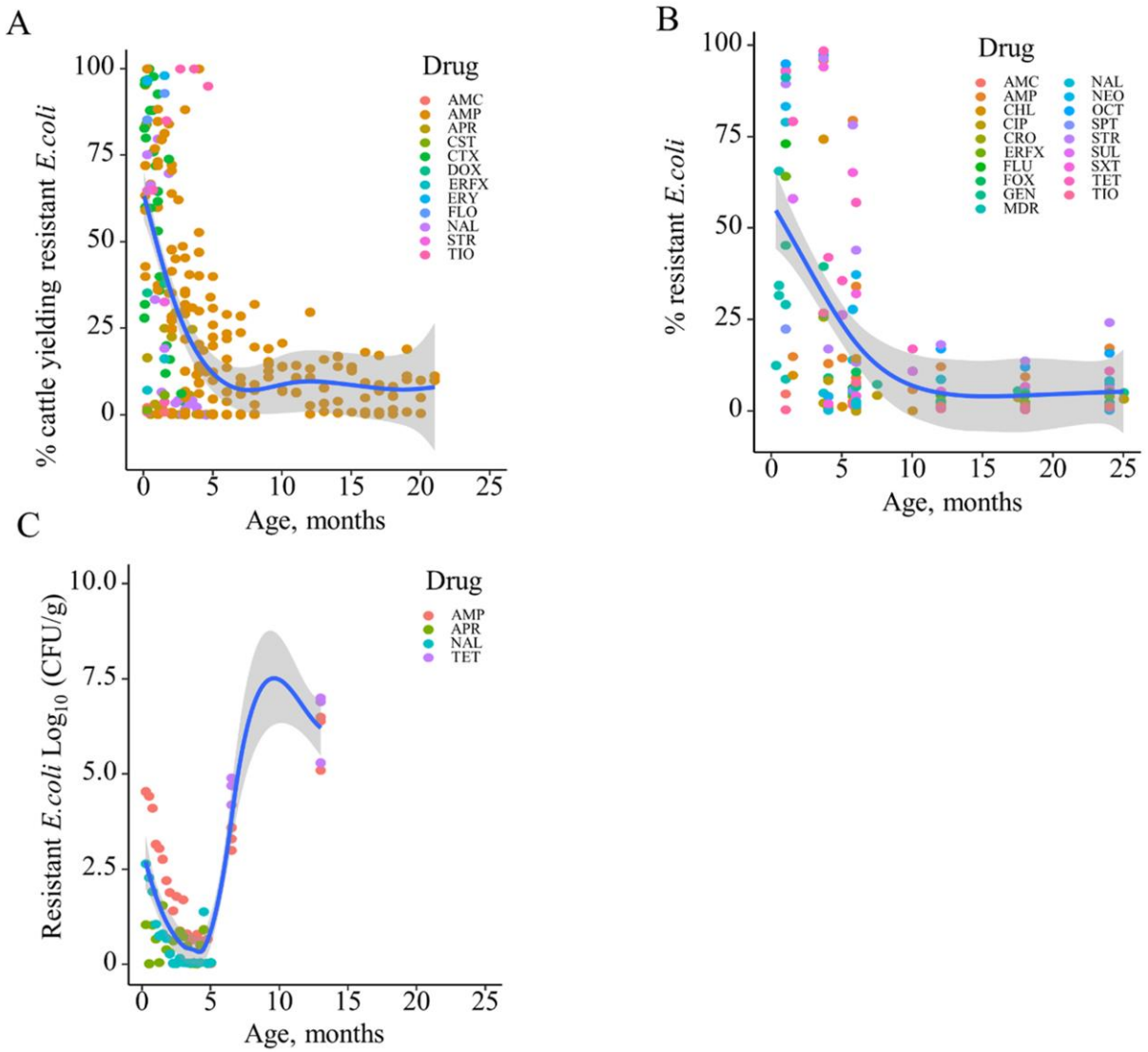

Figure 3.4. Age-dependent antimicrobial resistance dynamics in cattle. (A) Percentage of cattle yielding antimicrobial-resistant fecal *E. coli* by age (data from *n*=10 studies). (B) Percentage of antimicrobial-resistant fecal *E. coli* by age (data from *n*=7 studies). (C)

Abundance of antimicrobial-resistant fecal *E. coli* (CFU/g) by age (data from *n*=2 studies). Plotted individual observations represent antimicrobial resistance to individual drugs (AMC, amoxicillin-clavulanic acid; AMP, ampicillin; APR, apramycin; CHL, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; CST, colistin; CTX, cefotaxime; DOX, doxycycline; ERFX, enrofloxacin; ERY, erythromycin; FLO, florfenicol; FLU, flumequine; FOX, cefoxitin; GEN, gentamicin; NAL, nalidixic acid; NEO, neomycin; OCT, oxytetracycline; SPT, spectinomycin, STR, streptomycin; SUL, sulfamethoxazole; SXT, trimethoprim-sulfonamides; TET, tetracycline; TIO, ceftiofur); MDR— multidrug resistance (to ≥3 drug classes). The blue trend lines with the confidence bands (the gray area around the blue line) are shown in A-C; these in each case were estimated using locally weighted scatterplot smoothing (LOESS).

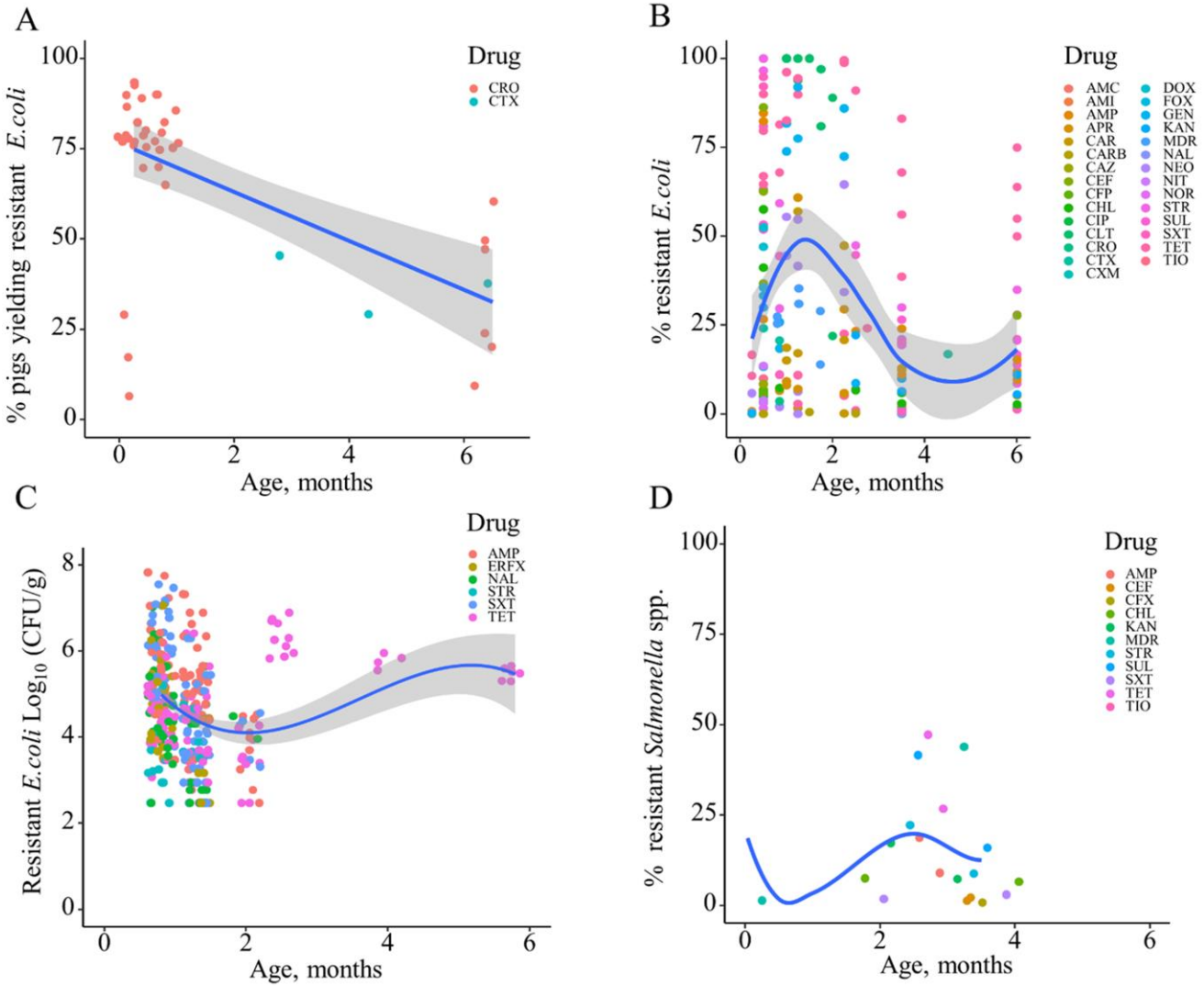

Figure 3.5. Age-dependent antimicrobial resistance dynamics in production pigs. (A) Percentage of pigs yielding antimicrobial-resistant fecal *E. coli* by age (data from *n*=2 studies). (B) Percentage of antimicrobial-resistant fecal *E. coli* by age (data from *n*=13 studies). (C) Abundance of antimicrobial-resistant fecal *E. coli* (CFU/g) (data from *n*=2 studies). Plotted individual observations represent

antimicrobial resistance to individual drugs (AMC, amoxicillin-clavulanic acid; AMI, amikacin; AMP, ampicillin; APR, apramycin; CAR, carbadox; CARB, carbapenem; CAZ, ceftazidime; CEF, cephalothin; CFP, cefoperazone; CHL, chloramphenicol; CIP, ciprofloxacin; CLT, chlortetracycline; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; DOX, doxycycline; ERFX, enrofloxacin; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NEO, neomycin; NIT, nitrofurantoin; NOR, norfloxacin; STR, streptomycin; SUL, sulfamethoxazole; SXT, trimethoprim-sulfonamides; TET, tetracycline; TIO, ceftiofur). MDR—multidrug resistance (to ≥3 drug classes). The blue trend lines with the confidence bands (the gray area around the blue line) are shown in A-C; these in each case were estimated using locally weighted scatterplot smoothing (LOESS).

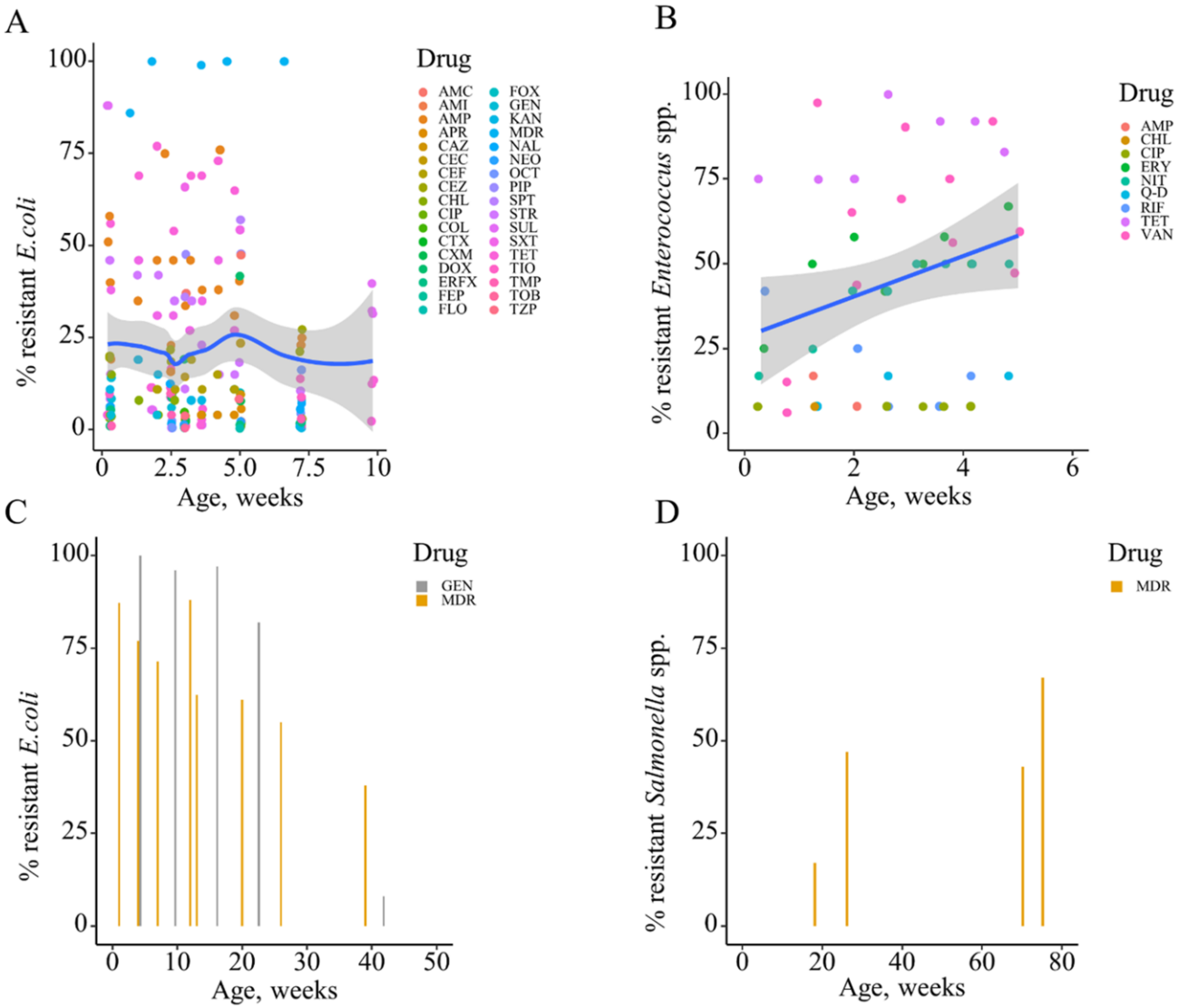

Figure 3.6. Age-dependent antimicrobial resistance dynamics in poultry (broiler, layer, and turkey). (A) Broiler chicken, percentage of antimicrobial-resistant fecal *E. coli* (data from *n*=6 studies). (B) Broiler chicken, percentage of antimicrobial-resistant fecal Enterococcus

spp. (data from *n*=3 study). (C) Turkey and layer, percentage of antimicrobial-resistant fecal *E. coli* (data from *n*=2 study). (D) Turkey and layer, percentage of antimicrobial-resistant fecal *Salmonella* spp. (data from *n*=2 study). Plotted individual observations represent antimicrobial resistance to individual drugs (AMC, amoxicillin-clavulanic acid; AMI, amikacin, AMP, ampicillin; APR, apramycin; CAZ, ceftazidime; CEC, cefaclor, CEF, cephalothin; CEZ, cefazolin; CHL, chloramphenicol; CIP, ciprofloxacin; COL, colistin; CTX, cefotaxime; CXM, cefuroxime; DOX, doxycycline; ERFX, enrofloxacin; FEP, cefepime; FLO, florfenicol; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; NEO, neomycin; OCT, oxytetracycline; PIP, piperacillin; Q-D, quinupristin–dalfopristin; RIF, Rifampicin; SPT, spectinomycin; STR, streptomycin; SUL, sulfamethoxazole; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TIO, ceftiofur; TMP, trimethoprim; TOB, tobramycin; TZP, piperacillin-tazobactam); MDR— multidrug resistance (to ≥3 drug classes). The blue trend lines with the confidence bands (the gray area around the blue line) are shown in A-B; these in each case were estimated using locally weighted scatterplot smoothing (LOESS).

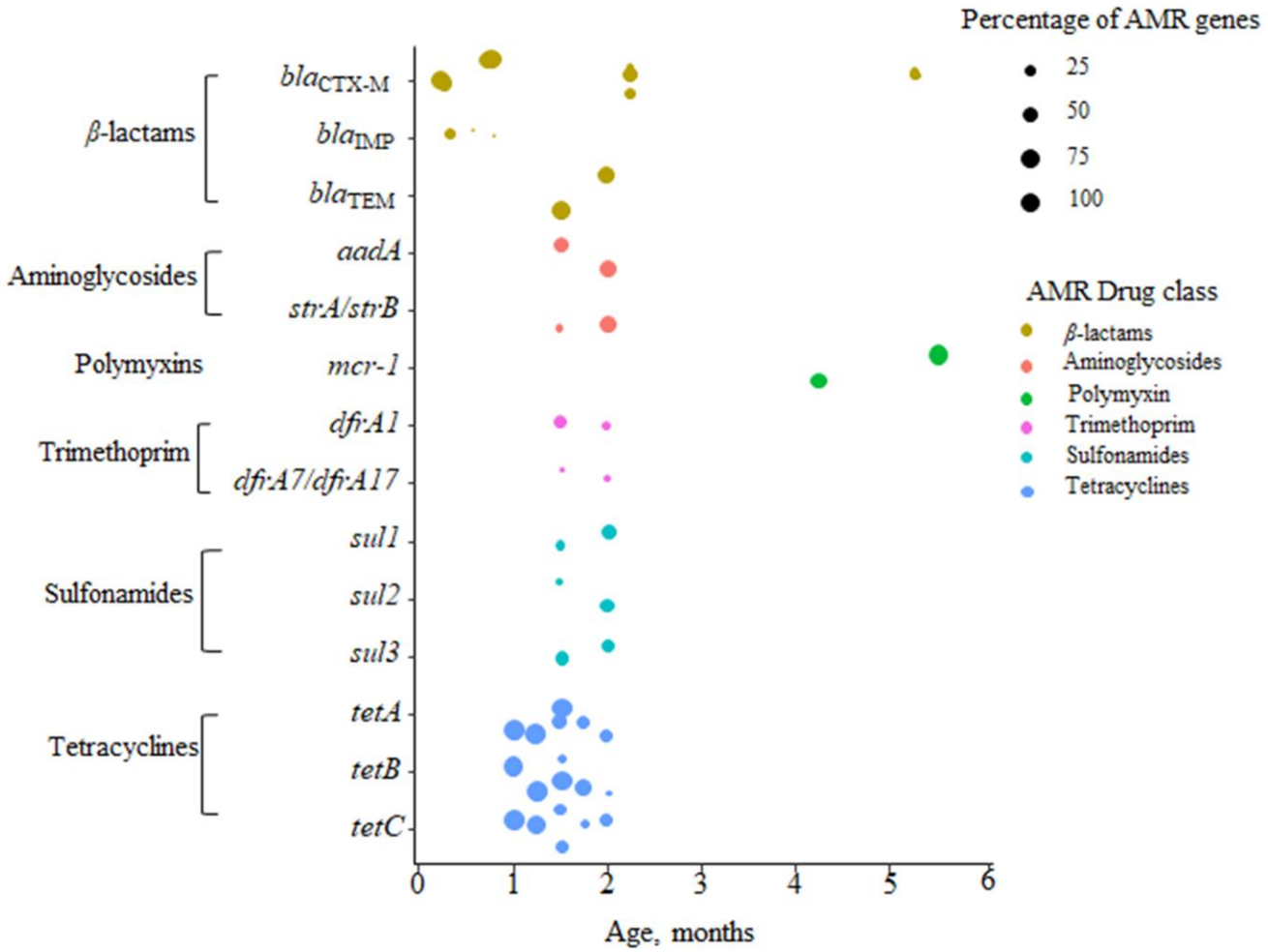

Figure 3.7. The average percentages of antimicrobial resistance genes of individual classes in the resistome in fecal samples of production pigs by age (data from *n*=5 studies; the average in each study is plotted).

Supplementary materials

Table S 3.3

Characteristics of the individual studies included in the review (*n*=62).

Species (number of studies)	Author name (last), year	Country	Studied animal age	Study design	Study type	Bacteria tested	Sample	AMR analysis
Cattle (*n*=22)	Mir et al., 2018	USA	Birth-12 months	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic
	Pereira et al., 2018	USA	Birth-6 weeks	Trial	Experimental	Microbiota	Fecal, individual	Microbiome taxa
	Maynou et al., 2017	Spain	6 weeks and 1 year	Trial	Experimental	*E. coli*	Fecal, individual	Phenotypic
	Adler et al., 2017	Israel	≤ 4 months, 5-10 months, 11-24 months, ≥ 25 months	Cross-sectional	Observational	*E. coli*	Fecal, individual	Phenotypic
	Ohta et al., 2017	USA	Sampling days (0, 4, 8, 14, 20, 26)	Trial	Experimental	*Salmonella* spp.	Fecal, individual	Phenotypic
	Hutchinson et al., 2017	USA	110 days-6 months	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic
	Catry et al., 2016	Belgium	Calves (4 and 23 weeks), beef cattle (6-24 months), dairy cattle	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic
	Pereira et al., 2015	USA	3-5 months and 14-19 months	Cross-sectional	Observational	*E. coli*, *Salmonella* spp.	Fecal	Phenotypic
	Brunton et al., 2014	UK	Birth-13 weeks	Trial	Experimental	*E. coli*	Fecal, individual	Phenotypic, genotypic
	Mazurek et al., 2013 *	Poland	Cattle (beef and dairy); Pig (6 to 8 weeks)	Cross-Sectional	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic

	Watson et al., 2012	UK	Day 1-day 161	Longitudinal	Observational	*E. coli*	Fresh floor fecal samples	Phenotypic, genotypic
	Alexander et al., 2011	Canada	Beef cattle sampling days (1-175 days)	Trial	Experimental	AMR genes	Fecal, individual	Genotypic
	Sharma et al., 2008	Canada	6.5 months-11 months	Trial	Experimental	*E. coli*	Fecal, individual	Phenotypic, genotypic
	Berge et al., 2006	USA	4 weeks	Trial	Experimental	*E. coli*	Rectal fecal samples, individual	Phenotypic
	Liebana et al., 2006	UK	~ 1 months	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic
	Hoyle et al., 2006	UK	Calves (1-8 weeks); Cattle (≤30 months and ≥ 30 months)	Longitudinal	Observational	*E. coli*	Fecal, individual, and environmental	Phenotypic, genotypic
	Donaldson et al., 2006	USA	2.5-19 weeks	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic
	Berge et al., 2005	USA	Birth-6 weeks	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic
	Hoyle et al., 2005	UK	1-21 weeks	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic
	Hoyle et al., 2004	UK	Birth-21 weeks	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic
	Hoyle et al., 2004	UK	1-8 months	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic
	Khachatryan et al., 2004	USA	Pre-weaned calves	Cross-sectional	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic
Pig (*n*=24)	Ciesinsk et al., 2018	Germany	24 days (one day before weaning), 38 days (two weeks after weaning), 52 days (four weeks after weaning)	Trial	Experimental	*E. coli*	Fecal, individual	Phenotypic

Randall et al., 2018	UK	8 weeks, 17 weeks, 24 weeks, 20 months	Longitudinal	Observational	*E. coli and mcr-1 gene*	Fecal, individual	Phenotypic, genotypic
Mollenkopf et al., 2018	USA	Piglets to Finisher	Longitudinal	Observational	*Enterobacteriaceae/bla*$_{IMP-6}$	Fecal, Individual	Genotypic
Cameron-Veas et al., 2018	Spain	Day 7, 9, 14 and 187	Longitudinal	Observational	*Salmonella* spp.	Fecal, individual	Phenotypic, genotypic
Lynch et al., 2018	Ireland	Piglet	Longitudinal	Observational	*Salmonella* spp.	Fecal, individual	Phenotypic
Græsbøll et al., 2017	Denmark	Nursery (4 to 7 weeks)	Randomized trial	Experimental	*E. coli*	Fecal, individual	Phenotypic
Dohmen et al., 2017	The Netherlands	Birth, 6, 12, 18 months	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic
Pruthvishree et al., 2017	India	1 month, ~2-month, 2-3 month	Cross-sectional	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic
Fernandes et al., 2016	Portugal	Piglet, weaner, finisher, sows	Longitudinal	Observational	*Salmonella* spp.	Fecal swab, individual	Phenotypic, genotypic
Cameron-Veas et al., 2016	Spain	Piglets (7-6 days) and finisher	Trial	Experimental	*E. coli*	Fecal swab, individual	Phenotypic
von Salviati et al., 2014	Germany	Fattening pig	Longitudinal	Observational	*E. coli*	Fecal, individual and pooled	Phenotypic, genotypic
Hansen et al., 2013	Denmark	Piglet, nursery, weaner, finisher, sows	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic
Quintana-Hayashi et al., 2012	USA	Nursery, finisher, farrowing	Longitudinal	Observational	*Campylobacter* spp.	Fecal, individual	Phenotypic
Rosengren et al., 2008	Canada	Nursery, grower-finisher, and sows	Cross-sectional	Observational	*Salmonella* spp.	Fecal,	Phenotypic
Alali et al., 2008	USA	Farrow-finish	Longitudinal	Observational	*E. coli*	Composite fresh samples, pooled	Phenotypic
Kobashi, et al., 2008	Japan	4-6 weeks	Trial	Experimental	*E. coli*	Fecal, individual	Phenotypic, genotypic

	Dewulf et al., 2007	Belgium	Nursery, grower, finisher	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic
	Scott et al., 2005	USA	Farrow-finish	Longitudinal	Observational	*E. coli*	Composite fecal samples	Phenotypic
	Butaye et al., 1999	Belgium	Pig (piglet, grower/finisher, and sows); poultry (9 and 32 days)	Cross-sectional	Observational	*Entero-coccus* spp.	Fecal, individual	Phenotypic, genotypic
	Mathew et al., 1999	USA	Production pigs (7-63 days) and sows	Longitudinal	Observational	*E. coli*	Fecal swab, individual	Phenotypic
	Moro et al., 1998a	USA	Grower, finisher, gilt, and sows	Cross-sectional	Observational	*E. coli*	Fecal, individual	Phenotypic
	Mathew et al., 1998b	USA	Piglets (7-63 days) and sows	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic
	Langlois et al., 1988	USA	Weaner, grower, finisher, adults (11-24 months) and sows	Longitudinal	Observational	*E. coli*	Rectal swab, individual	Phenotypic
	Sogaard, 1973	Denmark	Piglet, finisher, sows	Cross-sectional	Observational	*E. coli*	Rectal swab, individual	Phenotypic
Poultry (broiler, layer or turkey, *n*=14)	Baron et al., 2018	France	2, 7 and 77 days	Longitudinal	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic
	Hume and	USA	Day 1-6 weeks (weekly samples)	Trial	Experimental	*Entero-coccus* spp.	Fecal, Cecum	Phenotypic
	Trung et al., 2017	Vietnam	20 weeks, > 20 weeks	Cross-sectional	Observational	*E. coli*	Fecal, individual	Phenotypic, genotypic
	Laube et al., 2013	Germany	1-35 days	Longitudinal	Observational	*E. coli*	Cloacal swab, Individual	Phenotypic, genotypic
	Schwaiger et al., 2013	Germany	21 days and 35 days	Cross-sectional	Observational	*E. coli*	Fecal, floor	Phenotypic, genotypic

	Ozaki et al., 2011	Japan	2 days-50 days	Longitudinal	Observational	*E. coli*	Fresh Dropping	Phenotypic
	da Costa et al., 2009	Portugal	2-33 days	Trial	Experimental	*E. coli and Enterococcus* spp.	Cloacal swab, individual	Phenotypic
	Garcia-Migura et al., 2007	UK	4 days-35 days	Longitudinal	Observational	*Enterococcus* spp.	Fecal, pooled	Phenotypic, genotypic
	Santos et al., 2007	USA	3 weeks and 9 weeks	Cross-sectional	Observational	*Salmonella* spp.	Fecal and litter sample	Phenotypic
	Li et al., 2007	USA	18-72 weeks	Cross-sectional	Observational	*Salmonella* spp.	Fecal, pooled	Phenotypic
	Welton et al., 1998b	USA	24 days-130 days	Longitudinal	Observational	*Enterococci* spp.	Cloacal, individual	Phenotypic
	Dubel et al., 1982	USA	1-28 weeks	Cross-sectional	Observational	*E. coli*	Cloacal, individual	Phenotypic
	Nakamura et al., 1982	Japan	5-360 days	Longitudinal	Observational	*E. coli*	Cloacal swab, individual	Phenotypic
	Hinton et al., 1982	UK	1-100 days	Trial	Experimental	*E. coli*	Cloacal swabs, individual	Phenotypic
Dog (*n*=2)	Bang et al., 2017	South Korea	3-6 weeks, 9-28 weeks, 2-6 years and ≥9 years	Cross-sectional	Observational	*Enterococcus* spp.	Fecal, individual	Phenotypic
	Siugzdaite et al., 2017	Lithuania	≤1 year, 1-5 years, 6-10 years, ≥10 years	Cross-sectional	Observational	*Staphylococci* spp.	Fecal, individual	Phenotypic, genotypic

Table S 3.4

Quality assessment of the individual studies included in the review (*n*=62).

Species (number of studies)	Author name (last), year	Country	1. Were the study objectives clearly stated?	2. Were the sampling methods clearly described?	3. Was the sample size calculated?	4. If the study was observational, were inclusion/exclusion criteria specified for subject selection?	5. If the study was experimental, were the groups (treatment and controls) specified?	5.1 Were sampling units randomly assigned to the treatment groups?	6. Were all procedures used in the study specified?	7. Is any bias present in the data collection?	8. Were potential biases and or confounders identified and adjusted or explained (outcome and analysis sections)?
Cattle (*n*=22)	Mir et al., 2018	USA	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Yes
	Pereira et al., 2018	USA	Yes	Yes	No	N/A	Yes	Yes	Yes	No	Yes
	Maynou et al., 2017	Spain	Yes	Yes	No	N/A	Yes	Unclear	Yes	No	Yes
	Adler et al., 2017	Israel	Yes	Yes	No	No	N/A	N/A	Yes	No	Yes
	Ohta et al., 2017	USA	Yes	Yes	No	N/A	Yes	Yes	Yes	No	Yes
	Hutchinson et al., 2017	USA	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Yes
	Catry et al., 2016	Belgium	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Partial
	Pereira et al., 2015	USA	Yes	Yes	Yes	Yes	N/A	N/A	Yes	No	Yes
	Brunton et al., 2014	UK	Yes	Yes	Yes	N/A	Yes	Unclear	Yes	No	Yes
	Mazurek et al., 2013*	Poland	Yes	Yes	No	Partial	N/A	N/A	Yes	Unclear	Partial
	Watson et al., 2012	UK	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Yes
	Alexander et al., 2011	Canada	Yes	Yes	No	N/A	Yes	Unclear	Yes	No	Partial
	Sharma et al., 2008	Canada	Yes	Yes	No	N/A	Yes	Yes	Yes	No	Partial
	Berge et al., 2006	USA	Yes	Yes	No	N/A	Yes	Unclear	Yes	No	Yes
	Liebana et al., 2006	UK	Yes	Yes	Yes	Partial	N/A	N/A	Yes	No	Partial
	Hoyle et al., 2006	UK	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Partial

	Donaldson et al., 2006	USA	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Yes
	Berge et al., 2005	USA	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Yes
	Hoyle et al., 2005	UK	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Yes
	Hoyle et al., 2004a	UK	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Yes
	Hoyle et al., 2004b	UK	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Yes
	Khachatryan et al., 2004	USA	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Partial
Pig (*n*=24)	Ciesinsk et al., 2018	Germany	Yes	Yes	No	N/A	Yes	Yes	Yes	No	Yes
	Randall et al., 2018	UK	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Yes
	Mollenkopf et al., 2018	USA	Yes	Yes	No	No	N/A	N/A	Yes	Unclear	Not Reported
	Cameron-Veas et al., 2018	Spain	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Yes
	Lynch et al., 2018	Ireland	Yes	Yes	No	No	N/A	N/A	Yes	No	Yes
	Græsbøll et al., 2017	Denmark	Yes	Yes	No	N/A	Yes	Yes	Yes	No	Yes
	Dohmen et al., 2017	The Netherlands	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Yes
	Pruthvishree et al., 2017	India	Yes	Yes	Yes	No	N/A	N/A	Yes	No	Yes
	Fernandes et al., 2016	Portugal	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Partial
	Cameron-Veas et al., 2016	Spain	Yes	Yes	No	N/A	Yes	Yes	Yes	No	Yes
	von Salviati et al., 2014	Germany	Yes	Unclear	No	Partial	N/A	N/A	Yes	Unclear	Partial
	Hansen et al., 2013	Denmark	Yes	Yes	No	No	N/A	N/A	Yes	No	Yes
	Quintana-Hayashi et al., 2012	USA	Yes	Yes	Yes	Yes	N/A	N/A	Yes	No	Yes
	Rosengren et al., 2008	Canada	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Yes
	Alali et al., 2008	USA	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Yes
	Kobashi, et al., 2008	Japan	Yes	Yes	No	N/A	Yes	Unclear	Yes	No	Yes

	Dewulf et al., 2007	Belgium	Yes	Yes	Yes	Yes	N/A	N/A	Yes	No	Yes
	Scott et al., 2005	USA	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Yes
	Butaye et al., 1999ǂ	Belgium	Yes	Yes	No	Partial	N/A	N/A	Yes	Unclear	Partial
	Mathew et al., 1999	USA	Yes	Yes	No	Partial	N/A	N/A	Yes	Unclear	Partial
	Moro et al., 1998a	USA	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Not Reported
	Mathew et al., 1998	USA	Yes	Yes	No	Partial	N/A	N/A	Yes	Unclear	Yes
	Langlois et al., 1988	USA	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Partial
	Sogaard, 1973	Denmark	Unclear	Unclear	No	No	N/A	N/A	Unclear	Unclear	Not Reported
Poultry (broiler, layer or turkey, *n*=14)	Baron et al., 2018	France	Yes	Yes	No	No	N/A	N/A	Yes	Unclear	Not Reported
	Hume and Dunskey, 2017	USA	Yes	Yes	No	N/A	Yes	Unclear	Yes	No	Partial
	Trung et al., 2017	Vietnam	Yes	Yes	No	Yes	N/A	N/A	Yes	No	Yes
	Laube et al., 2013	Germany	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Partial
	Schwaiger et al., 2013	Germany	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Partial
	Ozaki et al., 2011	Japan	Yes	Yes	No	No	N/A	N/A	Yes	No	Yes
	da Costa et al., 2009	Portugal	Yes	Yes	No	N/A	Yes	Unclear	Yes	No	Yes
	Garcia-Migura et al., 2007	UK	Yes	Yes	No	No	N/A	N/A	Yes	No	Not Reported
	Santos et al., 2007	USA	Yes	Yes	No	No	N/A	N/A	Yes	Unclear	Partial
	Li et al., 2007	USA	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Partial
	Welton et al., 1998	USA	Yes	Yes	No	Partial	N/A	N/A	Yes	No	Partial
	Dubel et al., 1982	USA	Yes	Yes	No	No	N/A	N/A	Yes	Unclear	Not Reported
	Nakamura et al., 1982	Japan	Unclear	Unclear	No	No	N/A	N/A	Yes	Unclear	Not Reported
	Hinton et al., 1982	UK	Yes	Yes	No	N/A	Unclear	Unclear	Yes	No	Not Reported
Dog (*n*=2)	Bang et al., 2017	Korea	Yes	Yes	No	No	N/A	N/A	Yes	No	Yes

Siugzdaite et al., 2017	Lithuania	Yes	Yes	No	No	N/A	N/A	Yes	Unclear	Yes

Yes- quality criteria met; No- quality criteria not met; Partial- not entirely mentioned; Unclear- insufficient information to evaluate quality criteria; N/A- not applicable, *n*-number of studies

Table S 3.5

List of studies that were retained (*n*=62) for analysis in the scoping review.

Species (number of studies)	References	Comments
Cattle (*n*=22)	(Hoyle et al., 2004a; Khachatryan et al., 2004; Hoyle et al., 2004a; Berge et al., 2005; Hoyle et al., 2005; Berge et al., 2006; Donaldson et al., 2006; Hoyle et al., 2006; Liebana et al., 2006; Sharma et al., 2008; Alexander et al., 2011; Watson et al., 2012; Mazurek et al., 2013*; Brunton et al., 2014; Pereira et al., 2015; Catry et al., 2016; Adler et al., 2017; Hutchinson et al., 2017; Maynou et al., 2017; Ohta et al., 2017; Mir et al., 2018; Pereira et al., 2018)	*cattle and pig
Pig (*n*=24)	(Sogaard, 1973; Langlois et al., 1988; Mathew et al., 1998; Moro et al., 1998; Butaye et al., 1999*; Mathew et al., 1999; Scott et al., 2005; Dewulf et al., 2007; Alali et al., 2008; Kobashi et al., 2008; Rosengren et al., 2008a; Quintana-Hayashi and Thakur, 2012; Hansen et al., 2013; von Salviati et al., 2014; Cameron-Veas et al., 2016; Fernandes et al., 2016; Dohmen et al., 2017; Graesboll et al., 2017; Pruthvishree et al., 2017; Cameron-Veas et al., 2018; Ciesinski et al., 2018; Lynch et al., 2018; Mollenkopf et al., 2018; Randall et al., 2018)	*pig and poultry
Poultry (*n*=14)	(Dubel et al., 1982; Hinton et al., 1982; Nakamura et al., 1982; Welton et al., 1998b; Garcia-Migura et al., 2007; Li et al., 2007; Santos et al., 2007; da Costa et al., 2009; Ozaki et al., 2011; Laube et al., 2013; Schwaiger et al., 2013; Hume and Donskey, 2017; Trung et al., 2017; Baron et al., 2018)	
Dog (*n*=2)	(Bang et al., 2017; Siugzdaite and Gabinaitiene, 2017)	

Chapter 4 - A Longitudinal Study of the Gut Microbiome, Mycobiome, and Antimicrobial Resistance in Cohorts of Production Pigs and Breeding Sows

Abstract

The objectives were to compare the fecal microbiome and mycobiome taxonomic compositions and antimicrobial resistance (AMR) dynamics in fecal bacteria between cohorts of production pigs and breeding sows. The study included a cohort of production pigs (from 2 days to 6 months old and balanced by gender, *n*=12) and two cohorts of breeding sows (from 3 weeks old to first farrowing/weaning at 1-year-old, *n*=6, and *n*=12 cohorts). The cohorts were fed corn-soybean based diets (the diets varied in composition) and raised in several physical environments (barns) throughout their life-span. Fecal samples were collected *per rectum* from each animal at nine age-points. Taxonomic structures of the fecal microbiome and mycobiome were evaluated using 16S rRNA gene and internal transcribed spacer (ITS) sequencing, respectively (*n*=8 production pigs and *n*=8 breeding sows). The phenotypic AMR at each age-point was evaluated based on the growth of fecal bacteria (coliforms as indicator Gram-negative and enterococci as indicator Gram-positive bacteria) on selective agar media supplemented with antimicrobial drugs. In a sub-study (*n*=2 production pigs) the fecal resistome was also assessed using a shotgun metagenomic approach. Our data showed the age dynamics of the microbiome and mycobiome compositions and AMR were comparable between the cohorts of production pigs and breeding sows. The fecal microbiome composition dynamics and changes in the evaluated descriptors of antimicrobial susceptibility of fecal bacteria from early-life to young-adult appeared similar

between the cohorts. Thus, in pigs, age could be one of the major factors affecting the microbial diversity and composition and along the phenotypic and genotypic AMR of fecal bacteria in this monogastric host.

Introduction

Antimicrobial resistance presents one of the major global threats to public health. Exposure to antimicrobials increases the level of antimicrobial resistance (AMR) in the gut commensal flora of both humans and animals. Several studies have reported the age-related colonization and diversification of the microbiome in pigs, starting from early life (Inoue et al., 2005; Kim et al., 2012; Looft et al., 2012b; Mann et al., 2014; Mach et al., 2015). Similarly, age appears to be a dominant factor affecting gut colonization with AMR bacteria, with higher host-level prevalence and within-host abundance of AMR enteric/fecal bacteria at younger ages in cattle and pigs (Hoyle et al., 2004a; Hoyle et al., 2004b) and human (Robey et al., 2017a). In the early 1960s, (Smith, 1961) demonstrated that human fecal bacterial composition varies enormously with age, with the bacterial flora changing from *E. coli*, *Clostridium* spp., and *Streptococci* spp. to *Lactobacilli* and *Bacteroides*. Another study revealed an age related AMR gene abundance, number or types, and diversity in humans (Lu et al., 2014b).

Longitudinal studies of indicator fecal bacteria, such as *E. coli* and *Enterococcus* spp. in animals, have expanded our understanding of the temporal dynamics of animal-level prevalence and within-animal abundance of AMR in fecal bacteria (Berge et al., 2006; Adator et al., 2020). However, such studies may not fully represent the AMR dynamics in complex natural systems of microbial communities typically carried by the host populations. Several studies have shown the presence of AMR genes in the commensal gut microbiome in humans (Sommer et al., 2009a; Hu et al., 2013; Ravi et al., 2015) as well in pigs (Xiao et al., 2016b). However, the role of the microbiome structure in the evolution, persistence, and dissemination of AMR is poorly understood, and many key-related ecological questions remain unanswered. The recent advancement of metagenomics enables the detection of the majority of AMR genes present and

comparing the microbiome and AMR genes in different samples (Ma et al., 2016; Thomas et al., 2017). For instance, metagenomic approaches have been used to evaluate the entire microbiome and their associated AMR genes (i.e., resistome) in different livestock production environments (Agga et al., 2015a; Noyes et al., 2016b; Yang et al., 2016b; Rovira et al., 2019).

While several earlier studies have focused on the bacterial component of the fecal microbial community, recent studies show the fungal component—mycobiome—is also complex and multifaceted, and influences host health (Mukherjee et al., 2015). Previous studies showed dramatic shifts between birth to day 35 of age in both fecal bacterial and fungal communities in piglets (Summers et al., 2019; Arfken et al., 2020); although the bacterial community diversity is more predictable while the mycobiome composition demonstrated a significant discrease in diversity over time. Fungal taxonomic composition and diversity and their interaction with commensal bacterial communities in the gut of pigs are mostly unknown.

The culture-dependent and culture-independent methods are used for profiling microbial species and AMR in fecal samples. The culture-dependent methods have an advantage of isolating single microbial species and their individual strains for detailed analyses, but the data do not truly represent the entire target microbial community and AMR gene diversity in the source population. Whereas, culture-independent methods, such as shotgun metagenomics, in which the microbial community DNA from a fecal sample is extracted and sequenced, help to understand the full microbial community structure, and evaluate AMR gene diversity. However, the results derived with such techniques depend on the depth of sequencing and then available bioinformatic pipelines for processing of sequencing data. The limitations include not being able to detect bacterial genes, including AMR genes, carried by low abundant species in the microbial community (Zaheer et al., 2018). In our approach, metagenomics was coupled with culture-based

methods to evaluate the presence and composition of AMR in the fecal bacterial populations at an ecological level and provide a broader picture.

While extensive cross-sectional studies have been conducted to understand AMR gene abundance and diversity in different food animals (Pollock et al., 2020), these studies do not provide details of the dynamics of AMR gene diversity and composition in gut microbial communities in the different age groups of food animals. We hypothesized that fecal microbiome and mycobiome taxonomic compositions and AMR dynamics in a monogastric host are a function of the host age. The age is a stronger driver of the dynamics than the production environment, in the case of a farm-animal host. Therefore, the objectives of this study were to elucidate and compare the dynamics of fecal microbiome and mycobiome taxonomic compositions and phenotypic AMR in cohorts of production pigs and breeding sows.

Materials and methods

Study design and animals

A production pig cohort (2 days to 6 months old, *n*=12 balanced by gender), born and raised in the Swine Teaching and Research Center of Kansas State University, was followed throughout all the production stages. The pigs were fed a corn grain-based diet designed for each growth stage. The pigs were allowed to nurse from birth to 21 days of age and then had access to feed and water as *ad libitum*. A total of 108 fecal samples were collected longitudinally from 12 pigs. From each animal, a fecal sample was taken at each of the following age-points: day 2 (48 hours after birth in the farrowing barn), day 22 (weaning), and after moving to a nursery barn at 23 days, on day 26 (3 days after moving to the nursery barn and starting a nursery diet), day 40 (a day before the next diet change in the nursey barn), day 54 (14 days after the diet change), day 77 (a day before moving to a finisher barn), day 93 (14 days after moving to the finisher barn and

introduction of a finisher diet; diet was changed one more time at 114 days), day 128 (2 weeks after the diet change), and day 179 (day of harvest) (Figure 4.1).

Similarly, two cohorts of breeder sows (21 days to 371 days old, first cohort *n*=6; second cohort *n*=12) were followed from arrival in the Swine Teaching and Research Center of Kansas State University through the first farrowing and weaning. The pigs were fed mainly corn grain-based diet designed for each growth stage. A total of 162 fecal samples were collected longitudinally from 18 pigs. From each animal, a fecal sample was taken at each of the following age-points: day 21 (at the time of purchase/entering the first growing barn), day 42 days (21 days after entry into the first growing barn), day 70 (a day before moving to a second growing barn), day 84 (14 days after moving to the second growing barn), day 154 (a day prior to moving to the breeding barn), day 224 (at first breeding/artificial insemination – parity 1), day 343 (7 days prior to moving to the farrowing barn), day 350 (on the day of parturition) and day 371 (on the day weaning) (Figure 4.1). Fecal samples were kept on ice after each sampling, and whole feces and fecal aliquots mixed with 50% glycerol stored at -80°C until DNA extraction and quantification of total and antimicrobial-resistant coliforms and enterococci were performed.

Fecal DNA extraction

Fecal DNA was extracted using the protocol published by (Yu and Morrison, 2004) and (Korte et al., 2020). Briefly, fecal samples were transferred into round-bottom tubes (2 mL) containing 800 µL of lysis buffer and a single steel bead (0.5 cm diameter). Samples were then heated at 70°C for 20 minutes with vortexing, then homogenized using a TissueLyser II (Qiagen, Venlo, the Netherlands) for 3 minutes at 30 per second, and then centrifuged at 5000 × g for 5 minutes at room temperature. The supernatant then transferred into the new Eppendorf tube (1.5 mL), added with 200 µL of 10 mM ammonium acetate, incubated for 5 minutes on ice, and then

centrifuged at 5000 × g for 5 minutes. The supernatant (up to 750 µL) was mixed with an equal volume of isopropanol, incubated for 30 minutes on ice, and centrifuged at 16,000 × g at 4°C for 15 minutes. The recovered DNA pellet was washed and resuspended in 150 µL of Tris-EDTA. After the addition of proteinase-K (15 µL) and Buffer AL (200 µL) (DNeasy Blood and Tissue kit, Qiagen, Germany), samples were incubated at 70°C for 10 minutes. In each tube, 200 µL of 100% ethanol added, vortexed, transferred to a spin column and processed according to the manufacturer's instructions (Qiagen, Germany). The DNA concentration were measured via fluorometry (Qubit 2.0, Life Technologies, Carlsbad, CA) using Quant-iT broad range (or high sensitivity) dsDNA reagent kits (Invitrogen, Carlsbad, CA).

16S rRNA library preparation and sequencing

Extracted pig feces DNA were processed at the University of Missouri Metagenomics Center. The 16s rDNA amplicons (V4 region of the 16S rRNA gene) were created with universal primers (U515F/806R) (Caporaso et al., 2011; Walters et al., 2011) against the V4 region (flanked by Illumina standard adapter sequences (Illumina Inc CA, USA). Oligonucleotide sequences are available at proBase (database of rRNA-targeted oligonucleotide probes and primers) (Loy et al., 2007). Dual-indexed forward and reverse primers were used in all reactions. Metagenomic DNA (100 ng) was used, and PCR was performed in 50 µL reactions with primers, dNTPs and DNA polymerase. PCR plate was transferred to the thermocycler for amplification (98°C (3 minutes) + [98°C (15 seconds) + 50°C (30 seconds) + 72°C (30 seconds)]× 25 cycles+ 72°C (7 minutes). After amplification completion, amplicon pools (5 µL/reaction) were combined, mixed, and purified by adding of Axygen Axyprep MagPCR clean-up beads (50 µL beads were thoroughly mixed with 50 µL amplicons) and incubated for 15 minutes at room temperature. The plate was placed on the magnetic stand for 5 minutes until the supernatant was

cleared and then washed with 80% ethanol. The pooled amplicon was evaluated by using the Advanced Analytical Fragment Analyzer and quantified using Quant-iT HS dsDNA kits, and diluted based on Illumina's standard protocol for sequencing (MiSeq instrument).

Sequencing data processing and bioinformatic analysis

The amplicon sequencing data of taxonomic marker genes, such as the 16S rRNA gene in bacteria, internal transcribed spacer (ITS) region in fungi, and 18s rRNA gene in eukaryotes provides the community compositions (Callahan et al., 2016). The Division Amplicon Denoising Algorithm (DADA) enables taxonomic assignment without building into operational taxonomic units (OTUs), using a new quality-aware model of Illumina amplicon errors. This is termed the amplicon sequence variant (ASV) (also called exact sequence variants, ESVs) based analysis. The primers were designed to match to 5' ends of the forward and reverse reads. The Cutadapt (Martin, 2011) algorithm was used to remove the primers at 5'end of forward reads. Read pairs were rejected if one read or the other did not match a 5'primer, and an error rate of 0.1 was allowed. Two passes were made over each read to ensure the removal of the second primer.

Quality filtering, pairing, denoising, de-replication, and determination of count of ASVs was performed with the Division Amplicon Denoising Algorithm (DADA2) plugin (Callahan et al., 2016) in the QIMME2 platform. For quality trimming, forward and reverse reads were truncated to 150 bases and bacterial 16s rRNA gene were subsetted to retain only those sequences that are between 249 and 275 nucleotides inclusive. Taxonomy was assigned to the sequences using the SILVA database v132 (Pruesse et al., 2007) of 16S rRNA sequences of bacterial species of different taxonomy using the classify-sklearn procedure. The ASVs identified other than bacteria were removed from further analysis. For the rarification, if there were more 10,000 counts in one or more samples, they are rarefied to the value of the smallest

sample greater than 10,000 minus 1. Similarly, to identify fungal ASVs, the internal transcribed spacer (ITS) region was sequenced utilizing primers ITS3 and ITS4. The Cutadapt algorithm (Martin, 2011) was used to remove the primers at both ends of the contigs and cull contigs that did not contain both primers. The QIMME2 DADA2 plugin was used to perform similar quality filtering and ASV identification, similar to that described for the bacterial taxonomic assignment. Individual sample read count was normalized to 27,392 and used analysis of the gut microbiome and mycobiome taxonomic compositions.

Resistome analysis (pilot study)

Fecal samples (*n*=18) from two production pigs at nine age-points (days 2, 22, 26, 40, 54, 94, 128, and 179) were subjected to shotgun metagenomic sequencing for evaluating the age related dynamics of the fecal resistome in pigs.

Fecal DNA extraction, library preparation, and sequencing for resistome analysis

Fecal DNA was extracted from each sample using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to the manufacturer instructions. Briefly, 250 mg of the fecal samples were placed in power bead tubes and vortexed for 10 minutes at maximum speed and then centrifuged (15,000x *g* for 1 minute). After centrifugation, 600 µL supernatant was transferred into position 2 of the rotor adapter, which was then placed into the centrifuge of the Qiacube (Qiagen, Germany). All subsequent steps, including the removal of inhibitory substances, were performed on the Qiacube following the DNeasy PowerSoil Pro Kit (Qiagen, Germany) IRT method. DNA concentration and quality were determined by fluorometric quantification Qubit™ (Thermo Fisher Scientific, Germany), and quality was assessed by measuring the A260/A280 ratio using Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Germany). After DNA extraction, libraries were constructed using the Qiagen QIAseq

FX DNA Kit (Qiagen, Germany) with 100 ng individual DNA samples. Paired-end sequencing was performed on the NovaSeq system (Novogene, Inc., CA, USA).

Resistance gene analysis

The sequencing reads were aligned to the AMR genes database MEGAREs to identify the resistome composition using AMR++ pipelines (Lakin et al., 2017). Briefly, raw sequence reads were processed, and low-quality reads and adapter contamination were removed using Trimmomatic (Bolger et al., 2014) and merged reads were mapped using Burrows-Wheeler-Alignment (BWA) (Li and Durbin, 2009) and then converted to SAM formatted file using Samtools (Li et al., 2009) which was then analyzed through ResistomeAnalyzer. The counts of aligned sequence reads were recorded at the gene (e.g., TEM-77), group (gene level group for that sequence, e.g., SHV β-lactamase, MCR-1), the mechanism (the biological mechanism of resistance, e.g., penicillin-binding protein), and class level major antimicrobial chemical class, e.g., β-lactams, aminoglycosides). MEGARes, provide explicit annotation and facilitates hierarchical statistical analysis at the population level. It is count-based, similar to microbiome analysis, and the annotation graph contains no cycles and contains only three hierarchical levels (Lakin et al., 2016). The AMR gene sequencing depth and length were normalized before performing the statistical analysis on ARGs count data.

Phenotypic AMR in fecal bacteria

A total of 270 samples (n=108 fecal samples from production pigs (n=12/age-points), n=162 from breeding sows (n=18/age-points)) were subjected to bacteriological culture and quantified for coliforms and enterococci by spiral plating using an Eddy Jet 2 spiral plater (Neutech Group Inc., Farmingdale, NY, USA) as described previously (Chalmers et al., 2018). Briefly, the fecal sample was diluted in phosphate-buffered saline (PBS) at a 1:10 ratio. The

dilution was plated on MacConkey agar (Remel™, Lenexa, KS, USA) (MAC), and MAC supplemented with an antimicrobial drug (the drug concentrations are listed in Table 4.1). The dilution was also plated on m-Enterococcus agar (Remel™, Lenexa, KS, USA) (ENT), and ENT supplemented with an antimicrobial drug (the drug concentrations are listed in Table 4.1). The plates were incubated at 37° C for 18 hours for MAC and up to 48 hours for ENT. The bacterial density (CFU/mL) was determined by counting the colonies of bacteria.

Statistical analysis

Statistical analysis was performed to evaluate age related dynamics of fecal microbiome and mycobiome taxonomic compositions and AMR in cohorts of production pigs and breeding sows. Alpha diversity of microbiome and mycobiome as captured by taxa richness (Chao1 index), diversity (Shannon and Fisher alpha index) indices, and evenness were estimated on the rarefied microbiome and mycobiome taxonomic composition data using the PAleontological STatistics (PAST) software (Hammer-Muntz et al., 2001). A mixed-effects linear model (R package "l*me4*") with pig identity as the random-effect variable (accounting for the repeated measurement) was used to determine significant trends in alpha diversity with pig age (predictor variable). Non-metric multidimensional scaling (NMDS) ordination plots were made using R *vegan* package (Jari Oksanen, 2019) on Hellinger transformed (Legendre and Gallagher, 2001) bacterial and fungal taxonomic counts using Bray-Curtis dissimilarity distances and visualized using ggplot2 package (Wickham, 2009). The plot was used if stress < 0.2. The ASVs present in < 1% samples were discarded prior to analysis. The difference in beta diversity based on Bray-Curtis distance was evaluated by Permutational analysis of variance (PERMANOVA) with strata of individual pigs to account for the repeated measurement of individual pigs using the *adonis* in *vegan* package in R software (version 3.6). The permutational analysis of multivariate dispersion

(PERMDISP) was then performed to compare to the Bray-Curtis distance to group centroids among samples.

Similarly, the richness and diversity of the fecal resistome (gene encoding AMR mechanisms of AMR encoded by the present genes, and by drug classes) were also estimated. The Procrustes (Peres-Neto and Jackson, 2001) was performed to compared resistome AMR genes and microbial species composition ordination based on Bray-Curtis similarity metrics with M^2 statistic measure of fit and correlation (r).

The changes in the total and antimicrobial-resistant coliforms and enterococci (log-transformed) counts were analyzed using generalized linear mixed models ("*lme4*" package in R software) (R, 2019) on data from pigs that had at least one positive count for a given antimicrobial. The pig identity was used as a random effect to account for the lack of independence between the samples.

Results

Age-dependent dynamics of the pig fecal microbiome (bacteriome) and mycobiome

We profiled the dynamics of the fecal microbiome (bacteriome) and mycobiome taxonomic compositions in a longitudinal study of one cohort of production pigs and two cohorts of breeding sows. A cohort of production pigs (n=8 pigs) was sampled at nine different age-points from ~ 2 days old to the market age of 179 days (Figure 4.1). Two cohorts of breeder sows (n=6 and n=12 per cohort) were sampled from the time of purchasing at ~21 days of age until weaning the first litter at 371 days of age (Figure 4.1).

The results showed that the fecal microbial community was established rapidly after birth and the diversity increased with age. In the production pig cohort, measurements of alpha diversity of the species such as richness (Chao1 index; β_1=0.69, SE=0.2, p <0.001) and diversity

indices (Shannon index β_1=0.005, SE=0.002, p=0.01; Fisher alpha index β_1=0.12, SE=0.03, p <0.001) demonstrated a significant positive increase from day 2 to day 177 of age (Figures 4.2, 4.3 and 4.3, a). However, evenness did not significantly change with age (Figure 4.5, a). The pig housing environment (barn/facility) also significantly impacted alpha diversity (richness, diversity of bacterial taxa). For instance, richness and diversity significantly increased (p <0.05) after the pigs were moved from the nursery to the finisher facility.

The significant shift in the overall microbiome taxonomic composition from 2 days old through day 179 in the cohort of production pigs is visualized using non-metric multidimensional scaling (NDMS plots) based on the Bray-Curtis dissimilarity distance (PERMANOVA p < 0.001) (Figure 4.6 a). Further, each of the nine age-points showed unique clusters and centroids. There were significant separations in beta diversity among samples collected on days 2, 21 (end of weaning and moving to the nursery facility), and 26 (4 days in the nursery facility on a solid phase 2 diet) and the rest of the age-points. Variation of fecal microbiome composition among individual animals was highest at 2 days old, with a decrease and leveling of approximately 2 months of age (PERDISP, p <0.001) (Figure 4.6, d).

In contrast, alpha diversity of fecal mycobiome taxonomic composition at the species level demonstrated an overall decrease with age, as indicated by the decreasing richness (Chao1 index; β_1= −0.02, SE=0.007, p =0.03), diversity (Shannon diversity β_1= −0.007, SE=0.002, p =0.001; Fisher alpha diversity β_1= −0.005, SE=0.002, p =0.01), and evenness (β_1= −0.005, SE=0.002, p =0.02) indices from day 2 to day 179. the alpha diversity of the mycobiome showed a slight increase after weaning on day 22 and remained stable until day 54, then dropped by day 77 and again increased from days 93 to 128 (Figures 4.2, 4.3 and 4.4, b). The evenness of fungal species steadily decreased from birth to day 40 and then slightly increased and remained similar

until the harvest on day 179 (Figures 4.5, b). The NMDS plots based on the Bray-Curtis distances show significant shifts in the fungal community composition from day 2 to day 179 (PERMANOVA, p=0.001) (Figures 4.6, a). The variation of fecal mycobiome composition among individual animals was highest at 2 days old, with fecal samples at older ages becoming less variable in the mycobiome taxonomic composition (Figure 4.7, b). Variation of beta diversity of fecal mycobiome showed mixed trends, first increasing from day 2 to 40 (a day before the change from phase 2 to phase 3 diet in the nursery facility) and then gradually decreasing throughout the rest of the age-points until day 179 (PERMDISP; $p < 0.001$) (Figure 4.6, d).

In breeder sows, alpha diversity of fecal microbiome at the species level as captured by richness (Chao1 index; β_1= 0.21, SE=0.1, p =0.04) and diversity (Shannon index; β_1= 0.007, SE=0.002, p =0.001 and Fisher alpha index; β_1= 0.03, SE=0.016, p =0.05) indices also significantly increased with age and these dynamics were comparable to that of production pigs in this study (Figures 4.2, 4.3 and 4.4, c). In contrast, the evenness of taxa decreased with the age of the breeding sow (Figure 4.5, c). The richness and diversity of the microbial community increased starting from ~3 weeks old (at the time of purchase/entering the first growing facility) through day 154 and then leveled off. However, no significant changes in the evenness of taxa were observed (Figure 4.4, c). The significant shift in microbiome community composition and structure from days 21 to 371 (farrowing and weaning) were observed on the NMDS plots based on the Bray-Curtis distance (PERMNOVA, p <0.001) (Figure 4.6, b-c). Day 21 (time of entry at the farm, first growing facility) followed by day 42 (3 weeks after entry) were the most distinct from the rest of the age-points. Variation of beta diversity of fecal microbiome showed

decreasing dynamics from 3 weeks to farrowing (day 21-day 371) in breeder sows (PERDISP, p <0.001) (Figure 4.6, e-f).

In contrast, fecal mycobiome of breeding sows showed mixed temporal trends in alpha diversity over the nine age-points from day 21 through day 371 (end of weaning the first litter) (Figures 4.2, 4.3 and 4.4, c). Overall, alpha diversity showed decreasing trends with age but did not change significantly. For instance, richness (Chao1 index; $\beta_1 = -0.0013$, SE=0.006, p =0.4) showed decreasing trends from day 21 to day 70 and then slight increase on day 84, day 224 to day 371. The diversity (Shannon diversity; $\beta_1 = -0.002$, SE=0.001, p =0.29 and Fisher alpha diversity; $\beta_1 = -0.013$, SE=0.002, p =0.33) and evenness ($\beta_1 = -0.003$, SE=0.001, p =0.33) showed increasing trends from day 21 to day 154 and a slight decrease following day 224 and then remained at the same level.

However, non-metric multidimensional scaling (NMDS) plots based on the Bray-Curtis distance of mycobiome revealed a shift in the fungal community with the age of the breeding sow (PERMNOVA, p <0.001) (Figure 4.7, b-c). Further, the variation of fecal microbiome composition among individual animals showed a slight increase from day 21 to day 70 and then a decrease following day 84 and remained stable the rest of the age-points (PERMDISP; p < 0.001) (Figure 4.7, e-f).

Microbial community taxonomic composition

Taxonomic compositions of bacterial and fungal communities at the phylum, family, genus, and species levels were evaluated. A dynamic shift in bacterial and fungal taxa with the age of pigs were observed. For microbiome composition at the phylum level, *Bacteroidetes* was the most abundant phylum (34% of the total phyla) followed by *Fusobacter* (~24%) and *Firmicutes* (~23%) at the first sampling of piglets (2 days old). *Firmicutes* appeared to be the

most abundant phylum followed by *Bacteroidetes* from weaning on day 21 to the day of harvest on day 179 (Figures 4.8). The abundance of *Firmicutes, Tenericutes*, *Cyanobacteria*, *Kiritimatiellaeota, Patescibacteria* significantly increased with age (false discovery rate adjusted $p < 0.001$), while *Proteobacteria* and *Fusobacteria* significantly decreased with age in production pigs. At the family level, there was also a significant shift in bacterial taxa detected from day 2 to day 179. For instance, several bacterial families including, *Bacteroidales_RF16_group*, unidentified bacterium RF39, *Streptococcaceae*, *Anaeroplasmataceae*, *Saccharimonadaceae*, and others (uncultured bacterium) significantly increased with the age of pigs while an abundance of *Fusobacteriaceae* and *Enterobacteriaceae* decreased with the age of the pigs (Figures 4.10, a).

Similarly, in breeding sows, *Firmicutes* (~50%) and *Bacteroidetes* (~40%) were the two most dominant phyla across all age points (Figure 4.9). However, other less abundant phyla, such as *Fibrobacteres*, *Spirochaetes*, and *Kiritimatiellaeota,* significantly (p <0.001) increased with age, while *Chlamydiae* significantly decreased with the age of breeding sows. At the family level, *Prevotellaceae*, *Spirochaetaceae*, *Peptostreptococcaceae*, *Anaeroplasmataceae*, *Fibrobacteraceae, Clostridiaceae*_1, *Erysipelotrichaceae*, p251o5, and *Veillonellaceae* significantly increased (p <0.001) with age, while *Coriobacteriaceae* and *Chlamydiaceae* decreased with the age of breeding sows (Figure 4.10, b).

The mycobiome was composed of a lower number of phyla compared to the microbiome of the fecal samples. Among the fungal phyla, *Ascomycota* and *Basidiomycota* were the most abundant in production pigs and breeding sows throughout all the age-points. The relative abundance of *Basidiomycota* significantly decreased with age in production pigs ($p = 0.001$), while the phylum *Mucoromycota* significantly increased ($p = 0.001$) with age in breeding sows.

At the family level, the abundance of several fungal families, including *Nectriaceae*. *Mucoraceae, Diaporthaceae*, *Ustilaginaceae*, *Rhizopodaceae*, and *Aspergillaceae* significantly (p <0.05, adjusted p-value at $\alpha = 0.05$) increased with age, while *Didymellaceae* decreased with age in production pigs (Figure 4.11, a). Similarly, in breeding sows, the abundance of *Nectriaceae*, *Rhizopodaceae*, *Orbiliaceae*, and *Ceratostomataceae* significantly increased with age (Figure 4.11, b).

The overall dynamics of the pig phenotypic AMR in production pigs and breeding sows

The temporal fluctuations in abundance of AMR fecal coliforms and enterococci (Figures 4.12, 4.13, 4.14 and 4.15) in the presence of the resistant breakpoint concentrations of antimicrobials (Table 4.1) was observed in both cohorts of production pigs and breeding sows. Similarly, there was variability in pig yielding AMR fecal coliforms and enterococci (Table 4.2 and 4.3). The higher abundance of AMR coliforms and enterococci were detected in the earliest ages in both cohorts of production pigs and breeder sows.

Total coliforms and enterococci count (no antimicrobial) remained relatively stable over time and did not decline as the pigs aged in production pigs (total coliforms, p=0.16, enterococci p=0.6). There was no relationship between total coliforms or enterococci counts and being housed (different housing or changes in diet) in production pigs (p >0.1). Similarly, coliform counts ($\log_{10}$ CFU/g) in the presence of ceftriaxone (3rd generation cephalosporins), aminoglycosides (gentamicin and streptomycin), aminopenicillins, and macrolides (azithromycin) significantly declined with the pig age (p <0.05). The coliform counts ($\log_{10}$ CFU/g) in the presence of tetracyclines or sulfonamides also declined with the pig's age (p <0.1) but was lower than the above-mentioned antimicrobials. Similarly, enterococci count ($\log_{10}$

CFU/g) in the presence of tetracyclines, nalidixic acid (quinolones), and aminoglycosides (gentamicin and streptomycin) significantly declined with pig's age (p <0.05). However, enterococci count (log_{10} CFU/g) in the presence of aminopenicillins, lincomycin (lincosamides), and nitrofurans did not significantly change with the age of the pigs (p >0.05).

In breeding sows, the total counts (log_{10} CFU/g) of coliforms also remained relatively stable over time and did not decline with the pig's age (p = 0.16); however, total enterococci counts declined with the pig's age (p = 0.01). The coliforms in the presence of ceftriaxone (3rd generation cephalosporins) or tetracyclines significantly decreased with the pig's age (p >0.05). Similarly, coliforms in the presence of aminoglycosides or aminopenicillins declined with age, but this relationship was not significant (p >0.1). Similarly, enterococci count in the presence of aminoglycosides or aminopenicillins declined significantly (p < 0.05) with the pig age.

Our results showed that cohort pigs rapidly acquired the AMR coliform or enterococci shortly after birth. The abundance of fecal bacteria in the presence of the resistant breakpoint concentrations of antimicrobials within the cohort declined at the earliest age points over the study period (birth to harvest) in production pigs and breeding sows. The age-dependent dynamics of AMR abundance in fecal bacteria was the highest at the earliest age-points and decreased with age.

Resistome composition in production pigs

The shotgun metagenomic sequence analyses were used to assess the resistome dynamics. In production pigs (n=2/age-points), the highest diversity and abundance of AMR-genes occurred at the earliest age-points and then decreased with age. A total of 116 AMR genes, which represent 34 AMR mechanisms and 13 AMR classes in production pigs. Antimicrobial resistance genes predicted to confer resistance to tetracyclines represented a significant portion

of resistome (~50%), followed by rifampin (~20%), aminoglycosides (~11%), macrolides-lincosamides-streptogramin (~7%), multidrug resistance (~4%), β-lactams (1%), fluoroquinolones (1%), and cationic antimicrobial peptides (1%) (Fig 4-16). The total number within each AMR-gene class, such as with β-lactams, fluoroquinolones, tetracyclines, sulfonamides, trimethoprim, phenicols, and cationic antimicrobial peptides were relatively higher in earliest age-points, while other AMR genes such as macrolides, lincosamides, and streptogramins (MLS), increased with age. Similarly, the richness and diversity of genes encoding AMR, mechanisms, and drug classes revealed the fecal resistome changes over time in pigs (Figure 4.17 and 4.18). The Procrustes analysis based on the Bray-Curtis similarity metrics also showed a relationship (m^2=0.48, p=0.1) between AMR gene composition and microbial community composition when pigs were housed in the finisher facility.

Discussion

The composition, diversity and interactions of the gut microbial community of the pig from birth to the harvest age in production pigs and from early life through the first breeding, farrowing, and weaning in breeding sows are critical to the overall animal health and performance. Understanding these dynamics is also important for the overall epidemiology of AMR as microbial communities serve as a reservoir for AMR genes in which the AMR genes can be transferred between native and transient gut bacteria (Sommer et al., 2010; Hu et al., 2014). Our longitudinal study of the pig fecal microbiome, mycobiome, and AMR in a cohort of production pigs (from day 2 through day 179 of age, n=12 pigs) and two cohorts of breeding sows (from day 21 through day 371/1st farrowing and weaning, n=6 and n=12 sows) revealed similar microbiome alpha diversity trends between production pigs and breeding sows. However, we found different mycobiome alpha diversity trends between production pigs and breeder sows.

Also, for either production pigs or breeding sows, the mycobiome trends differed from the microbiome trends. Further, phenotypic AMR in indicator fecal bacterial (coliforms and enterococci) and the resistome demonstrated age-dependent dynamics, with the highest AMR at the earliest age-points sampled and a decrease in AMR with age in either production pigs or breeder sows.

Microbiome taxonomic composition, richness, and diversity

The observations of overall increasing diversity and richness of the fecal microbial species with the age of production pig are in agreement with the results of earlier studies (Chen et al., 2017; Lu et al., 2018; Wang et al., 2019). Similar dynamics of microbiome diversity were also noted in humans from early infants to adults (Kong et al., 2019). In our study, the bacterial species diversity (measured by Shannon index) and richness (measured by Chao1 index) was highest on day 98 and then plateaued until day 179. The diversity and richness demonstrated a sharp increase following the transition to a solid-phase diet around 3 weeks of age. Similar results of increasing bacterial diversity with age (birth, days 10, 21, 23, 62, 84, and market) were reported in production pigs (De Rodas et al., 2018). In the same study, they reported the effect of age on microbiome diversity and richness across all gut location samples (duodenum/jejunum, ileum, cecum, and colon) with a substantial shift in microbial community composition between days 21 and 33 of the pig's age.

Further, the beta diversity analysis of pig fecal microbiome composition showed clear clustering by age. Based on NMDS, bacterial communities appeared to separate on days 2, 22, and 26 and become more similar between individual pigs at later age-points. An earlier study also showed age related clustering of the microbial communities in pigs (Wang et al., 2019; Arfken et al., 2020) and calves (Hennessy et al., 2020). Fecal samples from piglets 48 hours after

birth could contain meconium; a study reported that meconium samples contain low but diverse microorganisms (Wang et al., 2019).

Firmicutes and *Bacteriodetes* were the most abundant fecal bacterial phyla detected throughout the study period, which agrees with an earlier study (Ke et al., 2019). The bacterial phyla, *Fusobacteria,* and *Proteobacteria* were dominant in early ages and decreased with the age of the production pigs. It has been reported that the phylum *Fusobacteria* was linked with the disease in the animal, suggesting that opportunistic pathogens are commonly found in nursing piglets (De Witte et al., 2017). The bacterial phyla such as *Firmicutes, Tenericutes*, *Kiritimatiellaeota*, and *Cyanobacteria* significantly increased with age, possibly because of the introduction of solid feed during nursery stages (after day 22). Interestingly, we also found that the relative abundance of bacterial families such as *Enterobacteriaceae* decreased with the pig's age. This finding indicates an age-specific change of the microbiome abundance in production pigs (Ke et al., 2019).

Similarly, the dynamics of alpha diversity patterns in breeding sows were similar in the cohort of production pigs (within overlapping age periods), with overall increasing diversity and richness of species. For example, Choa1 richness and Fisher alpha diversity increased from days 21 to 154 and remained stable until day 371. This indicated that the bacterial communities in breeding sows increased until day 154 (closed to first breeding period) and then stabilized at subsequent farrowing and weaning. These results were comparable to those of the production pigs cohort, suggesting that swine gut microbial communities fully develop and mature before the market (Wang et al., 2019). Similarly, beta diversity of the microbiome composition showed distinct clusters on days 21 (at the time of arrival at the farm) and 42, from the rest of the age-

points. Similarly, a study also reported a clear age-dependent succession of overall bacterial community structure in piglets and sows (Bian et al., 2016).

Similarly, in breeding sows, consistent with the previous findings (Mach et al., 2015), *Firmicutes* and *Bacteriodetes* were the two predominant phyla present in the longitudinal study. Interestingly, the relative abundance of bacterial phyla such as *Spirochaetes*, *Kiritimatiellaeota*, and *Fibrobacteres* increased with age while the relative abundance of *Chlamydiae* decreased with the age of breeding sows. Similarly, the relative abundance of bacteria family such as *Erysipelotrichaceae*, *Clostridiaceae1*, *Spirochaetaceae*, and *Peptostreptococcaceae* increased with age, and *Chlamydiaceae* and *Coriobacteriaceae* significantly decreased with age. Han et al. (Han et al., 2018) also reported similar relative abundances of phyla at various growth stages in pigs (day 10 to day 147 of age).

Domestic pigs have a life expectancy of 15 to 20 years but were only studied until 1 year of age. Therefore, whether the pig fecal bacterial diversity remains stable after the 1-year age-point or how the gut microbiome composition changes during aging cannot be inferred. However, results showed that between early-life and 1 year of age, the dynamics of richness and diversity of the fecal microbiome composition were similar and comparable among the production pigs and breeding sow cohorts, which suggested that age is a major driver of the microbiome composition.

Mycobiome taxonomic composition, richness, and diversity

In contrast, the fecal mycobiome in production pigs showed the richness (measured by Chao index) and diversity indices (Shannon index and Fisher alpha index) of fungal taxa remained relatively steady until the age of weaning and then slightly increased post-weaning (day 26) and then remained relatively unchanged. Interestingly, evenness was reduced from birth

to day 40 (2 weeks after diet changed in nursery barn) and remained relatively stable until the day of harvest (day 179).

Further, the diversity and richness decreased on day 77 (*i.e.*, a day before moving the nursery to the finisher barn). This indicates a potential fluctuation of fungal communities with age and when pigs moved to different facilities. Similarly, in breeding sows, the fecal mycobiome showed a reduction in Chao1 richness, Shannon diversity, and evenness of fungal taxa from day 21 through day 154 (a day prior to moving to the breeding barn) and remained stable until day 371. The similar dynamics of fungal diversity and richness were also observed in a previous mycobiome study in piglets from birth to weaning (Arfken et al., 2020) where the piglets were nursed with their mother until 21 days of age (weaned on day 21) and then received nursery diet 1 (days 21–28) followed by nursery diet 2 (days 29–35). No antibiotics, antifungals, or other additives were administered to the piglets at any time during the study period. The most abundant bacterial phyla in the GI tract and feces were *Bacteroidetes*, *Firmicutes*, and *Epsilonbacteraeota*, and the dominant fungal phyla were *Ascomycota* (90%) and *Basidiomycot*a (9%).

Similarly, low fungal diversity has been reported in healthy adult human fecal samples (Raimondi et al., 2019). The low fungal diversity in pigs corresponded to human findings as these samples are collected from apparently healthy animals. Data from the study showed a significant increase in the abundance of *Aspergillaceae*, *Rhizopodaceae*, *Nectriaceae*, and *Mucoraceae* with the age of pigs (both production and breeding sows). Interestingly, the abundance of *Didymellaceae* decreased with the age of the pigs. Further, there were compositional differences in early ages; however, the beta diversity indicated no distinct cluster of fungal taxa with the age of pigs (both production pigs and breeding sows). In addition to the

fungal taxa composition structure, greater variation among the fungal community was observed in both production and breeding sows. This indicates that the pig fecal mycobiome does not follow the defined pattern of colonization and succession that does a microbiome (Arfken et al., 2019; Summers et al., 2019).

AMR in enteric bacteria

In the present study, both abundance of fecal coliforms and enterococci (log_{10} CFU/g) in the presence of antimicrobials (resistant breakpoint concentrations) were higher at early ages in both production pigs and breeding sow cohorts. Several previous studies demonstrated carriage of AMR resistance bacteria associated with the age of food animals with a higher prevalence of AMR in fecal bacteria within the host shortly after birth and then a decline over time (Hoyle et al., 2004a; Hoyle et al., 2004b; Khachatryan et al., 2004; Berge et al., 2010; Edrington et al., 2012a; Mainda et al., 2015a), and pigs (Moro et al., 1998; Agga et al., 2014; Agga et al., 2015b). We also observed similar dynamics of high levels of abundance of AMR fecal bacteria (coliforms and enterococci) within the cohorts of production pigs and breeding sows were observed. This phenomenon could be associated with an age-dependent shift of gut microbial communities (Chen et al., 2017; Grosicki et al., 2018; Ke et al., 2019; Wang et al., 2019), which often serve as an AMR gene reservoir and can transfer between native and transient gut bacteria (Sommer et al., 2010; Hu et al., 2014).

To understand the age-dependent resistome dynamic, shotgun metagenomic sequencing was used for the analysis of the presence of AMR genes in production pigs (sub-study, *n*=2 pigs at 9 age-points). This was selected based on the fact that cohorts of pigs possess similar microbiome and mycobiome diversity and phenotypic AMR profiles at the early ages. In our preliminary study, we detected 116 AMR genes, which represented 34 AMR mechanisms and 13

AMR classes of drugs. This preliminary data indicated that the richness and diversity of the fecal resistome change overtime with pig age with the relatively highest diversity at an early age. The abundance of AMR gene-drug classes, such as β-lactams, fluoroquinolones, tetracyclines etc., were relatively higher in the earliest age-points. This age-dependent AMR in pig fecal bacteria could directly be related to the decrease in the relative abundance of specific microbial taxa, for instance, *Enterobacteriaceae* (i.e., significantly decreased with age in our pig fecal samples), which was considered to harbor the most AMR genes at an early age. However, resistance to MLS increased over time, indicating that not every AMR gene decreased in abundance with the pig's age. It has been suggested that the resistome is mainly structured by the bacterial phylogeny and bacterial taxa *Proteobacteria* were more likely to drive the resistance than other phyla (Forsberg et al., 2014). Interestingly, in the production pigs taxonomic data, the relative abundance of *Proteobacteria* (which includes *Enterobacteriaceae*) decreased with age. One study showed that mobile AMR genes were mainly present in four bacterial phyla: the *Proteobacteria, Firmicutes, Actinobacteria*, and *Bacteroidetes* (Huddleston, 2014). The same study showed that *Streptococcus agalactiae*, *E. coli*, and *Streptococcus suis* were the three most abundant bacterial species that are shared between animal and human intestines and consistently harbor known AMR genes.

Recent studies have shown the fecal resistome significantly changed over time in food animal (e.g. dairy calves), with the highest being at early ages and then a decrease over time in dairy calves (Liu et al., 2019) and pigs (Pollock et al., 2020). Further, it has been shown that the prevalence and abundance of AMR genes were not influenced by antibiotic use. AMR gene abundance was higher in nursing piglets with low microbiome diversity (Pollock et al., 2020). Thus, consistent with the hypothesis, our data suggest that the gut microbiome in pigs may serve

as a natural reservoir of AMR genes. In addition, the Procrustes (based on Bray-Curtis distance) analysis showed the marginally significant relationship (m^2=0.48, p=0.1) between AMR gene composition and microbial community composition when pigs were housed in a finisher facility). Furthermore, (Joyce et al., 2019) reported a significant positive correlation between the total resistome and the total microbiome from fecal samples of healthy pigs, further suggesting microbiome composition influenced the resistome composition.

The microbial community is dynamic in early life in food animals as well as humans; it is typically established immediately after birth, and the microbial population composition is in fluctuation until maturity is attained (Yatsunenko et al., 2012; De Rodas et al., 2018). Alteration to the microbial community during development may alter both its phylogenetic composition and its associated resistome.

Conclusions

In this study, the fecal microbiome, mycobiome, and AMR profiles (phenotypic and genotypic) at nine age-points using cohorts of production pigs (day 2 to harvest) and breeding sows (day 21 through first farrowing and weaning) were assessed. The results indicated that bacterial richness and diversity significantly increased with the age of pigs. The age-based dynamic shift of the ecological community in the swine gut microbiome was exhibited with *Firmicutes* as the dominant phylum in early life, followed by *Bacteroidetes* throughout the rest of the studied ages. The study also showed shifts in the fungal community composition, richness, and diversity in individual animals with age; however, the dynamics did not follow the same defined pattern as the bacterial colonization and succession and were less repeatable among the animals. Ascomycota was the most abundant fungal phylum at all ages, followed by Basidiomycota. The indicator fecal bacteria (coliforms and enterococci) in the pig cohorts

exhibited an age-dependent dynamic of AMR to many antimicrobial drug classes, including the third-generation cephalosporins, aminopenicillins, aminoglycosides, and macrolides, with the highest between-animal prevalence and within-animal AMR abundance in early ages, followed by a decrease with as the age of the pigs increased. The shotgun metagenomic approach revealed that the diversity of AMR genes was largest at earliest age-points and decreased with age. The variety of genes encoding resistance to each of the β-lactams, fluoroquinolones, tetracyclines, or cationic antimicrobial peptides was highest at the earliest age-points. The age-dependent dynamics of richness and diversity of the fecal microbiome and mycobiome taxonomic compositions, and phenotypic AMR in fecal bacteria were comparable among the production pig and breeding sow cohorts, indicating age rather than the production system was the bigger driver.

Table 4.1

Antimicrobial drug concentrations used to supplement the microbiological agar to evaluate the dynamics of the phenotypic AMR in production pigs and breeding sows.

Bacteria/Media	Antimicrobial (class) supplemented	Antimicrobial agent supplemented (Abbreviation)	Concentration (µg/mL) [a]
Coliforms			
MacConkey Agar (MAC)	Aminoglycosides	Gentamicin (GEN)	16
		Streptomycin (STR)	32
	3rd generation cephalosporins	Ceftriaxone (AXO)	4
	Sulfonamides/folate path inhibitors	Sulfamethoxazole (SMX)	512
	Macrolides	Azithromycin (AZI)	32
	Aminopenicillins	Ampicillin (AMP)	32
	Phenicols	Chloramphenicol (CHL)	32
	Fluoroquinolones	Ciprofloxacin (CIP	1 (and 0.25)
	Quinolones	Nalidixic acid (NAL)	32
	Tetracyclines	Tetracycline (TET)	16
	Fluoroquinolones	Enrofloxacin (ENR)	0.125*
Enterococci			
Enterococcus Agar (ENT)	Aminoglycosides	Gentamicin (GEN)	500
		Streptomycin (STR)	1024
	Lincosamides	Lincomycin (LIN)	8
	Macrolides	Erythromycin (ERY)	8
		Tylosin	32
	Nitrofurans	Nitrofurantoin (NIT)	128
	Aminopenicillins	Penicillin (PEN)	16
	Phenicols	Chloramphenicol (CHL)	32
	Fluoroquinolones	Ciprofloxacin (CIP)	4
		Enrofloxacin (ENR)	4
	Quinolones	Nalidixic acid (NAL)	32‡
	Tetracyclines	Tetracycline (TET)	16

[a] Breakpoints based on Clinical Laboratory Standards Institute guidelines (CLSI, 2008) and National Antimicrobial Resistance Monitoring System, * European Committee on Antimicrobial Susceptibility Testing (epidemiological cutoff value), ǂ adopted from *E. coli* break-point concentration

Table 4.2

Percentage (95% confidence intervals) of production pigs (*n*=12) yielding antimicrobial-resistant coliforms or enterococci.

Fecal bacteria		Age, days								
	Antimicrobials	2	22	26	40	54	77	93	128	177
Coliforms	Aminopenicillins	100 (73.5-100)	91.6 (61.5-99.7)	100 (73.5-100)	83.3 (51.5-97.9)	75 (42.8-94.5)	83.3 (51.6-97.9)	91.7 (61.5-99.8)	83.3 (51.6-97.9)	100 (73.5-100)
	3rd generation cephalosporins	83 (51.6-97.9)	91.6 (61.5-99.7)	58.3 (27.6-84.8)	33.3 (9.9-65.1)	75 (42.8-94.5)	16.7 (2.1-48.4)	33.3 (9.9-65.1)	25 (5.5-57.2)	83.3 (51.6-97.9)
	Tetracyclines	100 (73.5-100)	100 (73.5-100)	100 (73.5-100)	83.3 (51.5-97.9)	91.7 (61.5-99.8)	91.7 (61.5-99.8)	100 (73.5-100)	100 (73.5-100)	100 (73.5-100)
	Macrolides	75 (42.8-94.5)	0	100 (73.5-100)	75 (42.8-94.5)	83.3 (51.6-97.9)	66.7 (34.9-90.1)	100 (73.5-100)	75 (42.8-94.5)	0
	Aminoglycosides	91.6 (61.5-99.7)	91.6 (61.5-99.7)	75 (42.8-94.5)	83.3 (51.6-97.9)	83.3 (51.6-97.9)	66.6 (34.8-90)	83.3 (51.5-97)	50 (21-78.9)	100 (73.5-100)
	Sulfonamides	91.6 (61.5-99.8)	100 (73.5-100)	83.3 (51-97.9)	83.3 (51.6-97.9)	83.3 (51.6-97.9)	83.3 (51.6-97.9)	91.6 (61.5-99.8)	75 (42.8-94.5)	100 (73.5-100)
	Phenicols	25 (5.4-57.2)	41.67 (15.2-72.3)	33.3 (9.9-65.1)	33.3 (9.9-65.2)	25 (5.5-57.2)	33.3 (9.9-65.1)	41.6 (15.2-72.3)	33.3 (9.9-65.1)	41.6 (15.6-72.3)
Enterococci	Aminopenicillins	58.3 (27.6-84.8)	50 (21-78.9)	25 (5.5-57.2)	0	0	0	0	0	50 (21-78.90)
	Quinolones	100 (73.5-100)	100 (73.5-100)	91.6 (61.5-99.8)	100 (73.5-100)	91.6 (61.5-99.78)	75 (42.8-94.5)	75 (42.8-94.5)	75 (42.8-94.5)	100 (73.5-100)
	Tetracyclines	100 (73.5-100)	100 (73.5-100)	100 (73.5-100)	100 (73.5-100)	91.6 (61.5-99.78)	91.6 (61.5-99.7)	91.6 (61.5-99.8)	100 (73.5-100)	100 (73.5-100)
	Macrolides	66.6 (34.8-90.07)	100 (73.5-100)	100 (73.5-100)	100 (73.5-100)	91.6 (61.5-99.78)	58.3 (27.6-84.8)	100 (73.5-100)	83. 3 (51.5-97.9)	100 (73.5-100)
	Aminoglycosides	75 (42.8-94.5)	16.7 (2-48.4)	58.3 (27.6-84.8)	83.3 (51.5-97.9)	50 (21-78.9)	66.6 (34.8-90)	75 (42.8-94.5)	66.6 (34.8-90)	0
	Lincosamides	75 (42.8-94.5)	100 (73.5-100)	83.3 (51.6-97.9)	33.3 (9.9-65.1)	75 (42.8-94.5)	50 (21-78.9)	50 (21-78.9)	58.3 (27.6-84.8)	100 (73.5-100)

Nitrofurans	66.6 (34.8-90)	8.33 (2-38.4)	41.6 (15.1-72.3)	41.6 (15.2-72.3)	8.3 (2-38.5)	0	16.67 (2-48.41)	33.3 (9.9-65.1)	0

*95% confidence interval (CI) is an exact confidence interval based on the binomial distribution

Table 4.3

Percentage (95% confidence intervals) of breeding sows (*n*=18) yielding antimicrobial-resistant coliforms or enterococci.

Fecal bacteria	Antimicrobials	Age, days								
		21	42	70	84	154	224	343	350	371
Coliforms	Aminopenicillins	100 (81.4-100)	94.4 (72.7-99.8)	100 (81.4-100)	66.6 (40.9-86.6)	94.4 (72-99.8)	100 (81.4-100)	100 (81.4-100)	88.8 (65.3-98.6)	100 (81.4-100)
	3rd generation cephalosporins	100 (81.4-100)	88.9 (65.3-98.6)	77.8 (52.3-93.5)	83.3 (58.6-96.4)	61.1 (35.7-82.7)	44.4 (21.5-69.2)	83. 3 (58.6-96.4)	61.1 (35.7-82.7)	77.7 (52.5-93.6)
	Tetracyclines	100 (81.4-100)	100 (81.4-100)	88.8 (65.3-98.6)	100 (81.4-100)	100 (81.4-100)	100 (81.4-100)	100 (81.4-100)	100 (81.4-100)	100 (81.4-100)
	Macrolides	77.8 (52.4-93.6)	77.7 (52.36-93.5)	61.1 (35.7-82.7)	44.4 (21.5-69.2)	44.4 (21-69.2)	83.3 (58.5-96.4)	72.2 (46.5-90.3)	72.2 (46.5-90.3)	88 (65-98)
	Aminoglycosides	100 (81.4-100)	88.9 (65.3-98.6)	83.3 (58.5-96.42)	88.8 (65.3-98.6)	72.2 (46.5-90.3)	94.4 (72.7-99.8)	100 (81.4-100)	94.4 (72.7-99.8)	38.9 (17.3-64.3)
	Sulfonamides	94.4 (72.7-99.8)	94.4 (72.7-99.8)	72.2 (46.5-90.3)	55.6 (30.7-78.5)	83.3 (58.6-96.4)	88.8 (65-98.6)	94.4 (72.7-99.8)	83.3 (58.6-96.4)	88.8 (65.3-98.6)
	Phenicols	77.8 (52.4-93.6)	66.6 (40.9-86.65)	50 (26-73.9)	27.7 (9.6-53.5)	0	16.6 (3.5-41.4)	38.8 (17.2-64.3)	22 (6.4-47.63)	72.2 (46.5-90.3)
Enterococci	Aminopenicillins	38.8 (17.3-64.3)	22.2 (6.4-47.6)	33.3 (13.3-59)	33.3 (13.4-59)	33.3 (13.3-59)	33.3 (13.3-59)	27.7 (9.7-53.5)	16.6 (3.5-41.4)	0
	Quinolones	94.4 (72.7-99.9)	83.3 (58.6-96.4)	94.4 (72.7-99.8)	61.1 (35.7-82.7)	61.1 (35.5-82.7)	77.7 (52.3-93.5)	88.8 (65.3-98.6)	95 (72.7-99.8)	83.3 (58.5-96.4)
	Tetracyclines	94.4 (72.7-99.8)	100 (81-100)	88.8 (65.3-98.6)	61.1 (35.7-82.7)	83.3 (58.6-96.5)	94.4 (72.7-99.8)	100 (81.5-100)	100 (81.5-100)	94.4 (72.7-99.8)
	Macrolides	100 (81-100)	77 (52.3-93.5)	77 (52.3-93.5)	88.8 (65.3-98.6)	66.6 (0.4-0.86)	77.7 (52.3-93.5)	38.8 (17.3-64.2)	33.3 (13.3-59)	50 (26-73.9)
	Aminoglycosides	100 (81-100)	88.8 (65.3-98.6)	83.3 (58.6-96.4)	88.8 (65.3-98.6)	72.2 (46.5-90.3)	100 (81.5-100)	77.7	72.2 (46.5-90)	83 (58.6-96)

Lincosamides	100 (81.5-100)	66.6 (40-86.7)	55.6 (30.7-78.6)	83.3 (58.58-96.4)	72.2 (46.5-90)	88.8 (65.3-98.6)	61.1 (35.7-82.7) (52.4-93.6)	66.6 (40-86.6)	100 (81.5-100)
Nitrofurans	33.3 (13.3-59)	50 (26-73.9)	50 (26-73.9)	38.8 (17.3-64.3)	22.2 (6.5-47.63)	27.7 (9.6-53.5)	27.7 (9.6-53.5)	33.3 (13.3-59)	50 (26-73.9)

*95% confidence interval (CI) is an exact confidence interval based on the binomial distribution

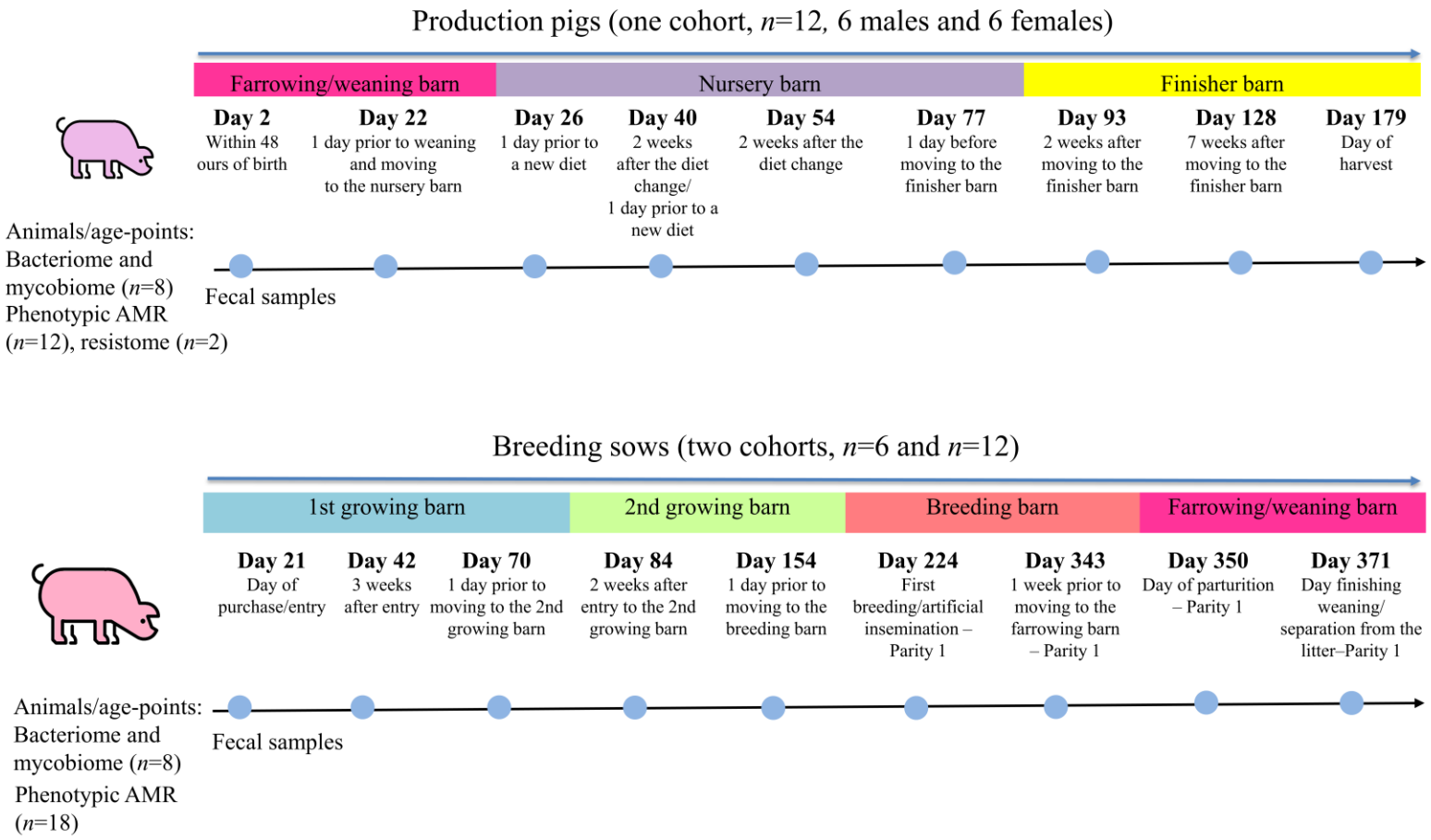

Figure 4.1. Schematic of the sampling design of the longitudinal studies of production pig and breeding sow cohorts. Fecal microbiome (bacteriome), mycobiome, and phenotypic AMR were evaluated at each age-point sampled. In the production pig cohort, 6 males and 6 females *n*= 12 animals were sampled at each of 9 age-points, from 2 days old to harvest at 6 months. In breeder sows, two cohorts of pigs (6 pigs in 1st cohort and 12 pigs in 2nd cohort) were sampled at each of 9 age-points from day 21 of age to first farrowing and weaning. Microbiome and mycobiome analysis were performed on the fecal samples from all 9 age-points per pigs from production pigs (4 males and 4 females, *n*=8 pigs, and *n*=72 fecal samples) and breeding sows (4 pigs cohort 1 and 4 cohort 2, *n*=8 pigs and *n*=72

fecal samples). Phenotypic AMR on fecal bacteria (coliforms and enterococci) were performed from the cohort of production pigs (*n*=12) at 9 age-points (*n*=108 fecal samples), and cohorts of breeding sows (*n*=18) at 9 age-points (*n*=162 fecal samples), and fecal samples (*n*=18) from the cohort of production pigs (*n*=2) at 9 age-points per pig were subjected to shotgun metagenomic sequencing for evaluating age related dynamic of fecal resistome.

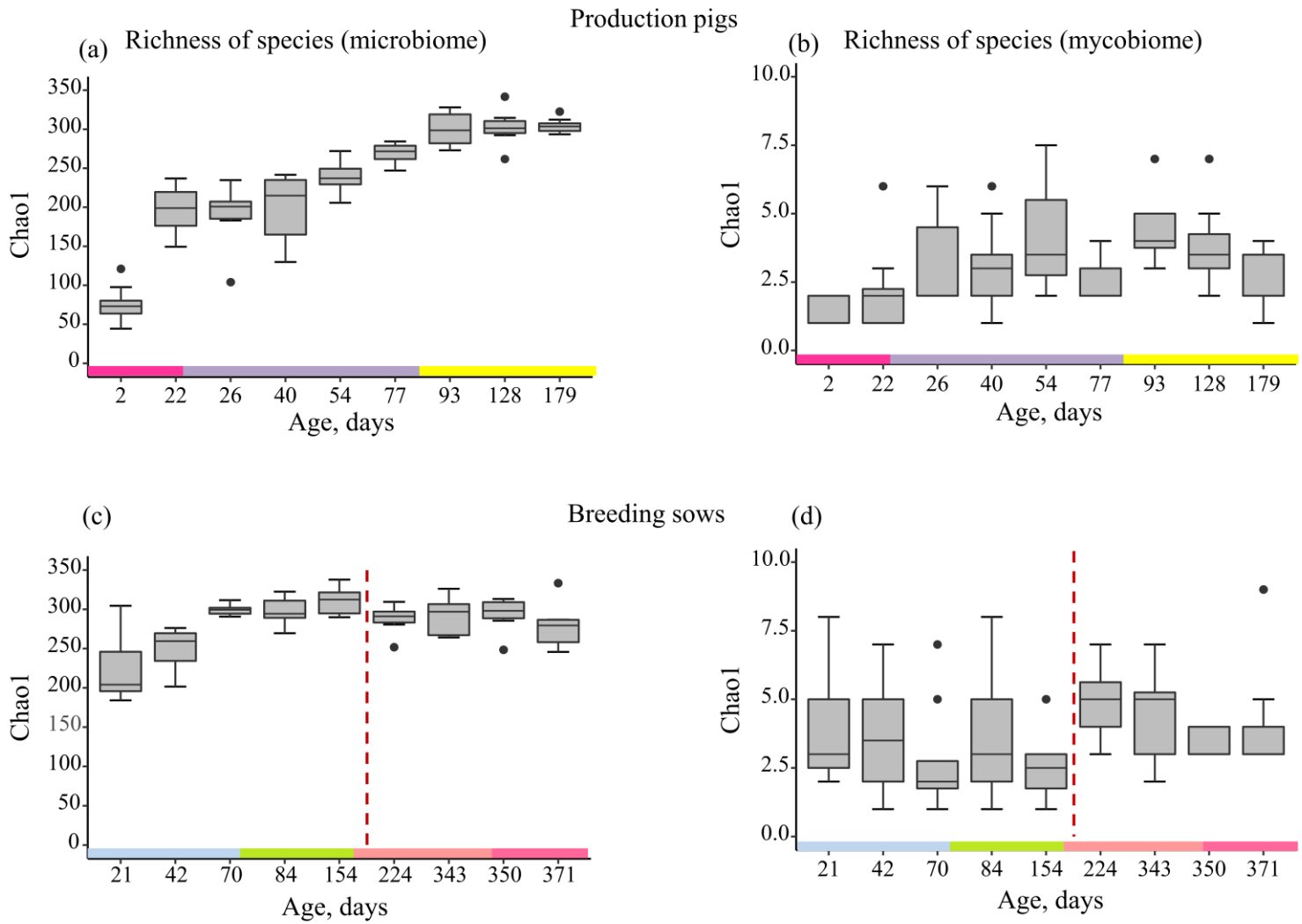

Figure 4.2. Alpha diversity described as richness (Chao1 index) of the fecal microbiome (a, c) and mycobiome (b, d) in production pigs (*n*=8) and breeding sows (*n*=8). The color bar above the X-axis represents the pig housing facility; for production pigs: pink—farrowing and weaning, light purple—nursery, and yellow—finisher; and for breeding sows: blue—1st growing facility, light green—2nd grower

facility, orange—breeding barn, and pink—farrowing and weaning barn. The red dotted line shows overlapping age-points between the cohorts of production pigs and breeding sows.

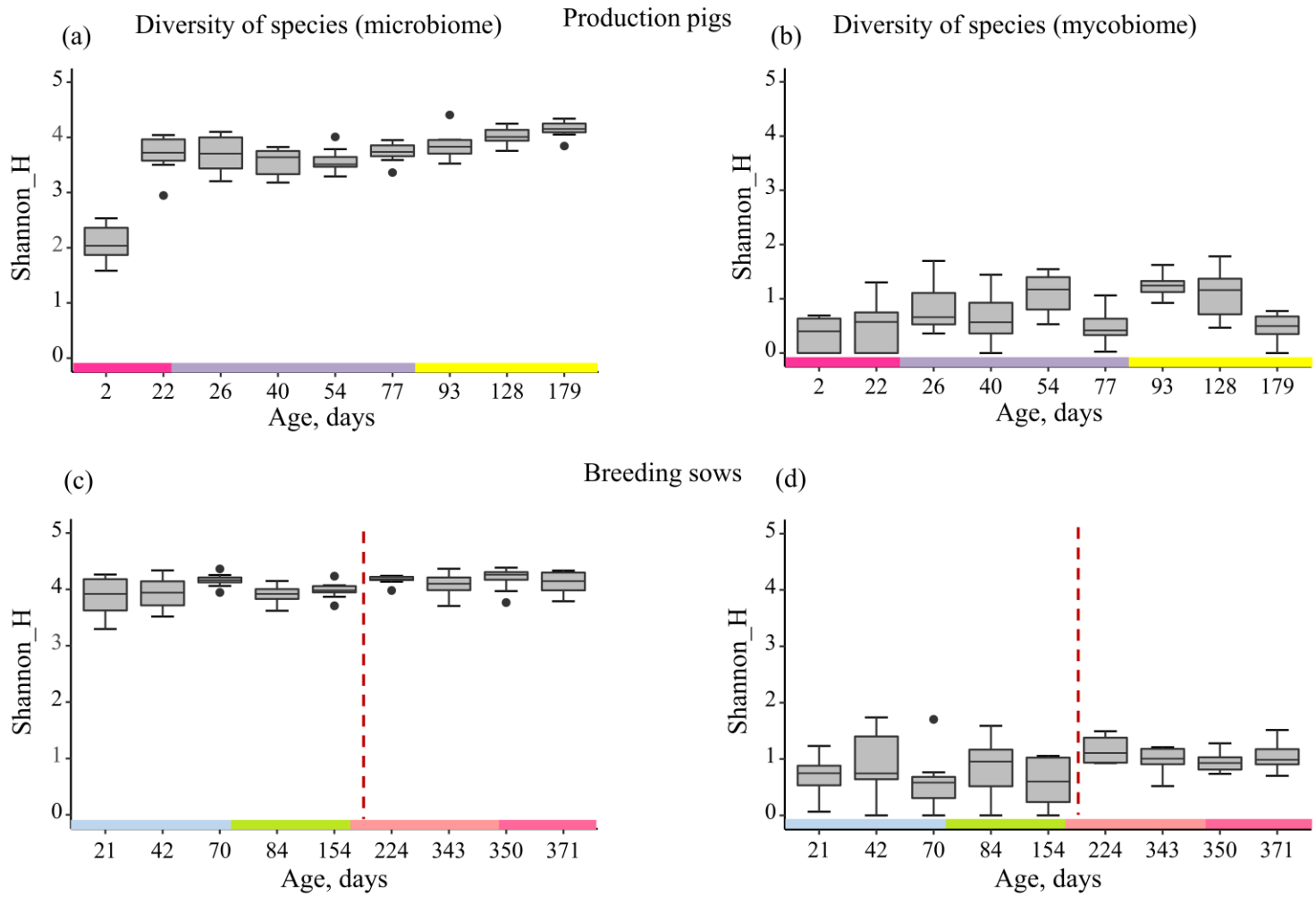

Figure 4.3. Alpha diversity described as diversity (Shannon index) of the fecal microbiome (a, c) and mycobiome (b, d) in production pigs (*n*=8) and breeding sows (*n*=8). The color bar above the X-axis represents the pig housing facility; for production pigs: pink—farrowing and weaning, light purple—nursery, and yellow—finisher; and for breeding sows: blue—1st growing facility, light green—2nd

grower facility, orange—breeding barn, and pink—farrowing and weaning barn. The red dotted line shows overlapping age-points between the cohorts of production pigs and breeding sows.

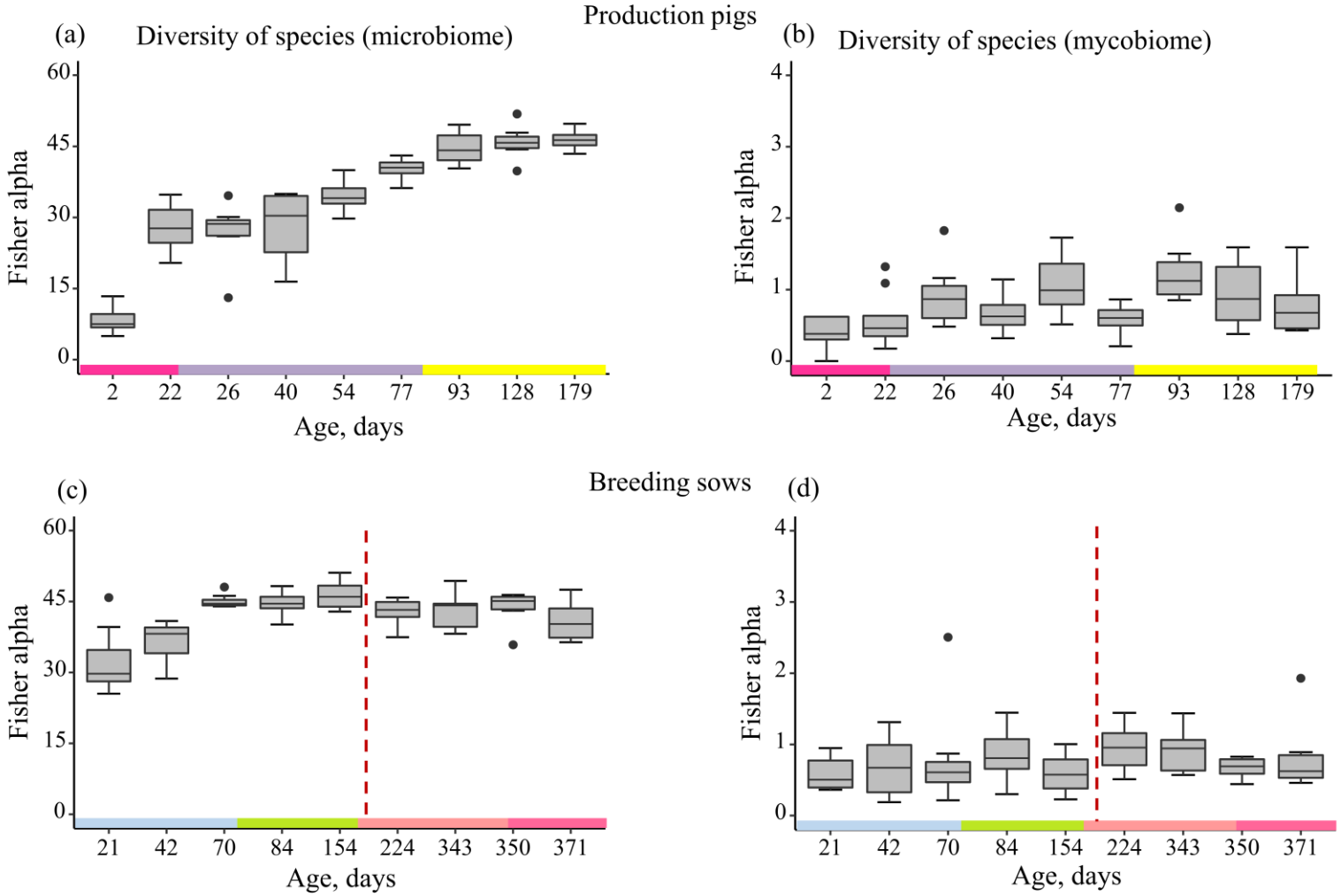

Figure 4.4. Alpha diversity described as diversity (Fisher alpha index) of the fecal microbiome (a, c) and mycobiome (b, d) in production pigs (*n*=8) and breeding sows (*n*=8). The color bar above the X-axis represents the pig housing facility; for production pigs: pink—farrowing and weaning, light purple—nursery, and yellow—finisher; and for breeding sows: blue—1st growing facility, light green—2nd

grower facility, orange—breeding barn, and pink—farrowing and weaning barn. The red dotted line shows overlapping age-points between the cohorts of production pigs and breeding sows.

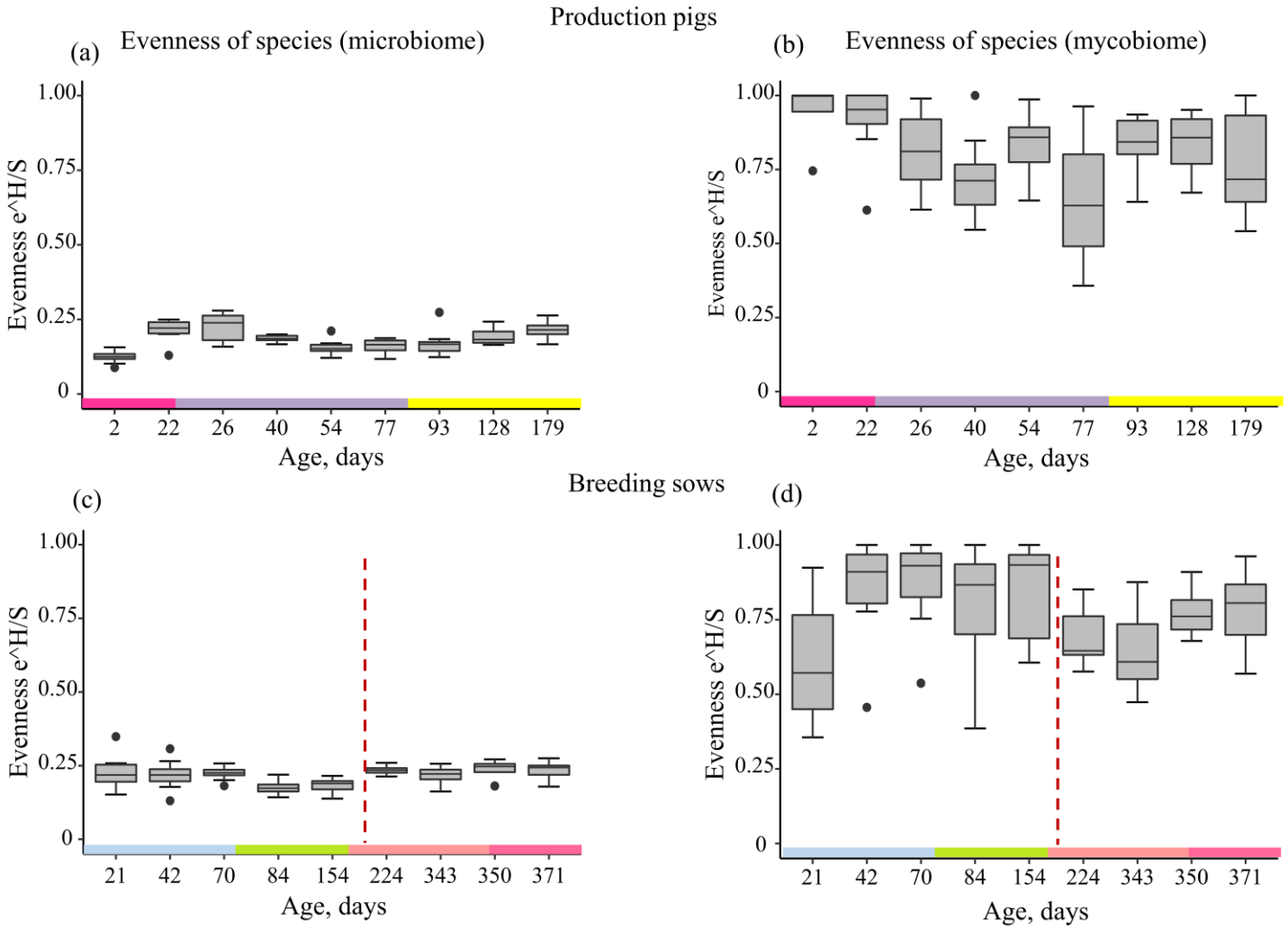

Figure 4.5. Alpha diversity described as evenness (evenness index) of the fecal microbiome (a, c) and mycobiome (b, d) in production pigs (*n*=8) and breeding sows (*n*=8). The color bar above the X-axis represents the pig housing facility; for production pigs: pink—farrowing and weaning, light purple—nursery, and yellow—finisher; and for breeding sows: blue—1st growing facility, light green—2nd

grower facility, orange—breeding barn, and pink—farrowing and weaning barn. The red dotted line shows overlapping age-points between the cohorts of production pigs and breeding sows.

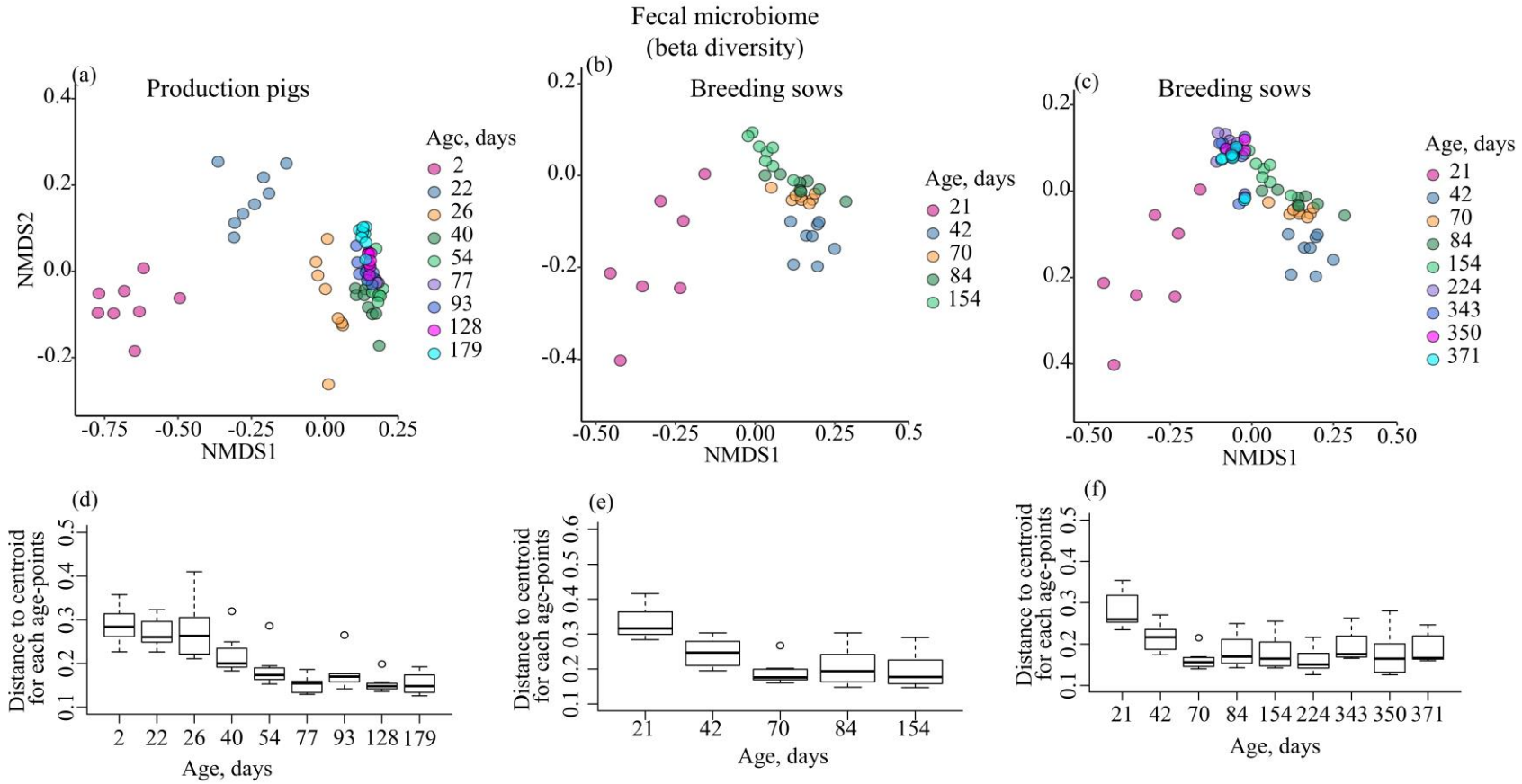

Figure 4.6. Beta diversity of fecal microbiome composition in production pigs (*n*=8) and breeding sows (*n*=8) by age. Non-metric multidimensional scaling (NMDS) plots based on the Bray-Curtis distances (a-c), and boxplot of the distances to age specific centroid for each of the age-points in production pigs (d), breeding sows (day 21 through day 154 of age, overlapping age-points with production pigs) (e), and breeding sows (day 21 through day 371)) (f).

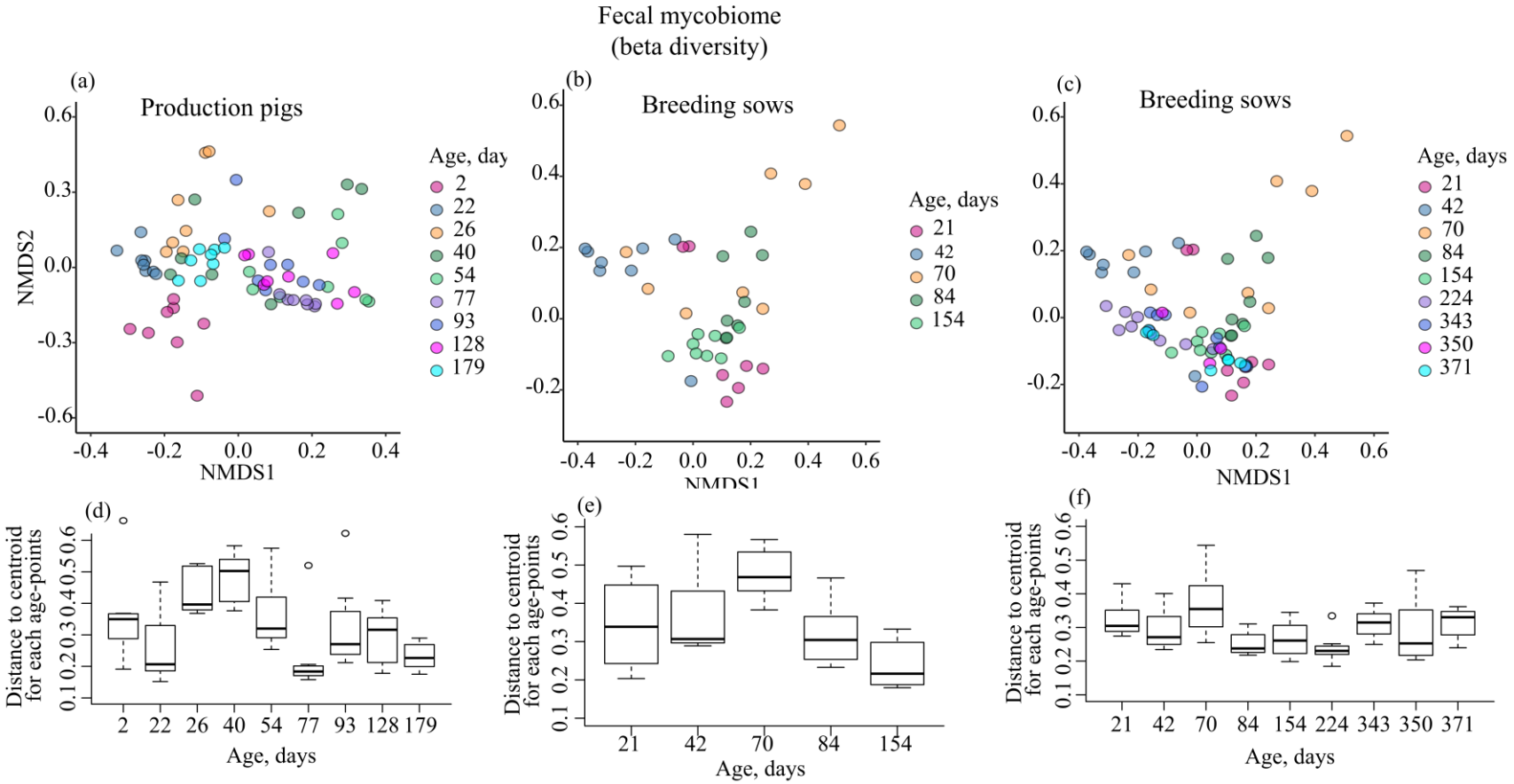

Figure 4.7. Beta diversity of fecal mycobiome composition in production pigs (*n*=8) and breeding sows (*n*=8) by age. Non-metric multidimensional scaling (NMDS) plots based on the Bray-Curtis distances (a-c), and boxplot of the distances to age specific centroid for each of the age-points in production pigs (d), breeding sows (day 21 through day 154 of age, overlapping age-points with production pigs) (e), and breeding sows (day 21 through day 371)) (f).

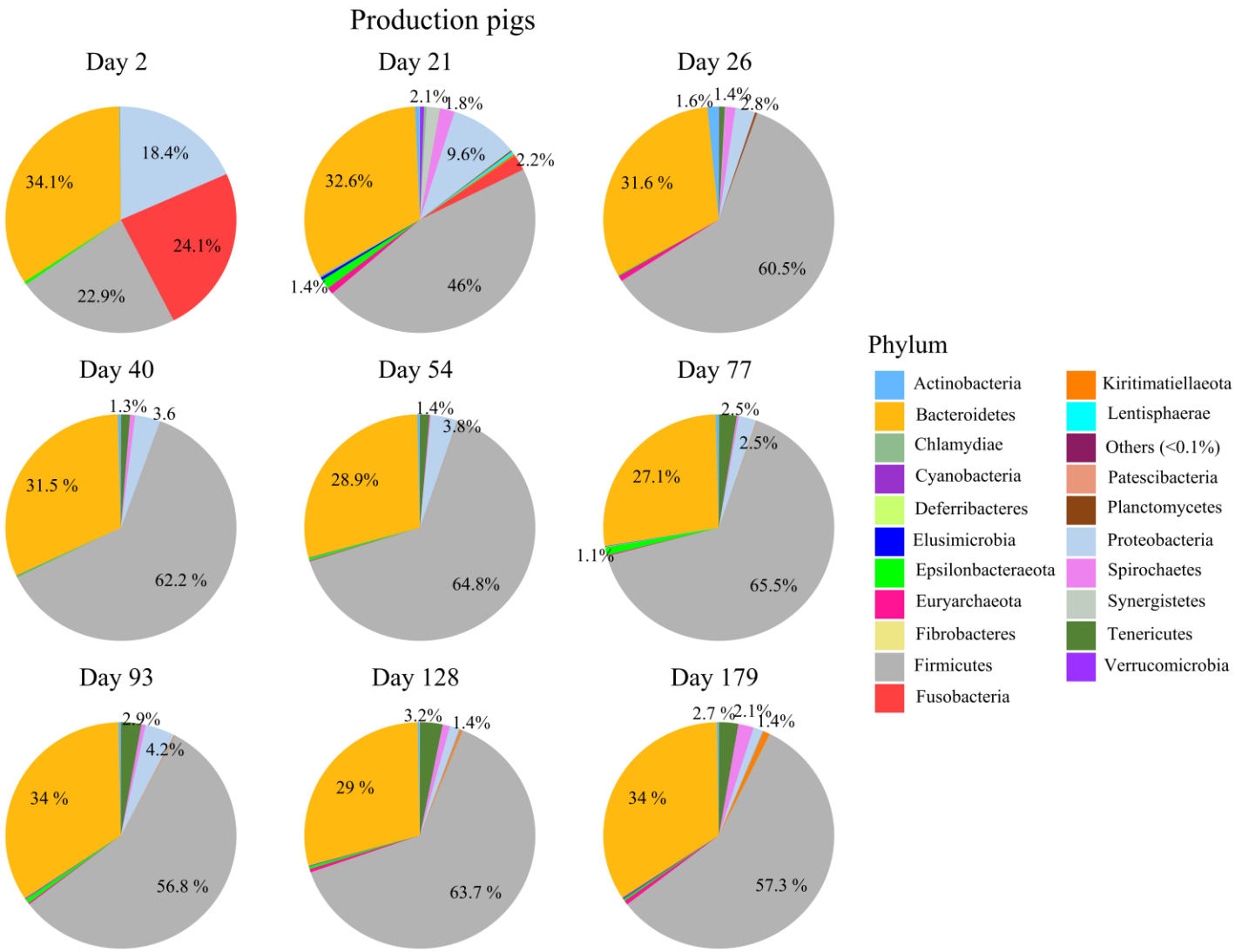

Figure 4.8. Taxonomic composition of the fecal microbiome (relative abundance of individual phyla) in production pigs (*n*=8) at nine age-points.

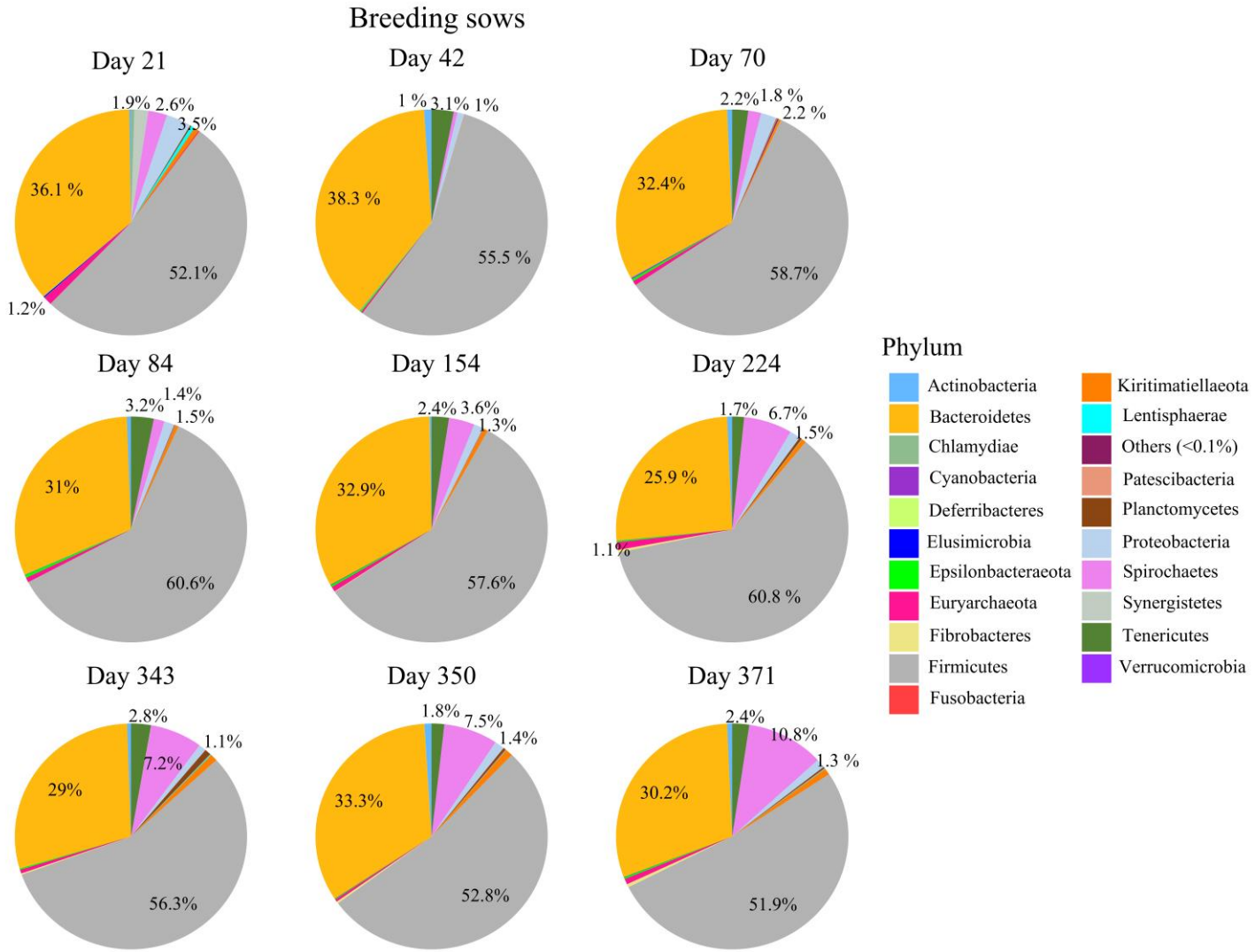

Figure 4.9. Taxonomic composition of the microbiome (relative abundance of individual phyla) in breeder sows (*n*=8) at nine age-points.

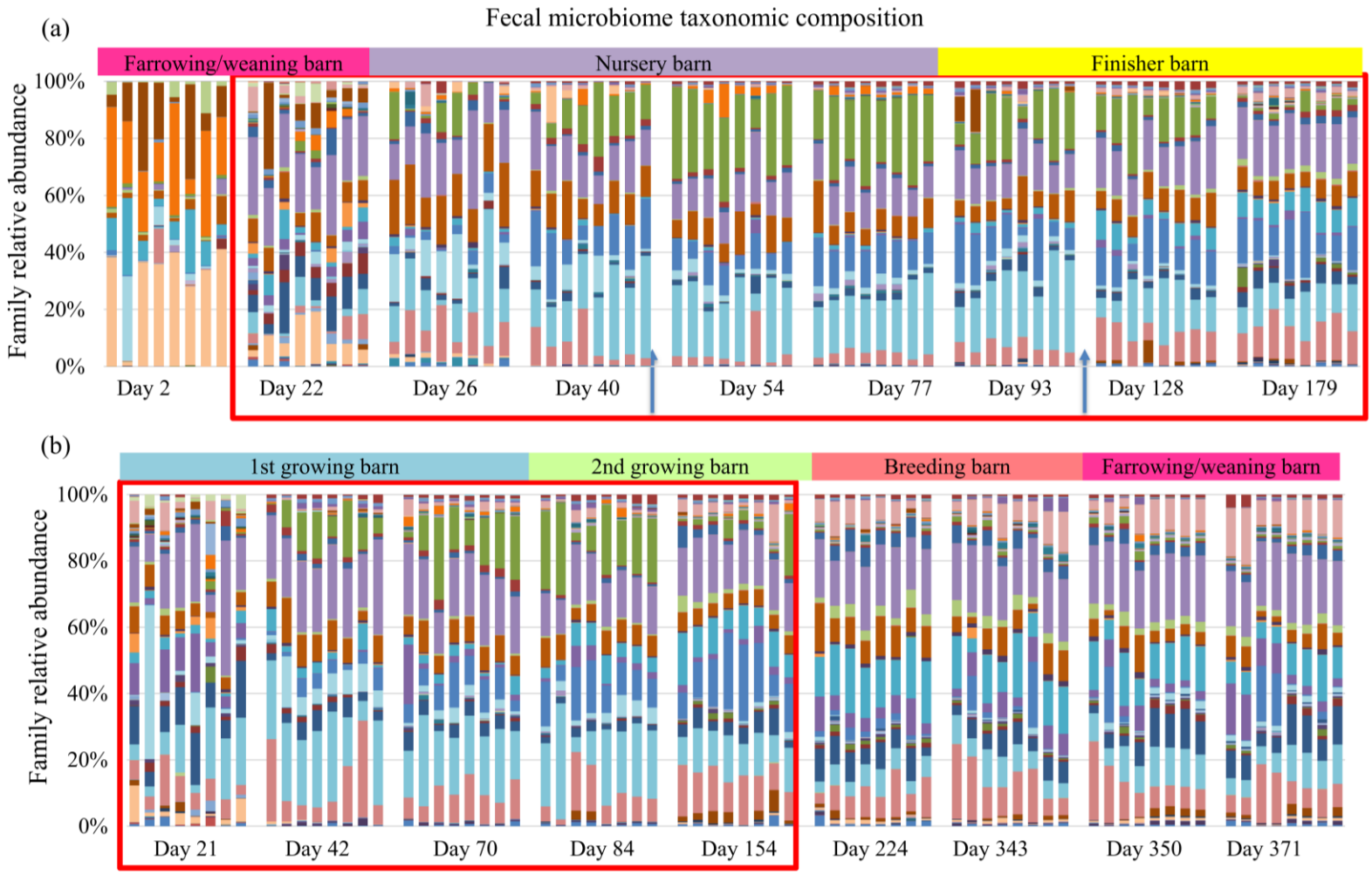

Figure 4.10. Taxonomic composition (relative abundance) of the microbiome at the family level in production pigs (a) and breeding sows (b). The rectangular red box represents the overlapping age-points between cohorts of production pigs (*n*=8) and breeding sows (*n*=8). The arrows indicate dietary changes during the production stage.

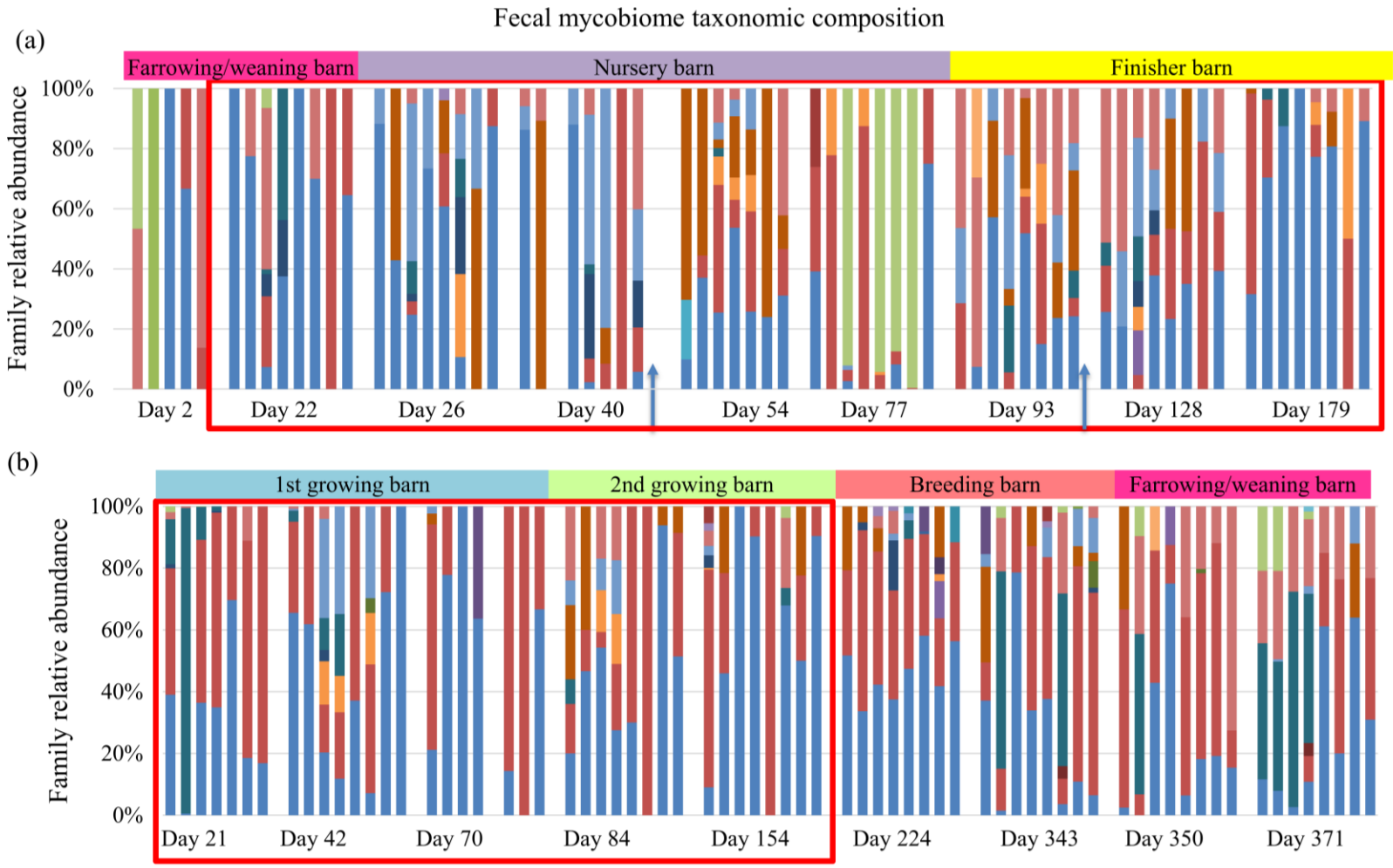

Figure 4.11. Taxonomic composition (relative abundance) of the mycobiome at the family level in production pigs (a) and breeding sows (b). The rectangular red box represents the overlapping age-points between cohorts of production pigs (*n*=8) and breeding sows (*n*=8). The arrows indicate dietary changes during the production stage

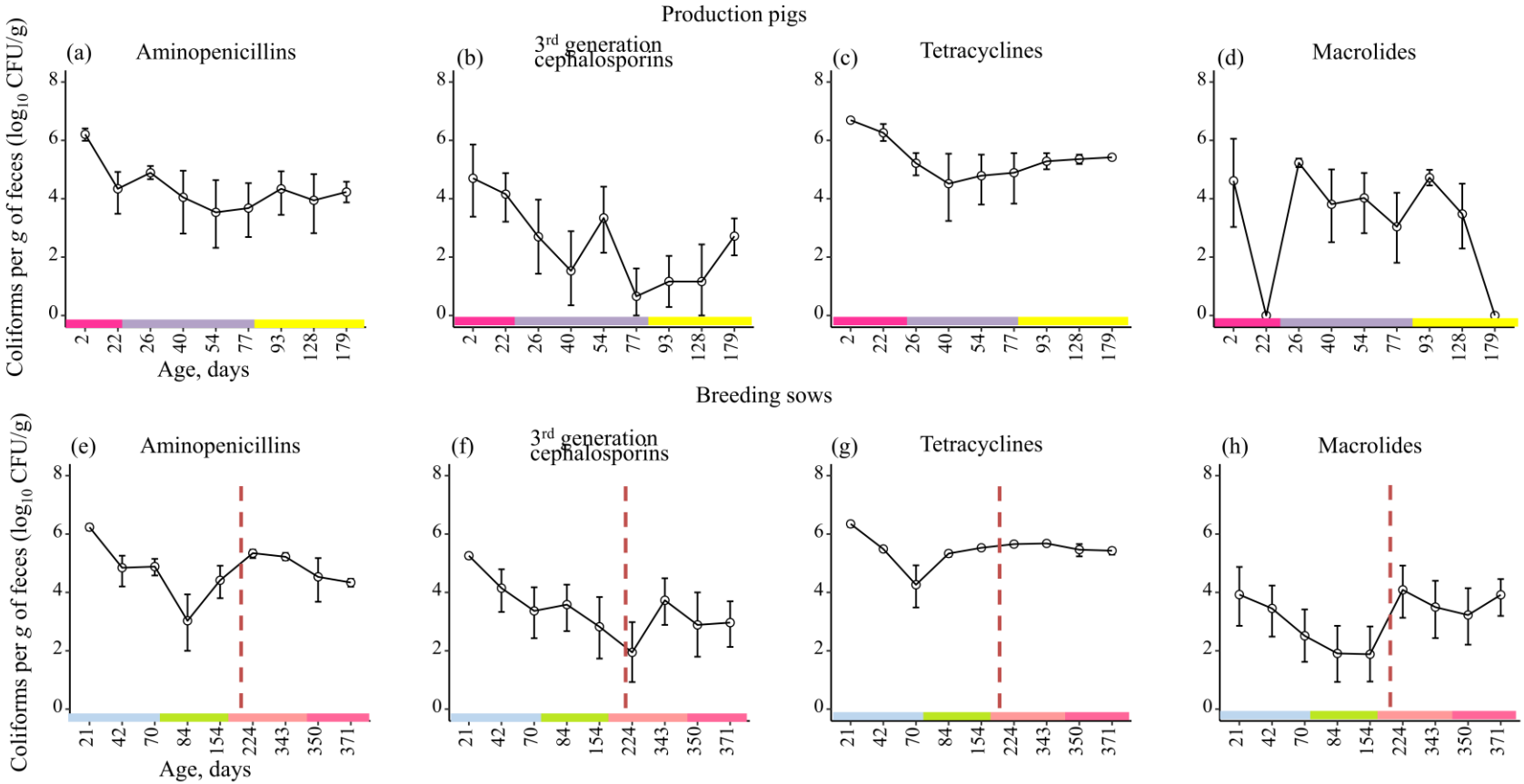

Figure 4.12. Phenotypically antimicrobial-resistant fecal coliforms (mean, 95% CI of the log-transformed viable coliform counts growing in the presence of the "clinical break-point" concentration of an antimicrobial of that class) for aminopenicillins, 3rd generation cephalosporins, tetracycline, and macrolides, as a function of pig age in production pigs (a-d) and breeding sows (e-h). The color bar above the X-axis represents the pig housing facility; for production pigs: pink—farrowing and weaning, light purple—nursery, and yellow—finisher; and for breeding sows: blue—1st growing facility, light green—2nd grower facility, orange—breeding barn, and pink—farrowing and weaning barn. The red dotted line shows overlapping age-points between the cohorts of production pigs and breeding sows.

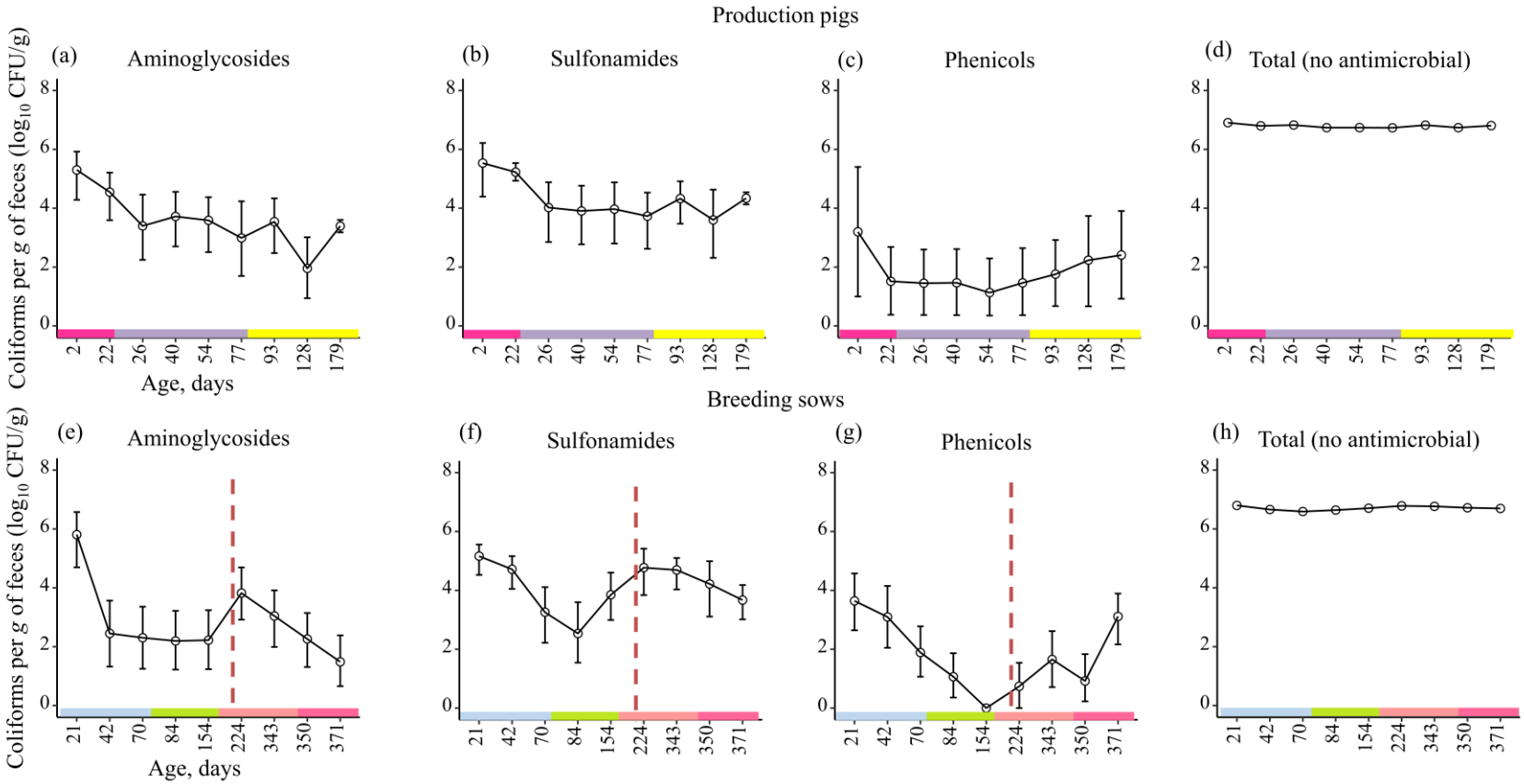

Figure 4.13. Phenotypically antimicrobial-resistant fecal coliforms (mean, 95% CI of the log-transformed viable coliform counts growing in the presence of the "clinical break-point" concentration of an antimicrobial of that class for aminoglycosides, sulfonamides, phenicols) and total coliforms (no antimicrobial) as a function of pig age in production pigs (a-d) and breeding sows (e-h). The color bar above the X-axis represents the pig housing facility; for production pigs: pink—farrowing and weaning, light purple—nursery, and yellow—finisher; and for breeding sows: blue—1st growing facility, light green—2nd grower facility, orange—breeding barn, and pink—farrowing and weaning barn. The red dotted line shows overlapping age-points between the cohorts of production pigs and breeding sows.

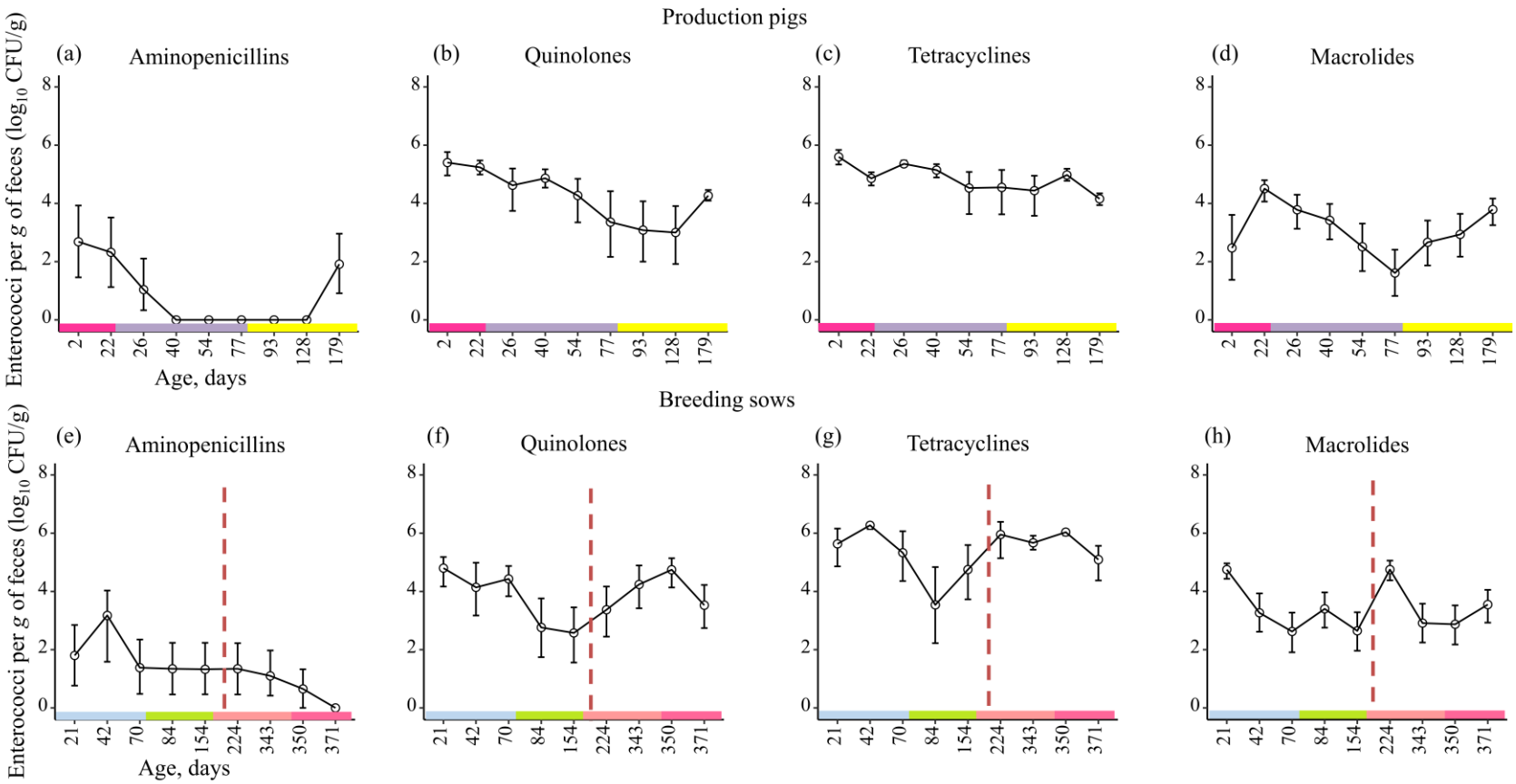

Figure 4.14. Phenotypically antimicrobial-resistant fecal enterococci (mean, 95% CI of the log-transformed viable enterococci counts growing in the presence of the "clinical break-point" concentration of an antimicrobial of that class for aminopenicillins, quinolones, tetracyclines, and macrolides) as a function of pig age in production pigs (a-d) and breeding sows (e-f). The color bar above the X-axis represents the pig housing facility; for production pigs: pink—farrowing and weaning, light purple—nursery, and yellow—finisher; and for breeding sows: blue—1st growing facility, light green—2nd grower facility, orange—breeding barn, and pink—farrowing and weaning barn. The red dotted line shows overlapping age-points between the cohorts of production pigs and breeding sows.

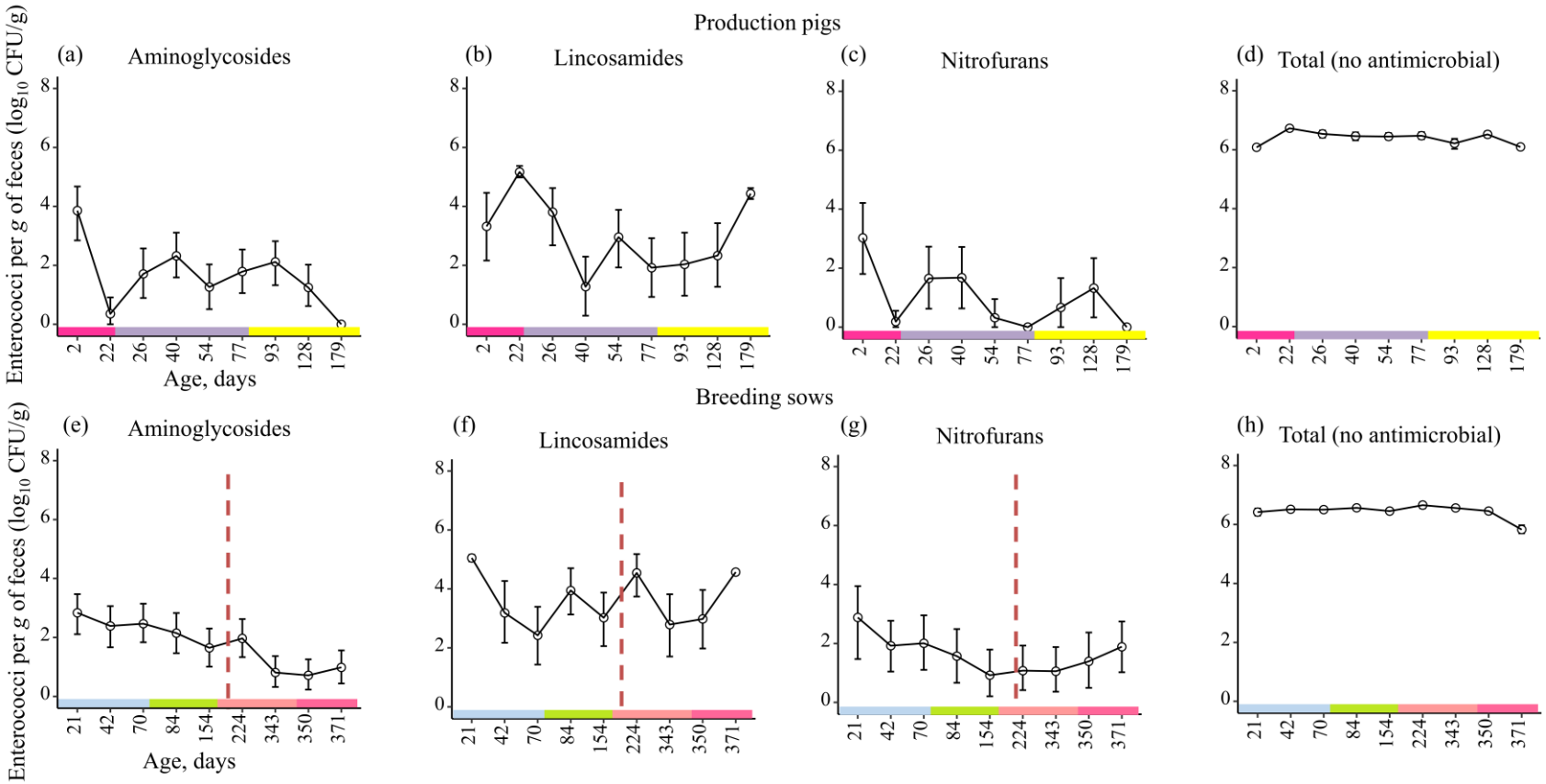

Figure 4.15. Phenotypically antimicrobial-resistant fecal enterococci (mean, 95% CI of the log-transformed viable enterococci counts growing in the presence of the "clinical break-point" concentration of an antimicrobial of that class for aminoglycosides, lincosamides, nitrofurans) and total enterococci (no antimicrobial) as a function of the age of production pigs (a-d) and breeding sows (e-f). The color bar above the X-axis represents the pig housing facility; for production pigs: pink—farrowing and weaning, light purple—nursery, and yellow—finisher; and for breeding sows: blue—1st growing facility, light green—2nd grower facility, orange—breeding barn, and pink—farrowing and weaning barn. The red dotted line shows overlapping age-points between the cohorts of production pigs and breeding sows.

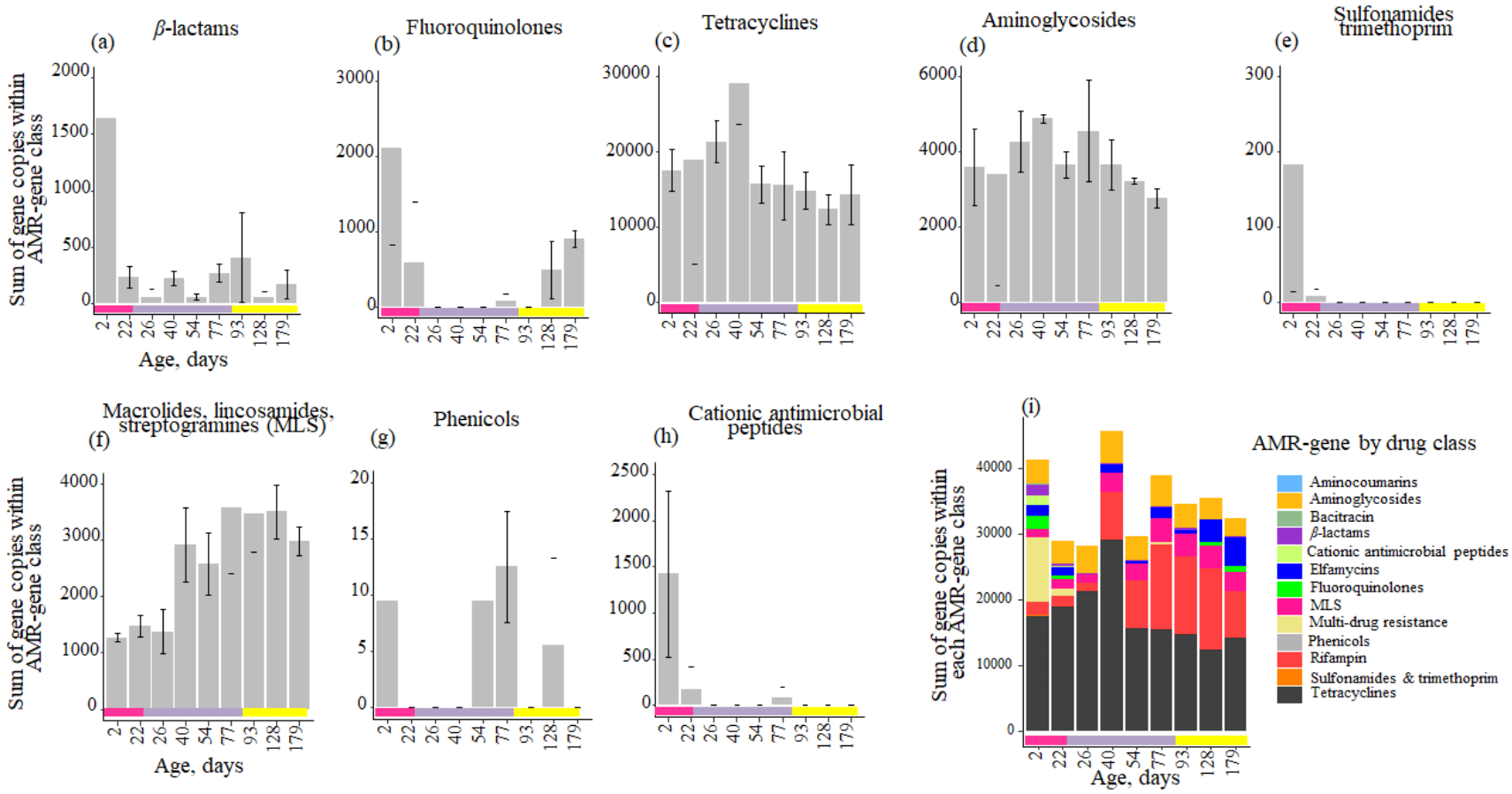

Figure 4.16. The age-dependent dynamics of fecal resistome in production pigs (*n*=2). The color bar above the X-axis represents the pig housing facility: pink—farrowing and weaning, light purple—nursery, and yellow—finisher.

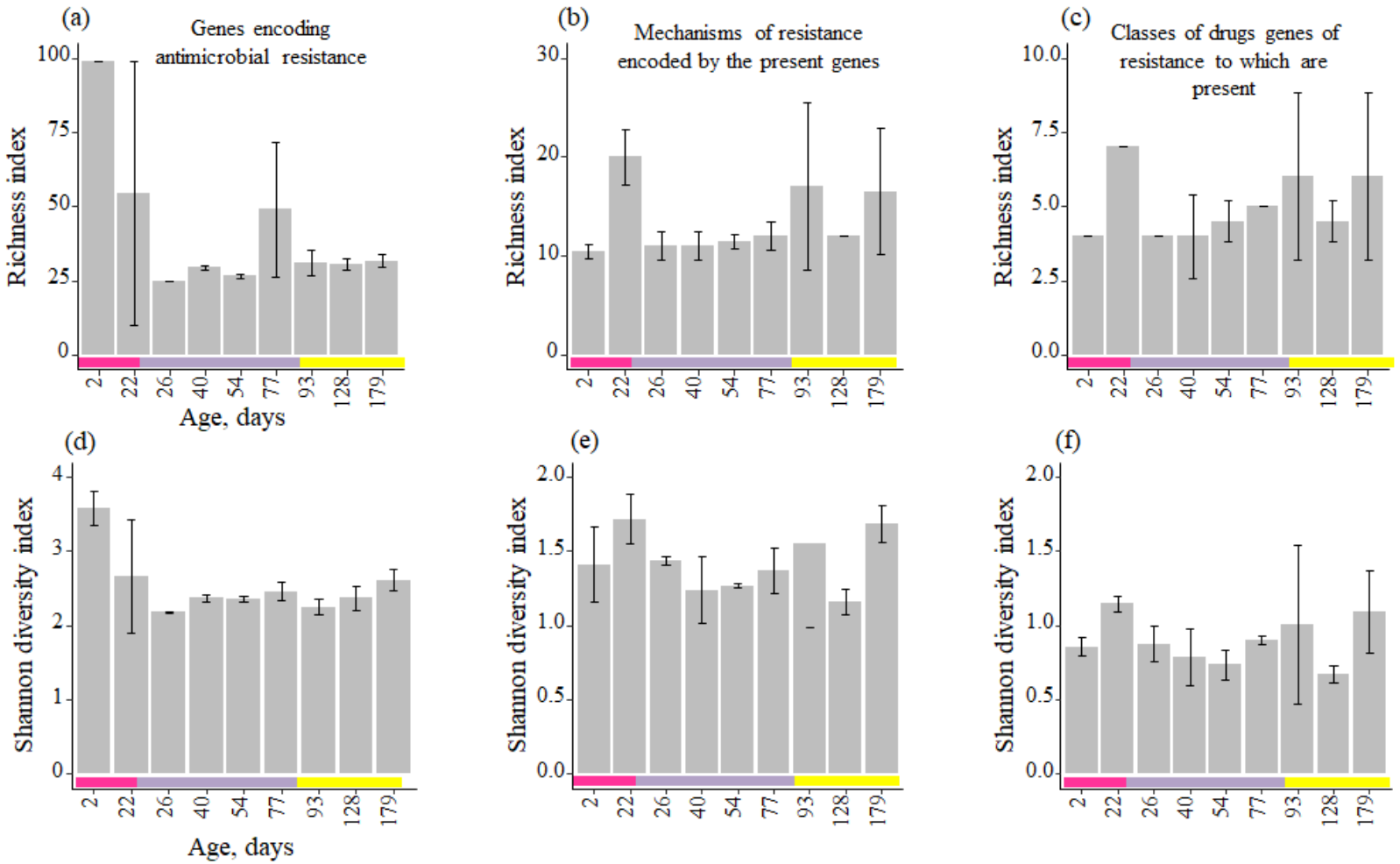

Figure 4.17. The richness (a-c) and diversity (d-f) of fecal resistome in production pigs (*n*=2) by age; genes encoding antimicrobial resistance (i.e., ARGs) (a, d), mechanisms of resistance encoded by the present genes (b, e) and classes of drugs genes of resistance to which are present (c, f). The color bar above the X-axis represents the pig housing facility: pink—farrowing and weaning, light purple—nursery, and yellow—finisher.

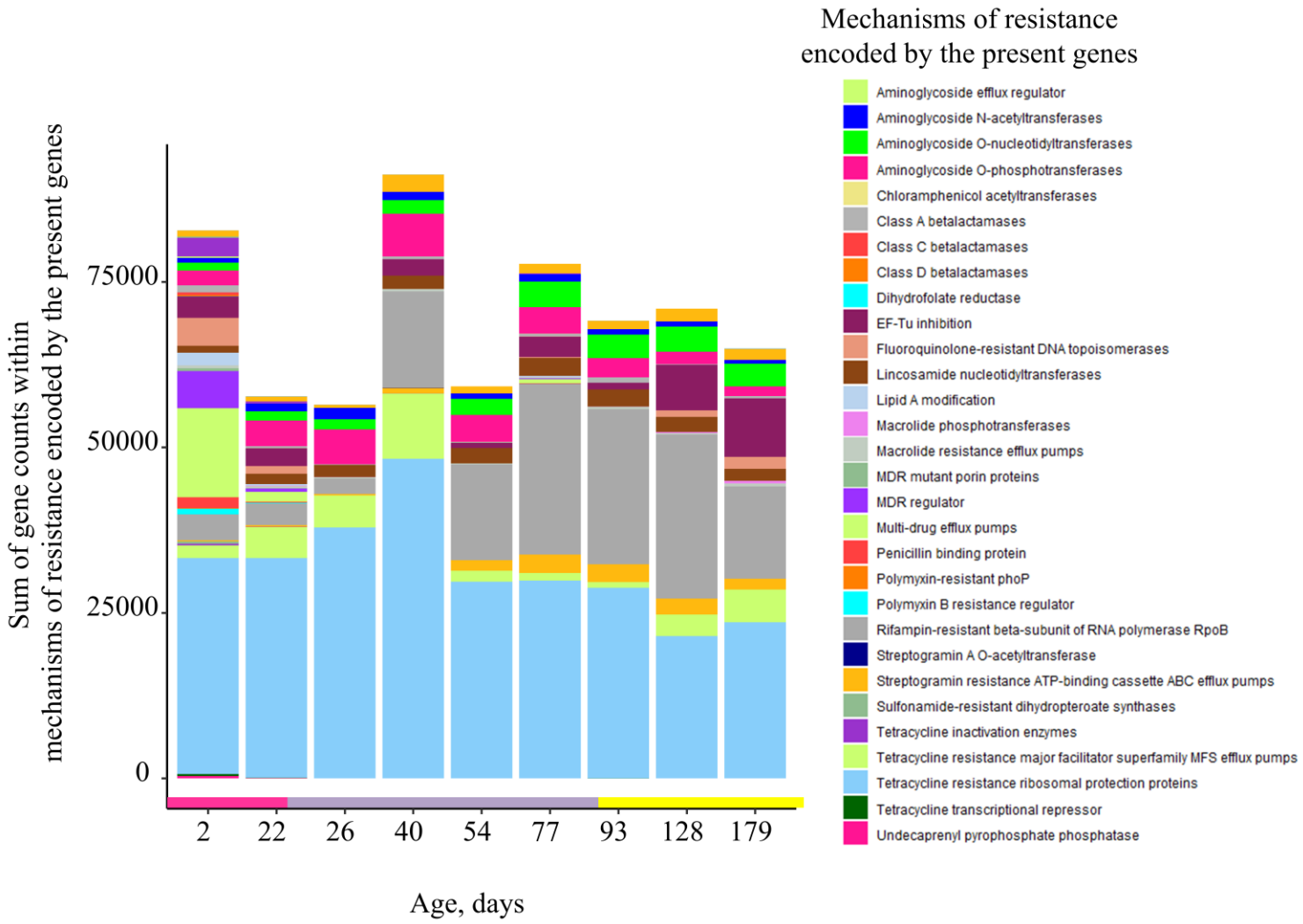

Figure 4.18. The mechanism of resistance encoded by the present AMR genes of fecal resistome in production pigs (*n*=2) by age; The color bar above the X-axis represents the pig housing facility: pink—farrowing and weaning, light purple—nursery, and yellow — finisher.

Chapter 5 - A Longitudinal Investigation of Age-related Dynamics and the Effects of Diet and Antimicrobial Treatments on the Fecal Microbiome and Antimicrobial Resistance of Finisher Pigs

Abstract

Age and diet are the main factors influencing the composition of the fecal microbiome. The use of antimicrobials can alter the taxonomic composition of bacterial communities. This study aimed to evaluate age-related dynamics, effects of the diet, and antimicrobial treatments on the fecal microbiome or antimicrobial resistance. Pigs at the finishing stage were randomly distributed to 36 pens, and the pens were randomly assigned to 1 of 3 dietary treatments with different levels and sources of fiber contents, a) control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), b) DDGS-distillers dried grains with solubles based diet (20% DDGS with 13.6% NDF and 3.1% crude fiber and primarily contains insoluble fiber), and c) sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber and primarily contains soluble fiber). Five finisher pigs from each treatment groups were selected randomly, and fecal samples were collected on day 98, 110, 144, and 179 (day of harvest). Also, fecal samples were collected from pigs that received injectable ceftiofur hydrochloride or penicillin G day 1 and day 3 after injection along with pen-mate untreated control. Fecal samples were subjected to 16s rRNA amplicon-based microbiome analysis and culture methods to quantify the abundance of total and antimicrobial-resistant coliforms and enterococci. The alpha diversity, such as species richness, significantly increased with pig age, and the overall bacterial composition changed with age (p <0.001) and diet (p < 0.001). A diet-

specific shift in the microbial community was observed. Neither ceftiofur nor penicillin G changed the richness, diversity, and evenness of the taxa; however, the antibiotics contributed to an altered overall fecal bacterial taxonomic composition and specific taxa. Our data suggest that age and diet affect antimicrobial resistance (AMR) in fecal bacteria.

Introduction

The composition of the gut microbial community is strongly influenced by several factors, including the age of animals, host immune and genetics factors, antimicrobial use (Looft et al., 2012b; Relman, 2012), and dietary effects (Scott et al., 2013). The study found that diet, especially crude fiber, was a significant factor shaping the pig gut microbiome (Wang et al., 2019). The microbial colonization of the pig intestine started at birth and developed during the neonatal period to weaning time with a sharp increase following the transition to solid food after weaning (De Rodas et al., 2018). However, the microbial ecosystem is dynamic and significantly altered after weaning, which can sometimes lead to enteric dysbiosis (Lalles et al., 2007; Mann et al., 2014).

The relative abundance of commensal bacteria such as *Lactobacillus* is reduced after weaning; this, in turn, allows pathogenic bacteria to adhere and proliferate in the intestine (Heo et al., 2013). Thus, antibiotics and minerals are often used in diets to prevent disease (Verstegen and Williams, 2002). However, use of antibiotics in a therapeutic setting can cause selective pressure that may drive the development of antimicrobial resistance (AMR) due to horizontal gene transfer or mutation (Martínez, 2008). Thus, due to the growing concerns over AMR, alternative approaches such as pre/probiotics and dietary interventions have been investigated. Therefore, studies investigating the role of dietary intervention in modulating the microbial population has received more attention. For instance, an increase in fiber in the diet has been shown to benefit gut microbial communities and improve the health and performance of pigs (Everts, 2010; Niu et al., 2019); these interventions could minimize AMR in animals. Lower fiber in the diet leads to a decrease in the microbiome diversity in humans (Flint et al., 2012). A

high fiber diet can significantly enrich *Bacteroidetes* and deplete *Firmicutes* (De Filippo et al., 2010).

Generally, microbes in animals at a young age were mainly fast-growing, grain fermenting types, while those in adult animals were fiber-digesting types (Huhtanen and Gall, 1953). In another study, De Rodas and colleagues (De Rodas et al., 2018) observed that the exposure to solid feed between days 21 and 33 had a more significant overall impact on microbial community structure than age and environment. Similarly, this study showed that the dietary interventions significantly reduced both the richness and diversity of the gut resistome in children (Wu et al., 2016). The study also showed that the moderate inclusion of dietary fiber in the diet—particularly protein levels of 20% crude protein (CP) supplemented with wheat bran and sugar beet pulp (2%)—increased production performance and gut maturation of the piglet with less incidence of diarrhea and need for antibiotic intervention (Hermes et al., 2009). Overall, these studies suggested that gut microbial colonization is influenced by diet and progresses with age.

The antibiotics commonly used in swine can markedly alter the microbial community. Examples include in-feed antimicrobials carbadox or a combination of chlortetracycline, sulfamethazine, and penicillin (Looft et al., 2012b). The third-generation cephalosporin ceftiofur is commonly used in swine as common preventive strategy for various bacterial diseases (Callens et al., 2012). An earlier study showed that the fecal microbiome of pigs that received ceftiofur returned to the initial microbiome two weeks after treatment (Zeineldin et al., 2018). However, the impact of antimicrobial treatment administration on the bacterial composition of pigs has not yet been clearly demonstrated, especially within a combination of dietary treatments. Here, we investigated age-related dynamics and the effects of diet (varying the fiber

contents) as well as the impact of a ceftiofur and penicillin G treatment (three-day treatment regimens). We characterize the longitudinal changes in the swine fecal microbiome with an abundance of AMR in coliforms and enterococci during the finishing stage.

Materials and methods

Study design and animals

The study was performed at the Kansas State University Swine Teaching and Research Center, Kansas State University Manhattan, Kansas. A total of 288 pigs (~36 kg starting body weight) from one finishing group were randomly distributed to 36 pens. Each pen was balanced by gender with 4 male and 4 female pigs. Pens were randomly assigned to 1 of 3 dietary treatments (different level and source of fiber): a) control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), b) distillers dried grains with solubles (DDGS)-based diet (20% DDGS with 13.6% NDF and 3.1% crude fiber and primarily contains insoluble fiber), and c) sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber and primarily contains soluble fiber). The dietary compositions are shown in (Table 5.1).

The diets were formulated and prepared at the Kansas State University O.H. Kruse Feed Technology Innovation Center (Manhattan, KS). All pigs of each experimental diet were followed throughout the finisher stage. When clinical signs of diarrhea, leg swelling, or other production-related diseases appeared, the pigs were treated with penicillin G or ceftiofur hydrochloride (Excenel®) by injection once a day for 1 to 3 days. The animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Kansas State University.

Longitudinal sampling of untreated pigs on the dietary treatments

Five pigs of each dietary treatment group (control, DDGS-based diet, and sugar beet pulp- based diet) were sampled longitudinally during the finishing phase. Fecal samples were collected per rectum from each of ~5 pigs at 4 age-points: at ~98 days (first week after moving into the finisher facility and associated diet change), 110 days (~ 2.5 weeks after moving into finisher facility), 144 days (~6 weeks after moving into finisher facility), and 177 days (day of harvest) (Figure 5.1) (Table 5.2). The feces and fecal aliquots mixed with 50% glycerol were frozen and stored at -80°C.

Ceftiofur and penicillin G treated pigs on the dietary treatments

The fecal samples were collected from ceftiofur hydrochloride- and penicillin G-treated pigs at two-time points of the 3-day treatment regimen. On days 1 and 3 of treatment, samples were collected approximately 6 hours (no later than 8 hours) after the intramuscular (IM) injection). A fecal sample was collected from one pen-mate untreated pig on day 1. However, some animals were only treated on days 1 and 2 when clinical improvement was seen; hence, only day 1 samples were collected.

A total of 40 fecal samples were collected from the ceftiofur-treated (n=25) and untreated pen-mate control (n=15) pigs. Specifically, on the control diet, 3 pigs were sampled on day 1 only and 3 pigs on both days 1 and 3 of the regimen along with 6 untreated pen-mate untreated pigs on day 1; on the DDGS based diet, 4 pigs were sampled on both day 1 and 3 of the regimen along with 4 untreated pen-mated pigs on day 1, and on the sugar beet pulp-based diet, 2 pigs were sampled on day 1 only and 3 pigs on both days 1 and 3 of the regimen along with 5 untreated pen-mated control pigs on day 1 (Figure 5.1) (Table 5.2).

Similarly, a total of 45 fecal samples were collected from the penicillin G treated (n=30 and untreated pen-mate control (n=15) pigs. Specifically, 5 pigs on each diet (control, DDGS

based diet, and sugar beet pulp based diet) were sampled on both day 1 and day 3 of the regimen along with 5 untreated pen-mated control pigs on day 1 on each diet (Figure 5.1) (Table 5.2). Fecal samples were transported to the laboratory for further processing, and the feces and fecal aliquots were mixed with 50% glycerol and stored at -80°C until DNA extraction, microbiome analysis, and quantification AMR was performed.

16s rRNA based microbiome analysis

Fecal DNA extraction

A total of 140 fecal samples (*n*=55 from pigs not treated with ceftiofur, *n*=40 from ceftiofur-treated pigs along with untreated pen-mate control, and *n*=45 from penicillin G treated pigs along with untreated pen-mate control) were collected. Fecal DNA was extracted using the protocol published by (Yu and Morrison, 2004) and (Korte et al., 2020). Briefly, fecal samples were transferred into round-bottom tubes (2 mL) containing 800 µL of lysis buffer and a single steel bead (0.5 cm diameter). Samples were then heated at 70°C for 20 minutes with vortexing, then homogenized using a TissueLyser II (Qiagen, Venlo, the Netherlands) for 3 minutes at 30 per second, and then centrifuged at 5000 × g for 5 minutes at room temperature. The supernatant then transferred into the new Eppendorf tube (1.5 mL), added with 200 µL of 10 mM ammonium acetate, incubated for 5 minutes on ice, and then centrifuged at 5000 × g for 5 minutes. The supernatant (up to 750 µL) was mixed with an equal volume of isopropanol, incubated for 30 minutes on ice, and centrifuged at 16,000 × g at 4°C for 15 minutes. The recovered DNA pellet was washed and resuspended in 150 µL of Tris-EDTA. After the addition of proteinase-K (15 µL) and Buffer AL (200 µL) (DNeasy Blood and Tissue kit, Qiagen, Germany), samples were incubated at 70°C for 10 minutes. In each tube, 200 µL of 100% ethanol added, vortexed, transferred to a spin column and processed according to the manufacturer's instructions (Qiagen,

Germany). The DNA concentration were measured via fluorometry (Qubit 2.0, Life Technologies, Carlsbad, CA) using Quant-iT broad range (or high sensitivity) dsDNA reagent kits (Invitrogen, Carlsbad, CA).

16S rRNA gene sequencing and bioinformatic analysis

Extracted pig feces DNA were processed at the University of Missouri Metagenomics Center. The 16s rDNA amplicons (V4 region of the 16S rRNA gene) were created with universal primers (U515F/806R) (Caporaso et al., 2011; Walters et al., 2011) against the V4 region (flanked by Illumina standard adapter sequences (Illumina Inc CA, USA). Oligonucleotide sequences are available at proBase (database of rRNA-targeted oligonucleotide probes and primers) (Loy et al., 2007). Dual-indexed forward and reverse primers were used in all reactions. Metagenomic DNA (100 ng) was used, and PCR was performed in 50 µL reactions with primers, dNTPs and DNA polymerase. PCR plate was transferred to the thermocycler for amplification (98°C (3 minutes) + [98°C (15 seconds) + 50°C (30 seconds) + 72°C (30 seconds)]× 25 cycles+ 72°C (7 minutes). After amplification completion, amplicon pools (5 µL/reaction) were combined, mixed, and purified by adding of Axygen Axyprep MagPCR clean-up beads (50 µL beads were thoroughly mixed with 50 µL amplicons) and incubated for 15 minutes at room temperature. The plate was placed on the magnetic stand for 5 minutes until the supernatant was cleared and then washed with 80% ethanol. The pooled amplicon was evaluated by using the Advanced Analytical Fragment Analyzer and quantified using Quant-iT HS dsDNA kits, and diluted based on Illumina's standard protocol for sequencing (MiSeq instrument).

Sequencing data processing and bioinformatic analysis

The amplicon sequence variant (ASV) (also called exact sequence variants, ESVs) based analysis. were performed. The Cutadapt (Martin, 2011) algorithm was used to remove the

primers at 5'end of forward reads. Read pairs were rejected if one read or the other did not match a 5'primer, and an error rate of 0.1 was allowed. Quality filtering, pairing, denoising, de-replication, and determination of the count of ASVs were performed with the Division Amplicon Denoising Algorithm (DADA2) plugin (Callahan et al., 2016) in the QIMME2 platform. For quality trimming, forward and reverse reads were truncated to 150 bases, and those with a read number of expected errors higher than 2 were discarded, and the bacterial 16s rRNA gene was subsetted to retain only those sequences that are between 249 and 275 nucleotides inclusive. Bacterial taxonomy was assigned to the sequences using the SILVA database v132 (Pruesse et al., 2007) of 16S rRNA sequences of bacterial species of different taxonomy using the classify-sklearn procedure. The ASVs identified other than bacteria were removed from further analysis. For the rarification, if there were more than 10,000 counts in one or more samples, they were rarefied to the value of the smallest sample greater than 10,000 minus 1. The total count from each sample was normalized to 38,628 prior to estimating alpha and beta diversity measures.

Phenotypic AMR in fecal bacteria

A total of 140 fecal samples (*n*=55 from pigs not treated with ceftiofur, *n*=40 from ceftiofur-treated pigs along with untreated pen-mate control, and *n*=45 from penicillin G treated pigs along with untreated pen-mate control) were subjected to culture method to quantify for coliforms and enterococci by spiral plating using an Eddy Jet 2 spiral plater (Neutech Group Inc., Farmingdale, NY, USA) as described previously (Chalmers et al., 2018). Fecal samples were diluted in phosphate-buffered saline (PBS) at a 1:10 ratio. The dilution was plated on MacConkey agar (MAC) to quantify coliforms, and MAC supplemented with an antimicrobial drug (the drug concentrations are listed in Table 5.3) to quantify antimicrobial-resistant coliforms. Similarly, the dilution was plated on m-Enterococcus agar (ENT), and ENT

supplemented with an antimicrobial drug (the drug concentrations are listed in Table 5.3) to quantify antimicrobial-resistant enterococci. The plates were incubated at 37° C for 18 hours for MAC and up to 48 hours for ENT. The coliform and enterococcus colonies were counted, and viable counts of the bacteria were estimated as colony-forming units per g (CFU/g) of feces, following the spiral-plater manufacturer's recommendations.

Statistical analysis

Microbiome data analysis

The alpha diversity, as captured by richness (Chao1 index), diversity (Shannon diversity index, Fisher alpha index), and evenness of taxa, was estimated from rarefied samples using the PAleontological STatistics (PAST) software (Hammer-Muntz et al., 2001). The associations of the age, diet, and their interaction (age x diet) with several alpha diversity indices (outcome variables) were evaluated using the generalized linear mixed-effects models ("*lmer"* in R software) (R, 2019). The pig identity was entered as a random effect to account for the lack of independence between samples.

Non-metric multidimensional scaling (NMDS) ordination plots were made using R package *vegan* (Jari Oksanen, 2019) on Hellinger transformed (Legendre and Gallagher, 2001) bacterial taxonomic counts data using the Bray-Curtis dissimilarity distances; they were visualized using ggplot2 package (Wickham, 2009). A two-dimension plot was used if stress was less than 0.2. The ASVs present in < 1% samples were discarded to avoid potential bias before analysis. The permutational multivariate analysis of variance (PERMANOVA) of the Bray-Curtis distance was performed using the *adonis* function in the R *vegan* package, with one or more of the explanatory variables (pig age and/or dietary treatment). The random effect of the individual pig was included in the model. The distances in the microbiome across individuals

within and between age groups receiving 1 of 3 dietary treatments were calculated using the Bray-Curtis matrices, and differences in variation between age groups and dietary treatments on the microbiome composition were tested with the multivariate homogeneity of group dispersion test (*Betadisper* function in the *vegan* package in R) (Jari Oksanen, 2019) followed by Tukey's honest significance difference method.

Phenotypic AMR data analysis

For the pigs sampled longitudinally and not treated with antimicrobial drugs throughout the finisher phase (*n*=5 sampled at 4 age-points), changes in the total AMR coliform and enterococcus populations (log_{10} CFU/g) by age were analyzed using the generalized linear mixed-effects model. For an individual antimicrobial drug, the data for all animals that had at least one positive sample (coliforms or enterococci growing on the agar supplemented with that drug) were used. The pig identity was used as a random effect to account for the lack of independence between the samples in the mixed-effects models. For the pigs treated by ceftiofur or penicillin G, analysis of variance was performed for the total and AMR coliform and enterococci populations to evaluate the effect of dietary treatment, antimicrobial exposure, and their interaction.

Results

The age-related dynamics and effect of dietary treatment on the taxonomic composition of fecal bacterial communities

We first investigated the dynamic fecal microbiome of untreated pigs on the dietary treatments (control diet; 8.7% NDF, 2.2 % crude fiber), DDGS-based diet (13.6% NDF and 3.1% crude fiber and primarily contains insoluble fiber), and sugar beet pulp-based diet (13.6% NDF and 5.1% crude fiber and primarily contains soluble fiber) (Table 5.1) by characterizing 55 fecal

samples collected on days 98 (moving to finisher facility) through 177 (market). The richness, diversity, and evenness of the fecal microbial taxa changed with age (days 98, 110, 144, and 177) of the pigs receiving 1 of 3 dietary treatments (Figure 5.2). The bacterial richness (Choa1 index of species richness) significantly increased with age ($p < 0.05$). Likewise, diversity indices (Shannon index and Fisher alpha index) also significantly increased ($p < 0.001$) from days 98 to 177 (market) of age. Similarly, the evenness of taxa also changed with age (p=0.004). However, there was no significant effect of diets ($p >$0.05) and interaction between age and dietary treatment ($p >$0.05) on the Chao1 richness, Shannon and Fisher alpha diversity indices, and evenness of taxa.

The non-metric multi-dimension scaling (NMDS) of the Bray-Curtis distances showed that age could lead to a significant variation in the fecal microbiome of pigs on dietary treatments (Figure 5.3). A significant shift in the overall microbial community composition with age was observed based on the Bray-Curtis distance; day 98 samples were unique from those of the other age-points (days 110, 144, and 177) (Figure 5.3, b-d). We further analyzed the taxonomic composition of bacterial communities with age and diet as well as their interaction using PERMANOVA (based on the Bray-Curtis distances), and we found that there was a significant difference in the overall taxonomic composition with age ($p <$0.001) and diet ($p <$0.001). However, we did not find enough evidence of interaction between age and diet (p=0.55). The degree of variation in the microbiome among individual pig was similar across ages and diet (multivariate homogeneity test of group dispersion; p=0.18, p=0.79, respectively).

The dynamic shift in the fecal microbiome taxonomic composition was further evaluated at the taxonomic level. Figure 5.4 shows the taxonomic composition of the fecal microbiome at the phylum level at four age points (days 98, 110, 144, and 177) on the dietary treatments.

Firmicutes followed by *Bacteroides* were the most dominant phyla across all age-points, but with slight variations between dietary treatments. On the control diet, the relative abundance of bacterial phyla such as *Spirochaetes*, *Epsilonbacteraeota*, and *Tenericutes* significantly increased ($p < 0.05$) with age. In contrast, the relative abundance of *Actinobacteria* and *Firmicutes* decreased with the age of pigs. However, dynamics were less distinct in those on the high fiber diets (sugar beet pulp based and DDGS based diets). Few phyla, such as *Epsilonbacteraeota* and *Proteobacteria,* significantly increased with age on the DDGS-based diet. However, there were no bacterial phyla associated with pig's age on the sugar beet pulp-based diet.

We further evaluated the relative abundance of bacterial taxa at the family level by fitting the DESeq2 algorithm (negative binomial with bias correction; false discovery rate based adjusted *p*-value at $\alpha=0.05$). On the control diet, the relative abundance of 2534 18B, *Bacteroidales* RF16, F082, *Barnesiellaceae*, *Bacteroidaceae*, *Spirochaetaceae*, *Rikenellaceae*, *Fibrobacteraceae*, *Tannerellaceae*, and *Clostridiales* taxonomy families significantly increased with the age of pigs; *Lactobacillaceae*, *Veillonellaceae*, *Coriobacteriaceae*, *Succinivibrionaceae*, *Eggerthellaceae*, *Lachnospiraceae*, *Atopobiaceae*, and *Bifidobacteriaceae* significantly decreased ($p <0.05$) with age. Similarly, on the DDGS based diet, the relative abundance of *Tannerellaceae*, F082, p 2534 18B5, *Bacteroidaceae*, *Bacteroidales* RF16, and *Campylobacteraceae* increased with age. However, on the same dietary group, relative abundance of *Veillonellaceae*, *Lactobacillaceae*, *Coriobacteriaceae*, *Streptococcaceae*, *Atopobiaceae*, *Eggerthellaceae*, and *Ruminococcaceae* significantly decreased ($p <0.05$) with age. On the sugar beet pulp-based diet, the relative abundance of *Bacteroidales* RF16, p 2534_18B5, and *Elusimicrobiaceae* increased with age. However, the relative abundance of

Streptococcaceae, *Atopobiaceae*, *Enterobacteriaceae*, and *Veillonellaceae* significantly decreased with age (Figure 5.5, a-c).

The effects of the dietary treatments and antimicrobial treatments on the richness, diversity, and taxonomic composition of bacterial communities

Of the pigs treated with ceftiofur, the richness of taxa (Chao1 index), diversity (Shannon index, Fisher alpha index), and evenness of taxa did not differ with those received ceftiofur treatment versus untreated pen-mate control, day of treatment (day 1 and day 3) and dietary treatments (p >0.10) (Figure 5.6 and 5.7). However, Fisher's alpha diversity was significantly higher ($p < 0.05$) on the sugar beet pulp-based diets compared to control diets. Similarly, there was no significant interaction between dietary treatments and ceftiofur treatment on richness, diversity, and taxa evenness. There was no significant effect of day of treatment (day 1 versus. day 3) or treatment effects (untreated versus ceftiofur treated).

Next, we performed a non-metric multi-dimension scaling (NMDS) of the Bray-Curtis distances to assess the effects of diet and antibiotics (ceftiofur-treated or penicillin G-treated group) on the overall bacterial taxonomic composition. The PERMANOVA results (based on Bray-Curtis distances) showed that both diets (p <0.001) and ceftiofur treatment (p=0.001) affected the overall bacterial composition (beta diversity) (Figure 5.8, a-d). However, we did not find sufficient evidence of the interaction of diet and ceftiofur treatment on bacterial community composition. Further, the degree of variation in the taxonomic composition among individuals pigs was similar across diet (multivariate homogeneity test of group dispersion; p=0.2) and antimicrobial treatment group and day of treatment (multivariate homogeneity test of group dispersion; p=0.48).

The relative abundance of the bacterial taxonomic composition (at the phylum level) is shown in Figure 5.9. There was no significant difference in the relative abundance of bacterial phyla in the ceftiofur-treated pigs receiving either a control diet or DDGS-based diet. However, on the sugar beet pulp-based diet, the relative abundance of *Kiritimatiellaeota* significantly increased ($p < 0.05$) in ceftiofur-treated pigs (days 1 and day 3) compared to the untreated pen-mate control (day 1). In the same sugar beet pulp dietary treatment, the relative abundance of *Pirellulaceae*, *Spirochaetaceae*, *Methanobacteriaceae*, and *Christensenellaceae* of bacterial families significantly increased (DESeq, adjusted $p < 0.05$) in the treated pigs compared to the untreated pen-mate control pigs. The relative abundance of *Bifidobacteriaceae*, *Prevotellaceae*, and *Chlamydiaceae* decreased in the ceftiofur treated pigs compared to untreated pen-mate control (Figure 5.11, a-c). However, we did not find a significant change in the relative bacterial abundance (at the family level) in those fed other control and DDGS-based diets.

In the penicillin G treated group, the richness of taxa (Chao1), diversity (Shannon, Fisher alpha), and evenness of taxa did not differ with penicillin G treated, day of treatment (day 1 vs. day 3) and dietary treatments (control, DDGS-based diet and sugar beet pulp-based diet). There was no interaction between dietary treatment and the effect of penicillin treatment on richness, diversity, and evenness of taxa (Figures 5.6 and 5.7). However, overall bacterial community composition (beta diversity) changed based on diet and day of penicillin G treatment (Figure 5.8, e-h). The PERMANOVA (based on Bray-Curtis distances) of taxonomic composition showed significant differences in the bacterial community composition based on diet (p=0.001) and penicillin G treatment (p=0.001). However, there was no significant interaction between dietary treatment and penicillin treatment effects on the taxonomic composition of bacterial communities.

The relative abundance of bacterial phyla did not vary with the penicillin G treatment group or across dietary treatments (Figure 5.10). However, the relative abundance of the bacterial family varied with dietary treatments (those received control diet). For instance, the relative abundance of *Peptostreptococcaceae* and *Enterobacteriaceae* significantly ($p < 0.05$) increased in pig treated with penicillin G while the relative abundance of *Streptococcaceae*, *Veillonellaceae*, and *Prevotellaceae* significantly decreased in the penicillin G treated pigs (Figure 5.11, d-f).

The abundance of phenotypically AMR fecal bacteria

We found that 49% and 46% of all samples (n=55) from untreated pigs (5 pigs/diet, day 98, 110, 144 and 177) on the dietary treatments (receiving 1 of 3 dietary treatments: control, DDGS-based diet or sugar beet pulp-based diet) carried coliforms and enterococci resistant to at least one antimicrobial drug, respectively (Table 5.3). The change in counts ($\log_{10}$ CFU/g) of the total and antimicrobial-resistant coliforms and enterococci in the pigs receiving different diets are shown in Figures 5.12 and 5.13. The total coliform counts did not change with age (day 98 through day 177/market) and dietary treatments. However, total enterococci counts did vary with dietary treatment (p=0.1) with lower in the DDGS-based diet compared to the control diet. Similarly, there were interactions between pig age and dietary changes (p=0.01) in total enterococci counts. Coliform resistant to ceftriaxone (3^{rd} generation cephalosporins), tetracyclines, macrolides, aminoglycosides, and sulfonamides did not vary with age or dietary treatments. Similarly, enterococci resistant to aminopenicillins, tetracyclines, macrolides, and lincosamides did not change with pig age or dietary treatment (and their interaction effects). However, nalidixic acid (quinolone)-resistant enterococci varied with dietary treatments (p = 0.1).

Of the ceftiofur-treated (day 1 and day 3 along with untreated pen mate control), 59% and 50% of all samples (n=40) carried coliforms and enterococci resistant to at least one of the tested antimicrobial drugs, respectively (Figures 5.14, 5.15 and 5.16).

Of the ceftiofur treated pigs, the total coliform counts did not change with the dietary treatments (ANOVA, p >0.05), and there was no interaction between the ceftiofur treatment and dietary treatments (ANOVA, p >0.05). Similarly, coliform resistant to ceftriaxone (3rd generation cephalosporins), tetracyclines, macrolides, aminoglycosides, and sulfonamides did not change significantly with both dietary treatment and ceftiofur treatment (ANOVA, p >0.05). However, only aminopenicillin resistant coliforms were significantly different across the dietary treatments (p = 0.03) (sugar beet pulp-based diet to control diet and sugar beet pulp to DDGS based diet). In the same ceftiofur treated pigs, enterococci resistant to aminopenicillins, quinolones, tetracyclines, macrolides, aminoglycosides, and lincosamides also did not change with either dietary treatment or ceftiofur treatment or their interaction effects.

Similarly, 62% and 48% of the penicillin G-treated (day 1 and day 3 along with untreated pen mate control), carried resistant coliforms and enterococci, respectively (to at least one antimicrobial drug tested (Table 5.3) (Figures 5.14, 5.15 and 5.16)). Total coliform counts did not change with the dietary treatments or antibiotic treatment (ANOVA, p >0.05). Coliform resistant to ceftriaxone (3rd generation cephalosporins), tetracyclines, macrolides, and sulfonamides also did not change significantly with either dietary treatment or penicillin treatment (ANOVA, p > 0.05). Enterococci resistant to aminopenicillins, tetracyclines, macrolides, aminoglycosides, and lincosamides also did vary with either dietary treatment or ceftiofur treatment or their interactions. However, quinolones resistant enterococci counts were

significantly varied with dietary treatments (sugar beet pulp-based diet versus control diet, adjusted *p*-value = 0.01).

Overall, higher variability in the abundance (log_{10} CFU/g) of AMR coliforms or enterococci were observed based on dietary treatment and antimicrobial treatment (either injectable ceftiofur or penicillin G).

Discussion

When designing this study, our research hypothesis was that there would be age-related dynamics and effects of the diet and antimicrobial treatments on the fecal microbiome or AMR. Thus, we modified dietary composition (with different levels and sources of fibers) to understand and measure the age-related change and effect of diet or antimicrobial use on the fecal microbiome and AMR. We targeted the treatment effect using ceftiofur and penicillin G, common antibiotics in swine production in the United States.

The alpha diversity and taxonomic composition changes significantly with age

The richness and diversity of the fecal microbiome increased with pig age. The alpha diversity indices such as richness and diversity (Fisher alpha index) of the taxa gradually increased from day 98 (first week after moving finisher/starting dietary treatments) until day 177 (market) on both control and sugar beet pulp-based diets. This increased trend with age was comparable to previous studies (Lu et al., 2018; Wang et al., 2019; Arfken et al., 2020). However, these dynamics dropped on day 177 (market) in pigs receiving DDGS-based diet indicating age and diet effects. Previous studies have shown that age, diet, and the use of antimicrobials can influence the swine gut microbial community (Ghanbari et al., 2019; Pollock et al., 2020).

The decreasing trend of microbiome diversity during the market age of production pigs was also reported in previous studies. For instance, Han and colleagues (2018a) studied microbiome diversity and composition of healthy pigs (*n*=32) at five age points (days 10, 21, 63, 93, and 147). They found that the intestinal microbiome changed with swine growth and reduced the alpha diversity on day 63 when antimicrobials were supplemented in the diet (Han et al., 2018). Similarly, De Rodas et al. (2018) reported that alpha diversity decreased in different locations of the GI tract in pigs from birth to day 84. This decreased trend was most noticeable in samples from the market age.

In our study, we modified the diet composition with varying levels and sources of fiber (higher in sugar beet pulp-based diet followed by DDGS-based diet compared to the control/corn-soybean based diet). We found both age and diet effects on the overall bacterial community structure. Similar age-and-diet related clustering of the bacterial community was reported in several studies of pigs (Frese et al., 2015b; Wang et al., 2019; Arfken et al., 2020) and cattle (Liu et al., 2019; Hennessy et al., 2020). Diet was one of the important factors in shaping the pig gut microbiome; a study showed that neutral detergent fiber is particularly impactful in shaping the pig gut microbiome (Wang et al., 2019) because the plant cells cannot be digested by the pig and thus they are passed to the colon for fermentation by the gut microbiome.

Similarly, Frese et al. (2015a) reported that the diversity of bacterial taxa increased with dietary changes from sows milk to a plant-based starter diet. The relative abundance of *Lactobacillaceae, Rumimococcaae, Veillonellaceae,* and *Prevotellaceae* increased in the weaned piglets. Recently, Zhang et al. (2016) reported that moderately increased fiber (both soluble and insoluble fiber) in the diet influenced the gut microbial composition in piglets fed with different

levels of fiber-containing diets compared to a control diet from postnatal days 7 to 22. In contrast, a study by Kraler et al. (2016) showed no difference in taxonomic composition related to diet modification (control diet and with low and high fiber diet). Other studies showed that dietary changes coupled with other management factors in the farms such as in-feed antimicrobials, prebiotics, and probiotics administration played an important role in shaping the gut microbial community in pigs (Bian et al., 2016; Guevarra et al., 2019).

Microbiome alpha diversity was not affected by antimicrobial exposure, but the community composition varied with diet and antimicrobial exposure

An earlier study showed impacts to the swine gut microbiome composition from antimicrobial exposure (Looft et al., 2014; Zhao et al., 2018). However, our data showed the alpha diversity indices of fecal microbiome were not affected by antimicrobial exposure (injectable ceftiofur and penicillin G). Similar findings were reported by Pollock et al. (2020) where higher levels of chlortetracycline and tylosin in the feed did not affect the fecal microbiome diversity in young pigs. Similarly, no impact by subtherapeutic effects of chlortetracycline and tylosin on alpha diversity were reported by (Holman and Chenier (2013).

In contrast, another study showed that the richness and diversity (Shannon index) of taxa differed in weaned pigs following oxytetracycline administration (Ghanbari et al., 2019). In our study, the taxa richness was less dispersed in ceftiofur-treated (both days 1 day 3) pigs across all diets compared to pen-mate untreated controls (day 1). Similarly, in the penicillin G-treated group, the fecal microbiome was more diverse (as measured by Shannon and Fisher alpha indices) in pigs receiving a sugar beet pulp-based diet followed by a DDGS-based diet compared to the control diet. These results indicated that despite penicillin G use, fecal bacterial richness and diversity increased based on the sugar beet-pulp and DDGS-based diet, suggesting that

penicillin G might have a minimal effect on gut microbial communities versus compared to that of ceftiofur. An earlier study also showed that the diversity of the microbiome taxa (measured by Shannon index) of untreated pigs was significantly lower compared to piglets treated with tulathromycin (Schokker et al., 2014). However, it is difficult to understand whether the short-term changes in the dynamics of microbial richness and diversity from antimicrobial administration could have any significant long-term impacts.

Similarly, the beta diversity of the fecal bacterial community structure revealed a shift in the composition of the bacterial community in both ceftiofur or penicillin G-treated pigs. Recently, Ruczizka et al. (2019) reported the effect of parenteral treatment of ceftiofur on the fecal microbiome in pigs from the suckling to growing stages (birth to day 97). They also found that ceftiofur administration disturbed the fecal microbiome composition as early as 12 hours postpartum. Ceftiofur is a common antibiotic in pigs and is administered intramuscularly. It is effective for at least 158 hours in plasma, but most of the drug is excreted as metabolites in the feces (Hornish and Kotarski, 2002; Ruczizka et al., 2019). This ceftiofur metabolite is then excreted via urine (~68%) and feces (~13%) in 10 days (Hornish and Kotarski, 2002) and may impact the gut microbial communities.

Of ceftiofur-treated pigs, no visible differences were seen in the abundance of the bacterial families in pigs fed either control or DDGS-based diet. However, on the sugar beet pulp-based diet, the most notable change in bacterial abundance was an increase in the relative abundance of the *Kiritimatiellaeota* phylum. Similarly, at the family level, the relative abundance of *Pirellulaceae*, *Spirochaetaceae*, *Methanobacteriaceae,* and *Christensenellacea* increased in the treated pigs compared to their pen-mate untreated pigs. Similarly, in the same dietary group, the relative abundance of *Bifidobacteriaceae*, *Prevotellaceae*, and *Chlamydiaceae*

significantly decreased in the treated group. In line with previous studies, our findings also showed the parental administration of ceftiofur in pigs reduced *Bifidobacteriaceae* in fecal samples (Ruczizka et al., 2019).

Of the penicillin treated pigs, there was no difference in the fecal microbiome at the phylum level across all dietary treatments following *p*-value correction. An earlier study also showed no significant difference in microbiome composition after day 55 of age between the treatment groups (those received tulathromycin at four days after birth and control) (Schokker et al., 2015). A similar finding was seen for the effect of antibiotic treatment on the fecal microbiome (Ruczizka et al., 2019). Furthermore, Kalmokoff et al. (2011) reported that the addition of either tylosin or virginiamycin to pig feed over 15 weeks did not affect the animal fecal microbiome (16S rRNA).

A great deal of variability was observed in the abundance of phenotypically AMR with age of pigs on the dietary treatment and antimicrobial exposure

Our data suggested age- and diet-related changes in an abundance of phenotypic AMR in fecal coliforms and enterococci of finisher pigs (days 98 to 177). The total coliforms or enterococci remained stable throughout the study period and across the dietary treatments. There was a noticeable fluctuation in the abundance of AMR fecal coliforms and enterococci with the age of pigs fed with different levels and sources of fiber; however, most marked changes were generally associated with the age of the pigs. A previous study showed that housing and dietary changes might affect the prevalence of AMR by changing the total coliforms in the gastrointestinal tract (Overland et al., 2000) and calves (Hoyle et al., 2004a). Recently, the rumen resistome composition was shown to be significantly related to diet and abundance. The diversity of AMR genes was higher in concentrated fed animals than forage-fed (Auffret et al., 2017). An

earlier study also showed that the fecal resistome significantly changed over time in animals (Liu et al., 2019) and is associated with diet.

Further, variability in the abundance of phenotypically AMR in coliforms or enterococci was not generally affected by antimicrobial exposure (injectable ceftiofur or penicillin G). An earlier study also showed that changes in the abundance of AMR genes were not associated with antimicrobial exposure (Pollock et al., 2020). This could be related to the relatively short sampling time (days 1 and 3 of the treatment regimen) or other factors affecting the entire farm, management, or environmental conditions (Mathew et al., 2003).

Conclusions

There is growing concern over antimicrobial resistance, and alternative approaches such as pre/probiotics and dietary interventions have been investigated. The benefits associated with the presence of fiber in the diet have been identified, including positive changes in the gut microbial community composition to a reduction in both richness and diversity of the gut resistome. In addition to diet, several factors such as animal age, antimicrobial use, and the environment could influence microbial diversity and antimicrobial resistance in fecal bacteria. Our data revealed that both pig age and diet are associated with bacterial taxonomic diversity, community composition and phenotypically AMR in fecal bacteria. Even short-term administration of ceftiofur and penicillin G changed the bacterial taxonomic composition. Our data further suggest an effect of age, diet, and antimicrobial use on the AMR in fecal bacteria.

Table 5.1

Formulation and chemical composition of dietary treatments.

Ingredients, %	Diet (Phase 1)			Diet (Phase 2)			Diet (Phase 3)		
	Control	DDGS	Sugar beet pulp	Control	DDGS	Sugar beet pulp	Control	DDGS	Sugar beet pulp
Corn	75.45	62.55	58.65	81.81	68.83	64.96	85.19	70.55	68.47
Soybean Meal, Dehull, Solvent Extracted	21.78	14.53	21.42	15.64	8.39	15.28	12.37	6.73	12.00
Sugar beet pulp	-	-	14.50	-	-	14.50	-	-	14.50
Choice White Grease	-	0.20	2.80	-	0.25	2.85	-	0.40	2.75
Calcium carbonate	0.92	1.05	0.60	0.92	1.07	0.62	0.92	1.07	0.62
Calcium phosphate (monocalcium)	0.55	0.25	0.62	0.40	0.10	0.45	0.35	0.00	0.37
Sodium chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
L- Lysine–HCl	0.30	0.45	0.30	0.30	0.45	0.30	0.30	0.40	0.30
DL- Methionine	0.06	0.02	0.11	0.03	-	0.08	0.01	-	0.06
L- Threonine	0.09	0.09	0.12	0.10	0.09	0.12	0.11	0.09	0.14
L- Tryptophan	0.01	0.03	0.01	0.01	0.03	0.02	0.02	0.03	0.02
L-Valine	-	-	0.04	-	-	0.03	-	-	0.03
Trace mineral premix	0.15	0.15	0.15	0.12	0.12	0.12	0.10	0.10	0.10
Vitamin premix without phytase	0.15	0.15	0.15	0.12	0.12	0.12	0.10	0.10	0.10
Ronozyme HiPhos 2700	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Corn DDGS, 7.5% Oil	-	20.00	-	-	20.00	-	-	20.00	-
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Chemical composition									
Digestible Lysine, %	0.95	0.95	0.95	0.80	0.80	0.80	0.72	0.72	0.72
Net energy, kcal/kg	2,487	2,487	2,487	2,528	2,529	2,530	2,549	2,549	2,547
NDF, %	8.7	13.6	13.6	8.7	13.7	13.7	8.8	13.7	13.7
Crude fiber, %	2.3	3.3	5.3	2.2	3.1	5.1	2.2	3.1	5.1
Crude protein, %	17.0	18.2	16.9	14.6	15.8	14.4	13.3	15.1	13.2

Crude fat, %	3.0	4.1	5.2	3.1	4.3	5.4	3.2	4.4	5.4
Calcium, %	0.59	0.59	0.59	0.54	0.54	0.54	0.51	0.51	0.51
Phosphorus, %	0.47	0.47	0.45	0.41	0.41	0.39	0.38	0.38	0.36
Digestible phosphorus, %	0.33	0.33	0.33	0.28	0.28	0.28	0.26	0.26	0.26

Dietary fiber includes soluble dietary fiber and insoluble dietary fiber; DDGS — distillers dried grains with soluble (by-product when corn is fermented to make ethanol), mainly contains an insoluble fiber; sugar beet pulp- mainly contains soluble fiber. NDF—Neutral Detergent Fiber, the pigs, were ~93 days old when they entered to finisher facility and started on dietary treatments (phase 1- day 93 to 103, phase 2- day 103 to 132, and phase 3- day 132 to 179).

Table 5.2

Numbers of pigs sampled on the dietary treatments.

Study group	Age, days (sampling time-points)	Dietary treatments			Total fecal samples
		Control	DDGS	Sugar Beet Pulp	
Untreated pigs	98	3	4	3	10
	110	5	5	5	15
	144	5	5	5	15
	177	5	5	5	15
Subtotal					*n*=55 samples
Ceftiofur treated pigs					
Ceftiofur treated	day 1 and day 3	3	4	3	10 pigs x 2 time-points=20 samples
	day 1 only*	3		2	5
Untreated pen-mate control	day 1	6	4	5	15
Subtotal					*n*=40 samples
Penicillin G treated pigs					
Penicillin G treated	day 1 and day 3	5	5	5	15 pigs x 2 time-points= 30 samples
Untreated pen-mate control	day 1	5	5	5	15
Subtotal					*n*=45 samples

DDGS—distillers dried grains with soluble, *pigs were only treated 1-2 days when clinical improvement was seen; hence, only day 1 sample was collected.

Table 5.3

Antimicrobial drug concentrations used to supplement microbiological agars to evaluate the dynamics of the phenotypic AMR in finisher pigs.

Bacteria/Media	Antimicrobial drug class	Antimicrobial drug supplemented (abbreviation)	Drug concentration (µg/mL) supplemented[a]
Coliforms MacConkey Agar (MAC)	Aminoglycosides	Gentamicin (GEN)	16
		Streptomycin (STR)	32
	3rd generation cephalosporins	Ceftriaxone (AXO)	4
	Sulfonamides/folate path inhibitors	Sulfamethoxazole (SMX)	512
	Macrolides	Azithromycin (AZI)	32
	Penicillin	Ampicillin (AMP)	32
	Phenicols	Chloramphenicol (CHL)	32
	Fluoroquinolones	Ciprofloxacin (CIP	1 (and 0.25)
	Quinolones	Nalidixic acid (NAL)	32
	Tetracyclines	Tetracycline (TET)	16
	Fluoroquinolones	Enrofloxacin (ENR)	0.125*
Enterococci spp. Enterococcus Agar (ENT)	Aminoglycosides	Gentamicin (GEN)	500
		Streptomycin (STR)	1024
	Lincosamides	Lincomycin (LIN)	8
	Macrolides	Erythromycin (ERY)	8
		Tylosin	32
	Nitrofurans	Nitrofurantoin (NIT)	128
	Aminopenicillins	Penicillin (PEN)	16
	Phenicols	Chloramphenicol (CHL)	32
	Fluoroquinolones	Ciprofloxacin (CIP)	4
		Enrofloxacin (ENR)	4
	Quinolones	Nalidixic acid (NAL)	32‡
	Tetracyclines	Tetracycline (TET)	16

[a] Breakpoints based on Clinical Laboratory Standards Institute guidelines (CLSI, 2008) and National Antimicrobial Resistance Monitoring System, * European Committee on Antimicrobial Susceptibility Testing (epidemiological cutoff value), ‡ adopted from *E. coli* break-point concentration

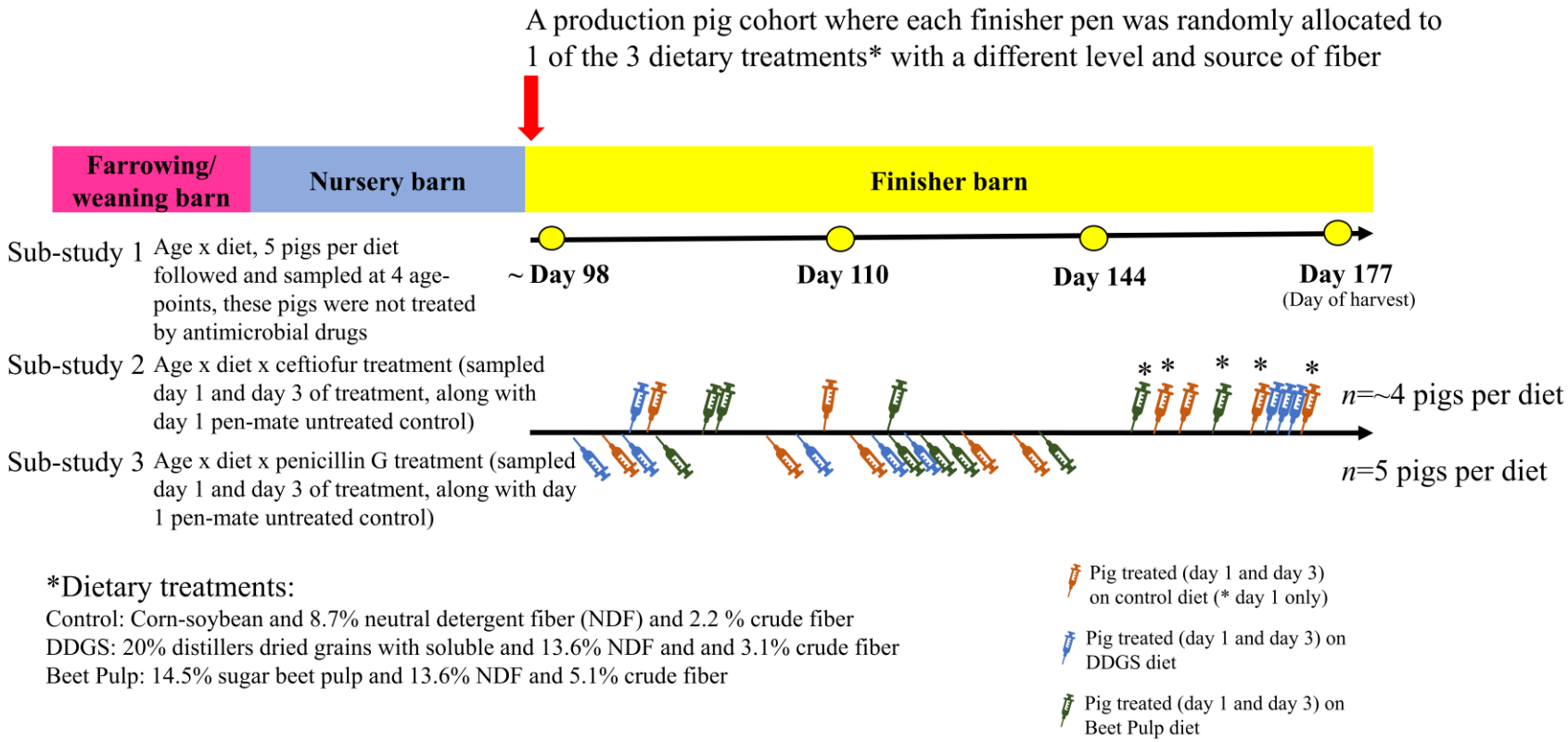

Figure 5.1. Schematic of the study design the age-related dynamics and effects of diet and antimicrobial treatments on the fecal microbiome taxonomic composition and AMR in finisher pigs. Sub-study 1, where fecal samples were collected longitudinally from individual pigs (*n*=5 pigs per diet, the animals were not treated with antimicrobial drugs during the finisher phase) at ~98 days (first week after moving into the finisher barn/facility and associated diet change), 110 days (~ 2.5 weeks after moving into finisher barn/facility), 144 days (~6 weeks after moving into finisher barn/facility), and 177 days (day of harvest). Sub-study 2, where fecal samples were collected from the individual ceftiofur-treated pigs (~ 4 pigs per diet, the animals were sampled on day 1 and day 3 of the treatment regimen; an untreated pen-mate control animal was sampled on day 1). Sub-study 3, where fecal samples were collected from

individual penicillin G treated pigs (~ 5 pigs per diet, the animals were sampled on day 1 and day 3 of the treatment; an untreated pen-mate control animal was sampled on day 1). The dietary treatments varied in fiber content and its source: control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with soluble (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

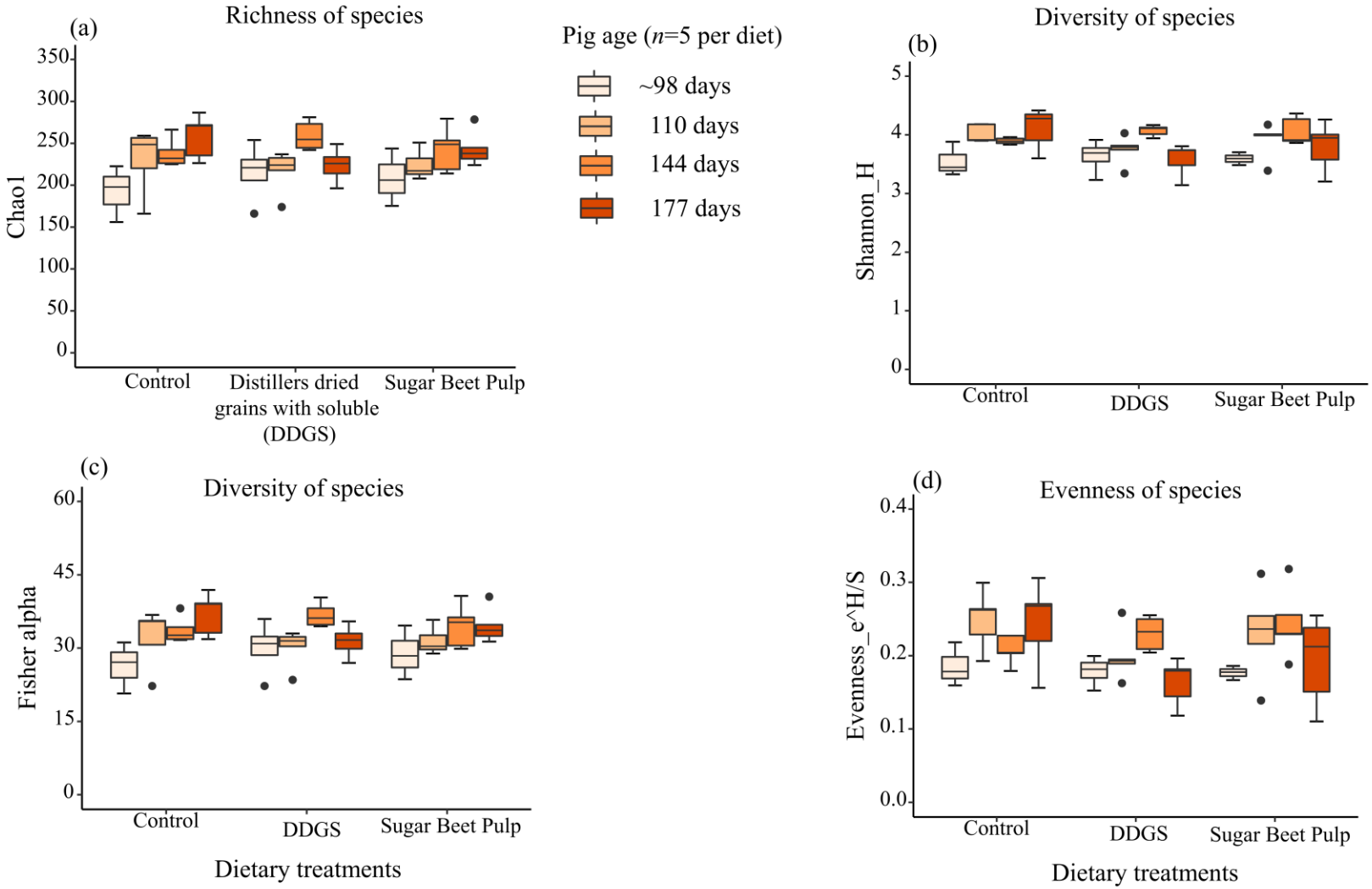

Figure 5.2. Age-dependent changes in alpha diversity of the fecal microbiome taxa depending on the dietary treatment in finisher pigs. The richness of bacterial species (Chao1 index (a)), diversity of species (Shannon index (b) and Fisher alpha index (c)), and evenness of species (d), from day 98 to day 177 of age. The dietary treatments varied in fiber content and its source: control diet (corn-soybean

based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

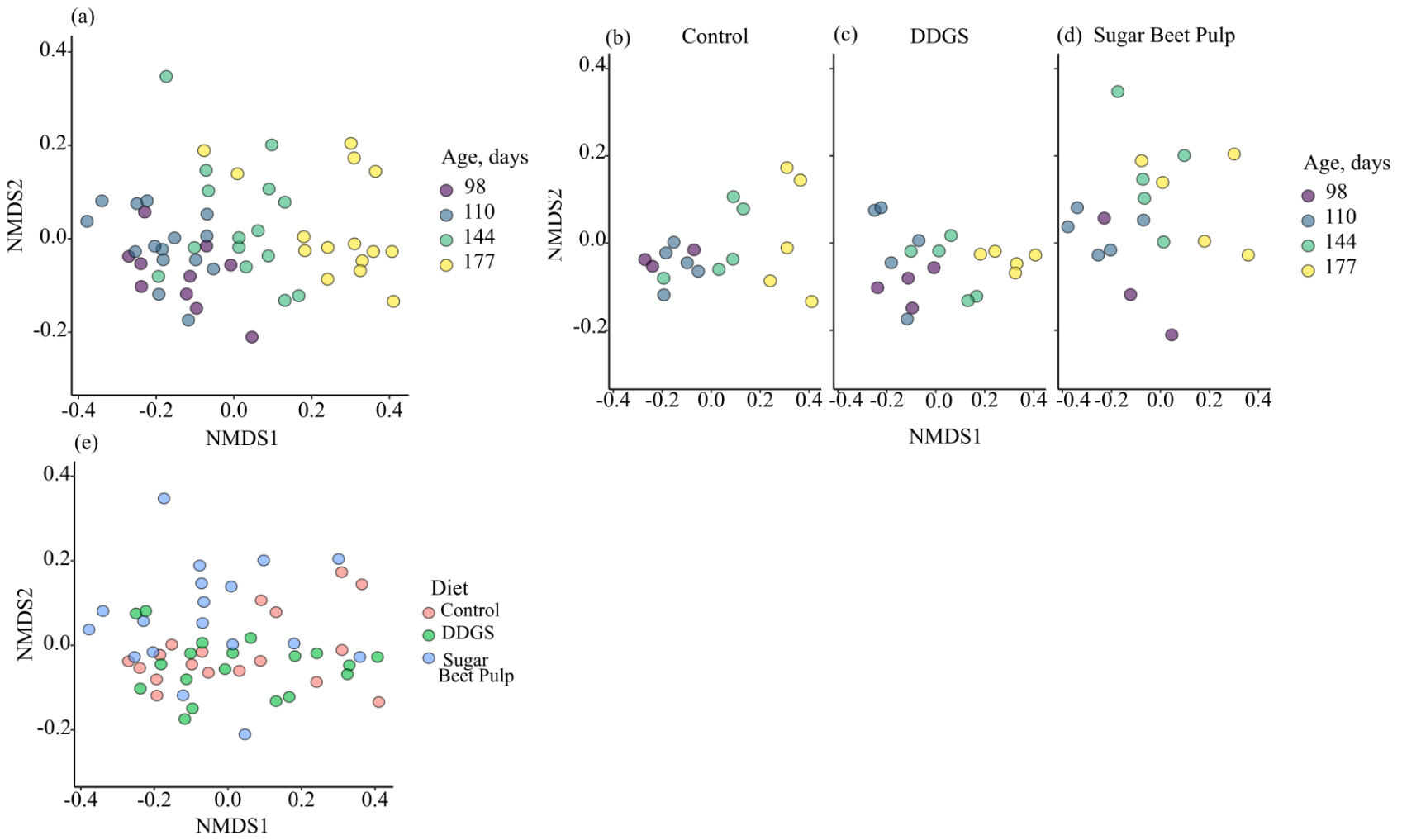

Figure 5.3. Beta diversity of the fecal microbiome taxa depending on the age and dietary treatment in finisher pigs. Non-metric multidimensional scaling (NMDS) plots are based on the Bray-Curtis distances by age (a), by age and dietary treatment (b-d), and by dietary treatment (e). The dietary treatments varied in fiber content and its source: control diet (corn-soybean based with 8.7% neutral

detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

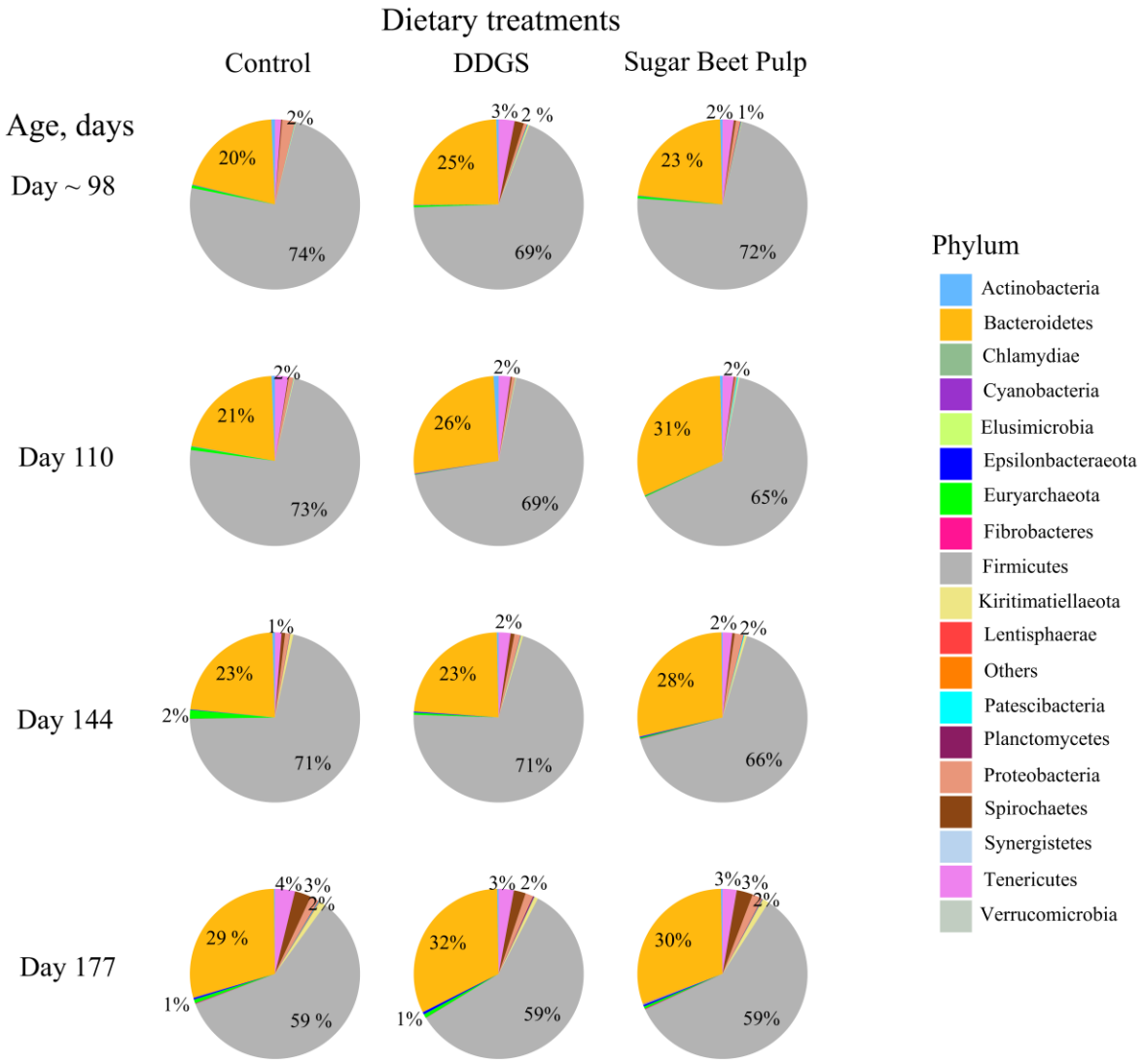

Figure 5.4. Pie chart depicting the relative abundance of individual phyla in the fecal microbiome by age of finisher pigs depending on the dietary treatment. The dietary treatments varied in fiber content and its source: control diet (corn-soybean based with 8.7% neutral

detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

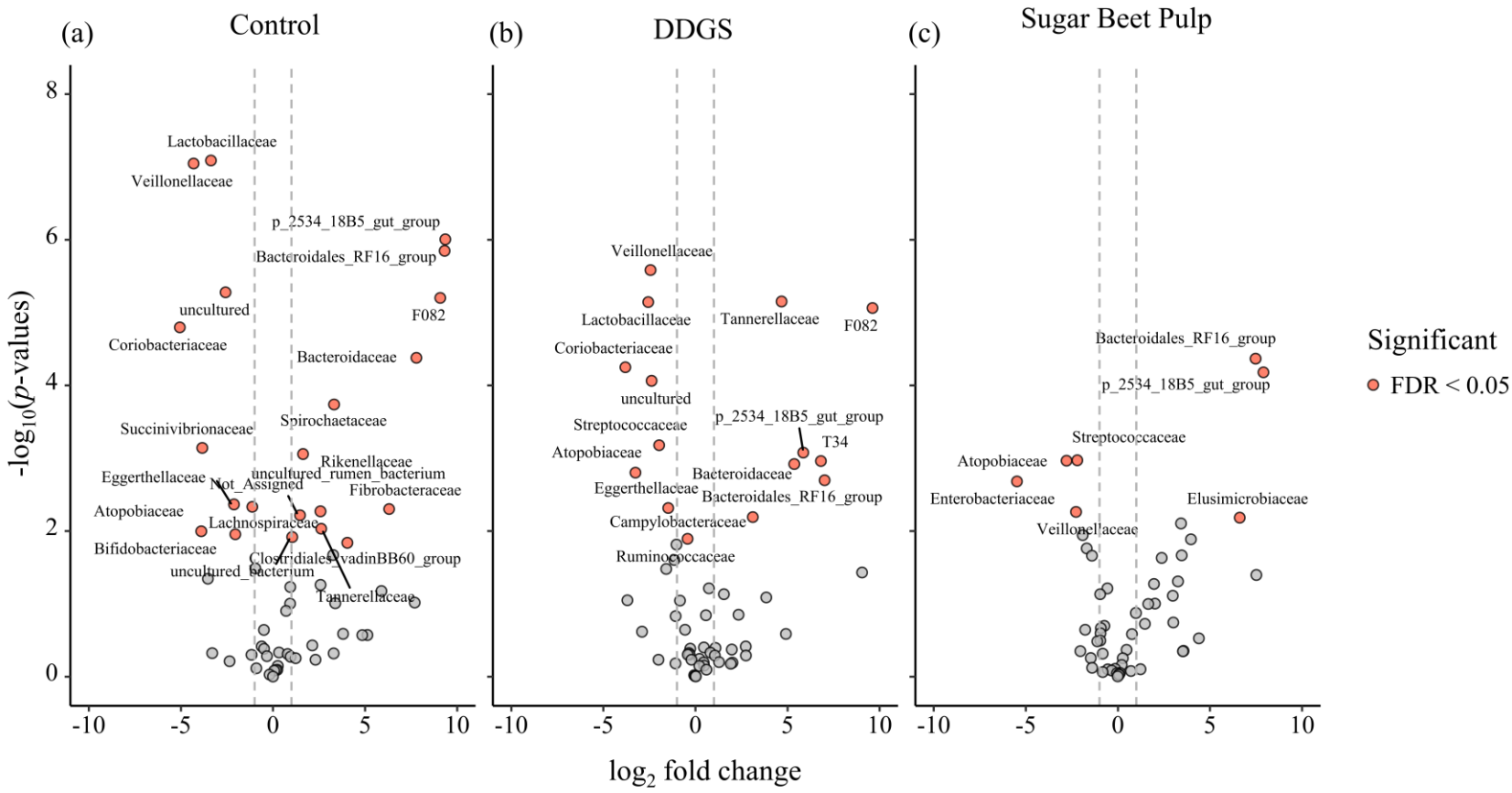

Figure 5.5. Age-dependent changes in absolute abundance of individual bacterial families in the fecal bacterial community in finisher pigs between days 98, 110, 144, and 177 of age depending on the dietary treatment. The dietary treatments varied in fiber content and its source: control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber). False discovery (FDR) adjusted *p*-value is reported (a-c). A positive/negative log-fold change indicates an increase/decrease in the family abundance with age. The vertical dotted lines indicated the log-fold changes between -1 to 1.

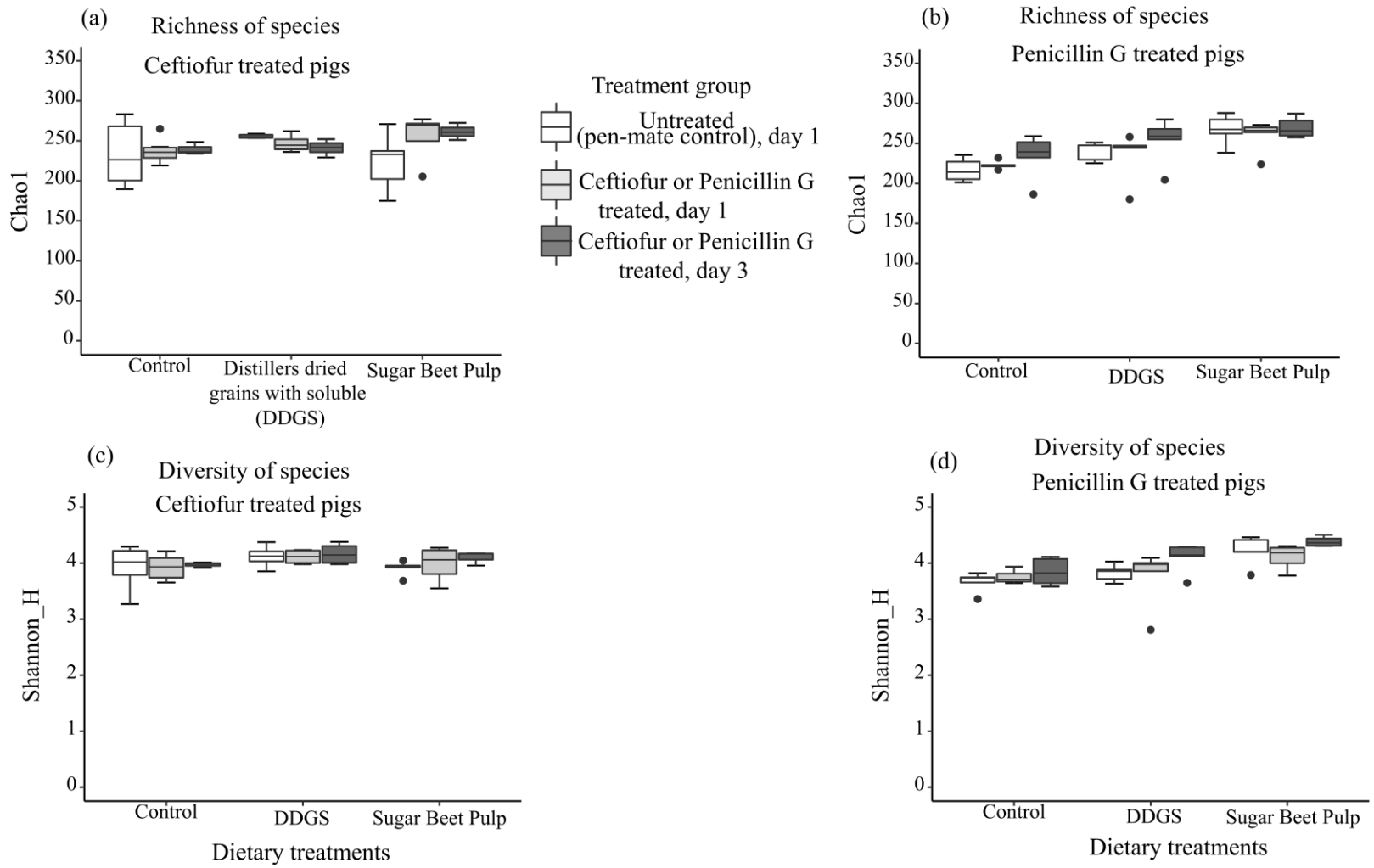

Figure 5.6. Alpha diversity of the fecal microbiome taxonomic composition across the dietary treatments and antimicrobial treatments in finisher pigs. The richness (Chao1 index) and diversity (Shannon index) in pigs receiving 1 of 3 dietary treatments and either no antimicrobial treatment or treated by ceftiofur (a, c) or penicillin G (b, d). The dietary treatments varied in fiber content and its source:

control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

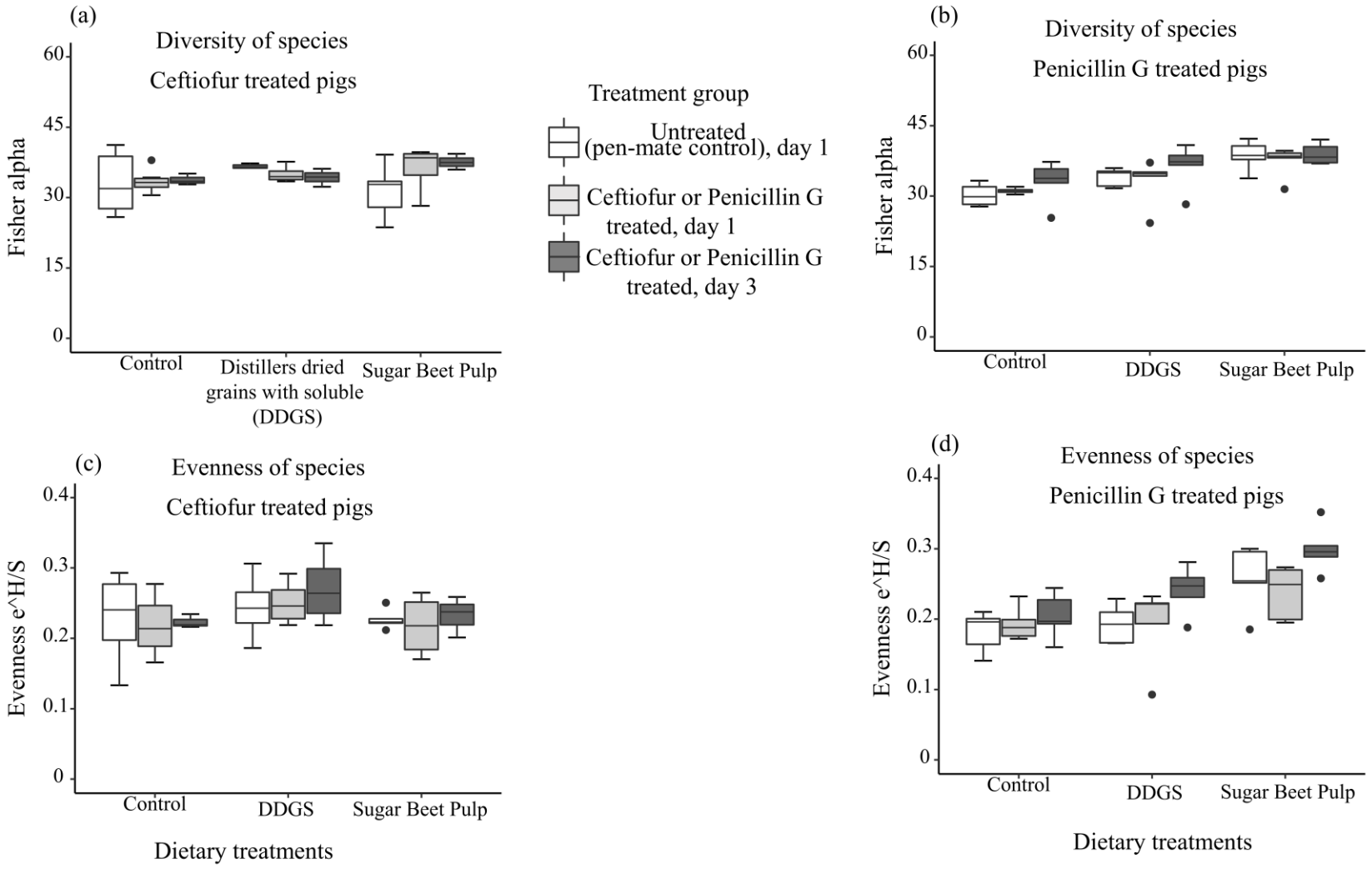

Figure 5.7. Alpha diversity of the fecal microbiome taxonomic composition across the dietary treatments and antimicrobial treatments in finisher pigs. The diversity (Fisher alpha index) and evenness (evenness index) in pigs receiving 1 of 3 dietary treatments and either

no antimicrobial treatment or treated by ceftiofur (a, c) or penicillin G (b, d). The dietary treatments varied in fiber content and its source: control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

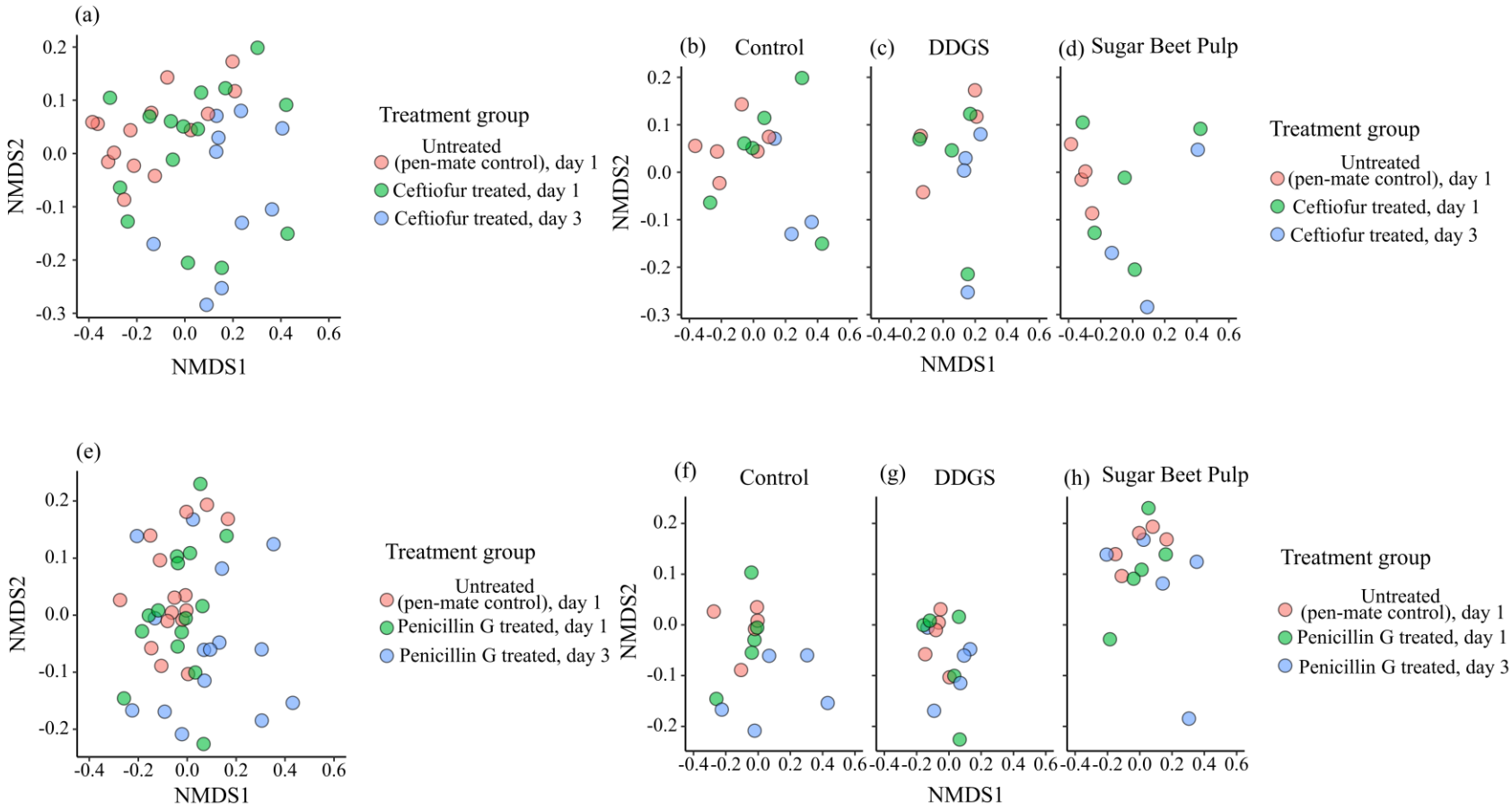

Figure 5.8. Beta diversity of fecal microbiome composition of pig receiving 1 of 3 dietary treatments and antimicrobial drugs. Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis distances by ceftiofur treatment group across the dietary treatment (a-c) by in penicillin G treated group across the dietary treatments (e-f). The dietary treatments varied in fiber content and its source: control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with

solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

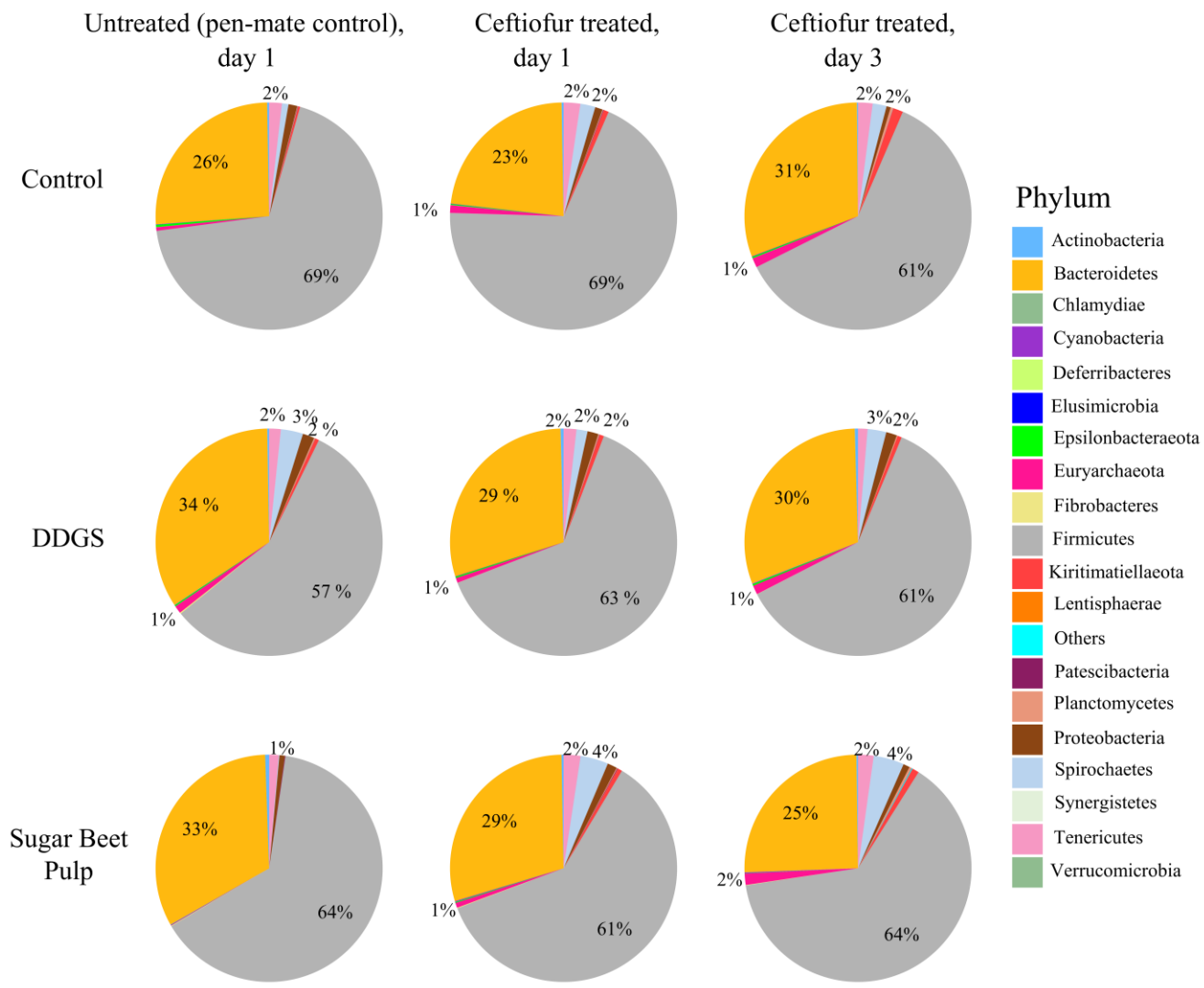

Figure 5.9. A pie chart depicting the relative abundance of the microbiome (phylum level) of pigs receiving 1 of 3 dietary treatment and receiving either no treatment or ceftiofur treatment on day 1 and day 3. The dietary treatments varied in fiber content and its source:

control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

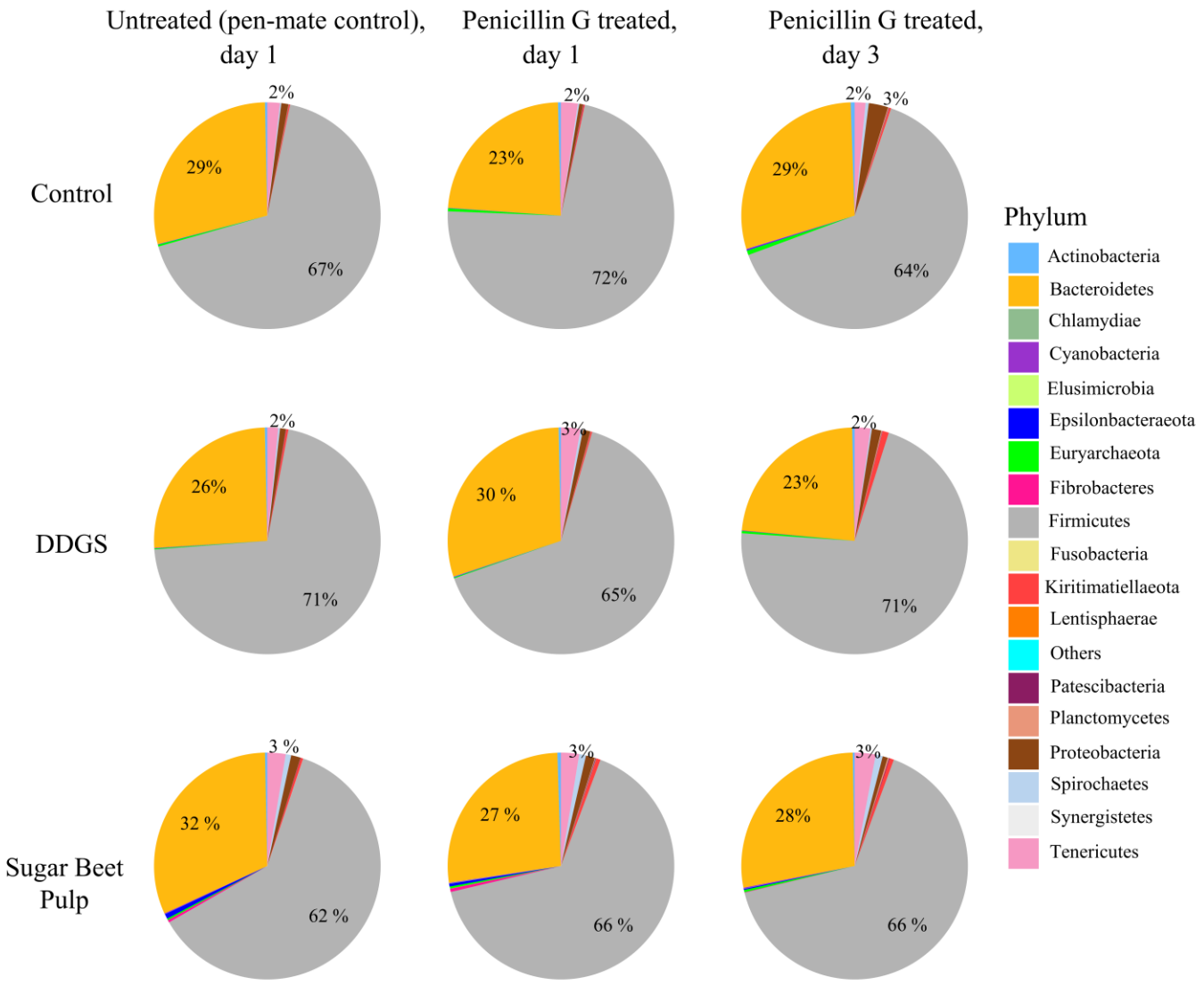

Figure 5.10. A pie chart depicting the relative abundance of the microbiome (phylum level) of pigs receiving 1 of 3 dietary treatment and receiving either no treatment or penicillin G treatment on day 1 and day 3. The dietary treatments varied in fiber content and its

source: control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

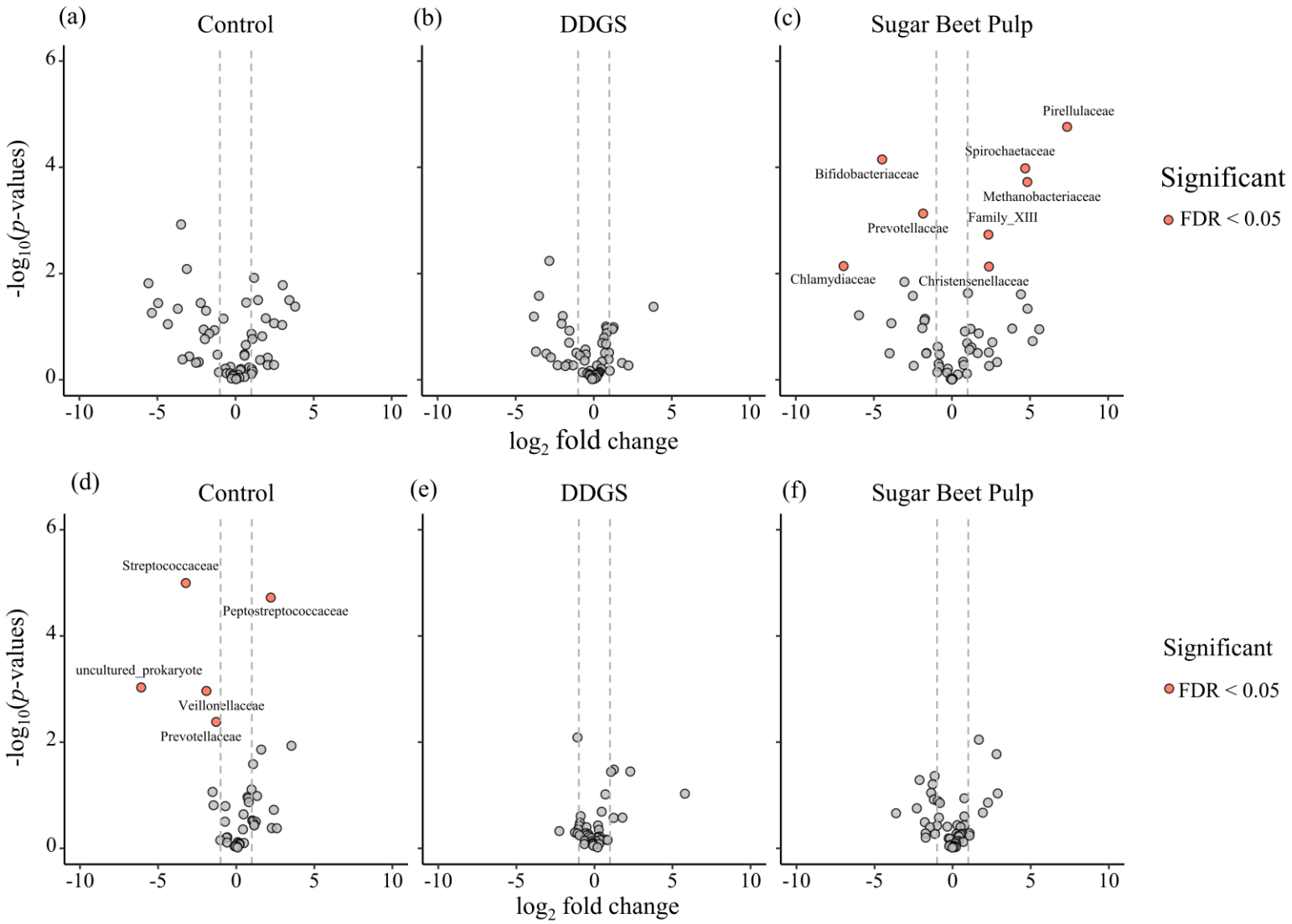

Figure 5.11. Bacterial taxonomic composition (at the family level) are showing significantly different abundance (adjusted *p*-value based on false discover rate (FDR)) in the ceftiofur treated group (a-c), and penicillin G treated group (d-f) receiving 1 of 3 dietary

treatment. The dietary treatments varied in fiber content and its source: control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber). Positive log-fold changes indicate an increase in abundance and negative log-fold changes indicate a decrease in abundance in the antimicrobial treated pigs compared to the control group. The vertical dotted line represents a range of log-fold change (-1 to 1).

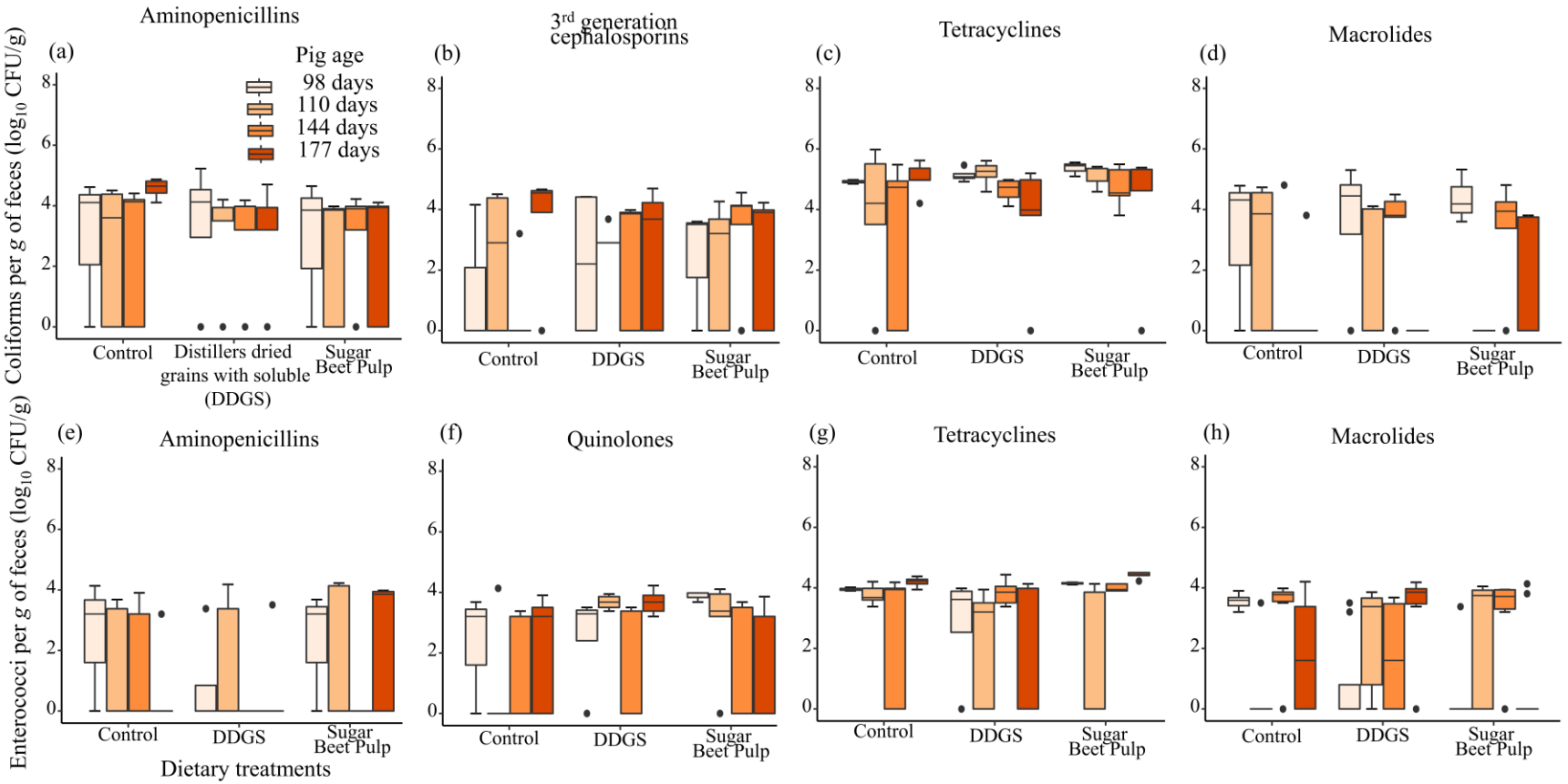

Figure 5.12. Boxplot of the abundance of fecal coliforms (a-d) or enterococci (log_{10} CFU/g) (e-h) growing in the presence of the clinical breakpoint concentration of a drug of that antimicrobial class, for aminopenicillins, 3rd generation cephalosporins, quinolones, tetracyclines, and macrolides. The data are for production pigs 98, 110, 144, and 177 days of age receiving 1 of 3 dietary treatments. The dietary treatments varied in fiber content and its source: control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

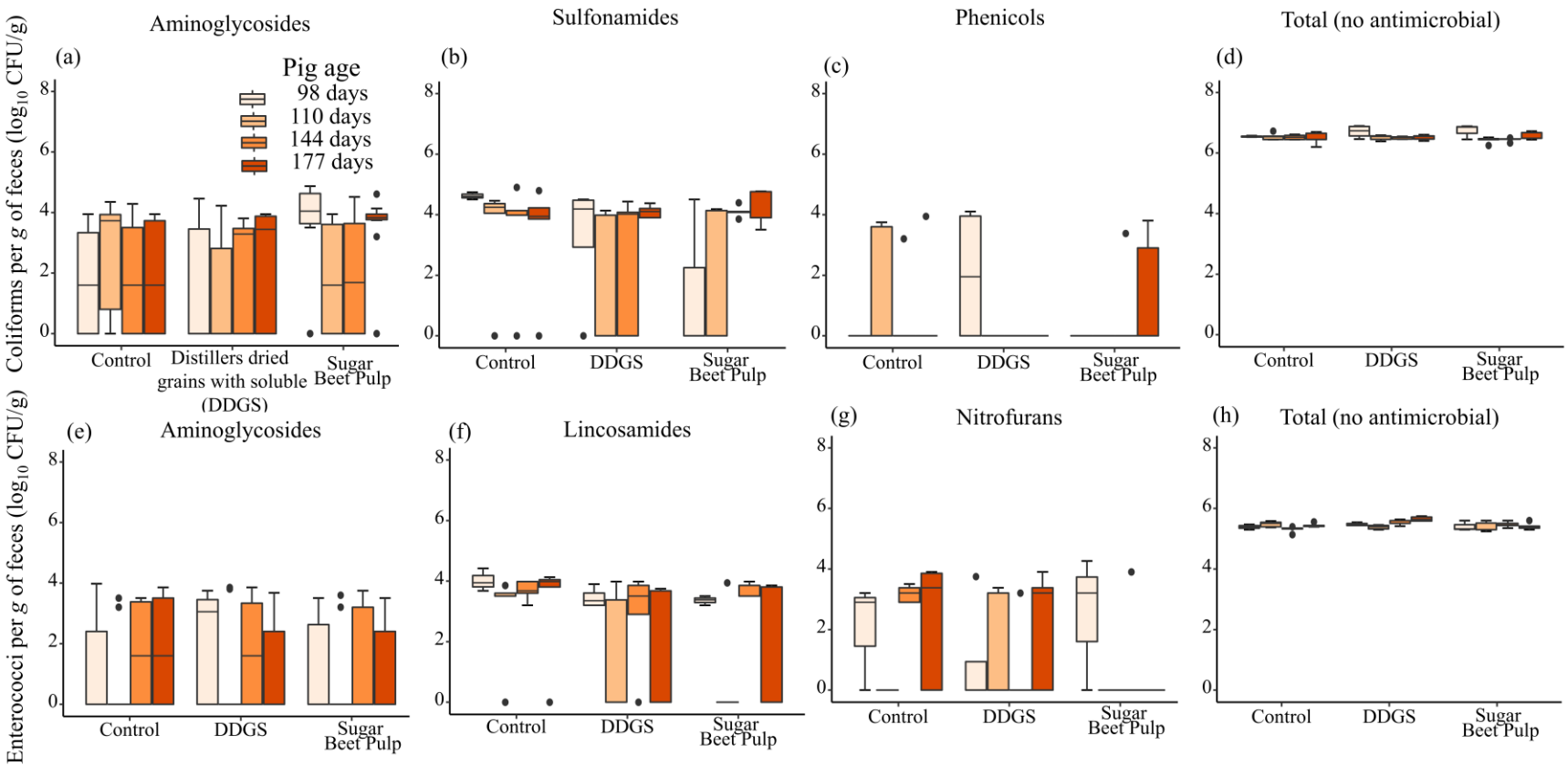

Figure 5.13. Boxplot of the abundance of fecal coliforms (a-d) or enterococci (log_{10} CFU/g) (e-h) growing in the presence of the clinical breakpoint concentration of a drug of that antimicrobial class, for aminoglycosides, sulfonamides, phenicols, lincosamides, and nitrofurans and without antimicrobial (d and h). The data are for production pigs 98, 110, 144, and 177 days of age receiving 1 of 3 dietary treatments. The dietary treatments varied in fiber content and its source: control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

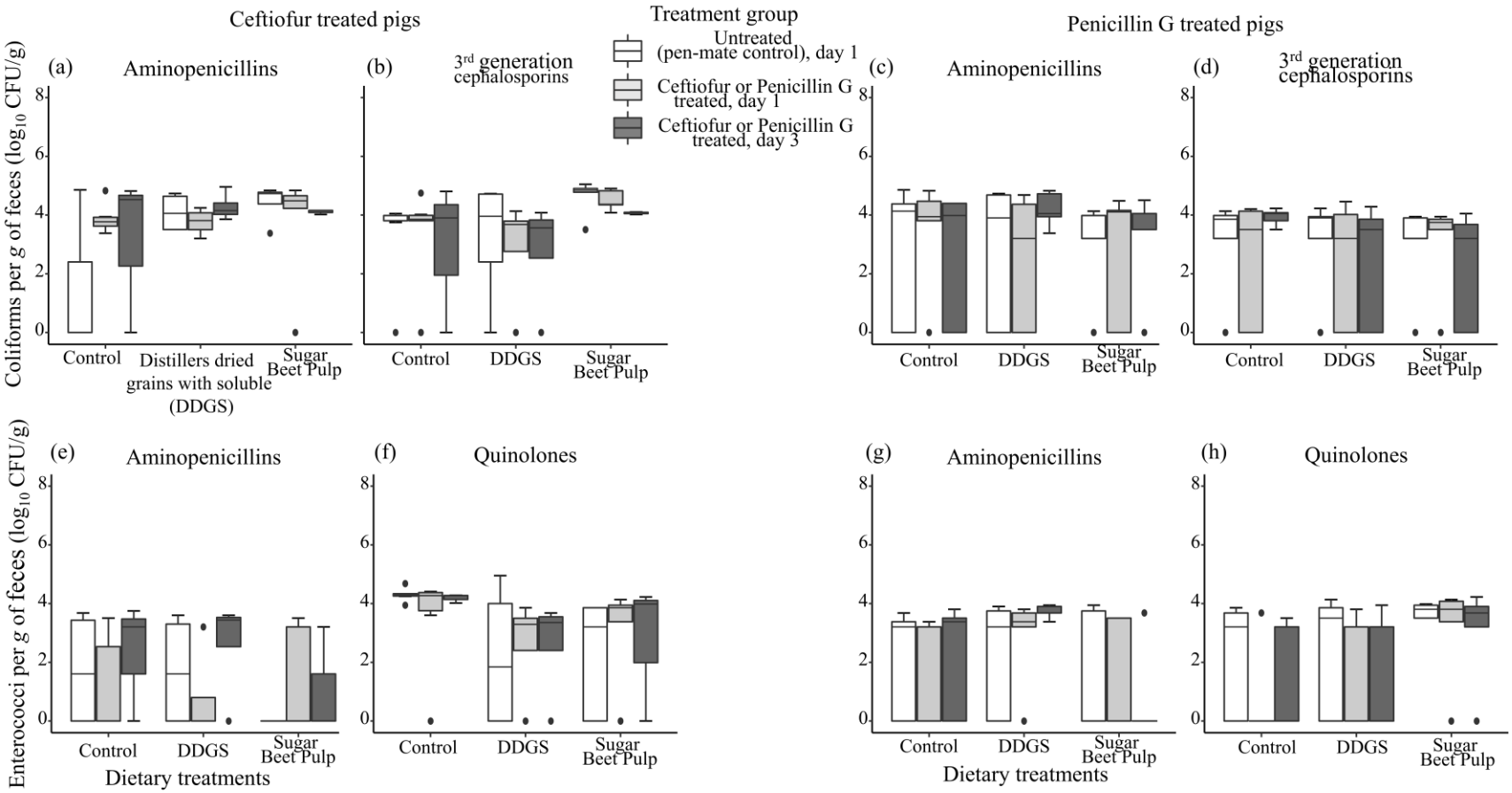

Figure 5.14. Boxplot of the abundance of fecal coliforms (a-d) or enterococci (c-d) (log_{10} CFU/g) growing in the presence of the clinical breakpoint concentration of a drug of that antimicrobial class, for aminopenicillins, 3rd generation cephalosporin and quinolones. The data are for finisher pigs receiving 1 of 3 dietary treatments and receiving either no antimicrobial treatment or treated by ceftiofur (a, b, e and f) or penicillin G (c, d, g, and h), for the days 1 and 3 of the treatment regimen. The dietary treatments varied in fiber content and its source: control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains

with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

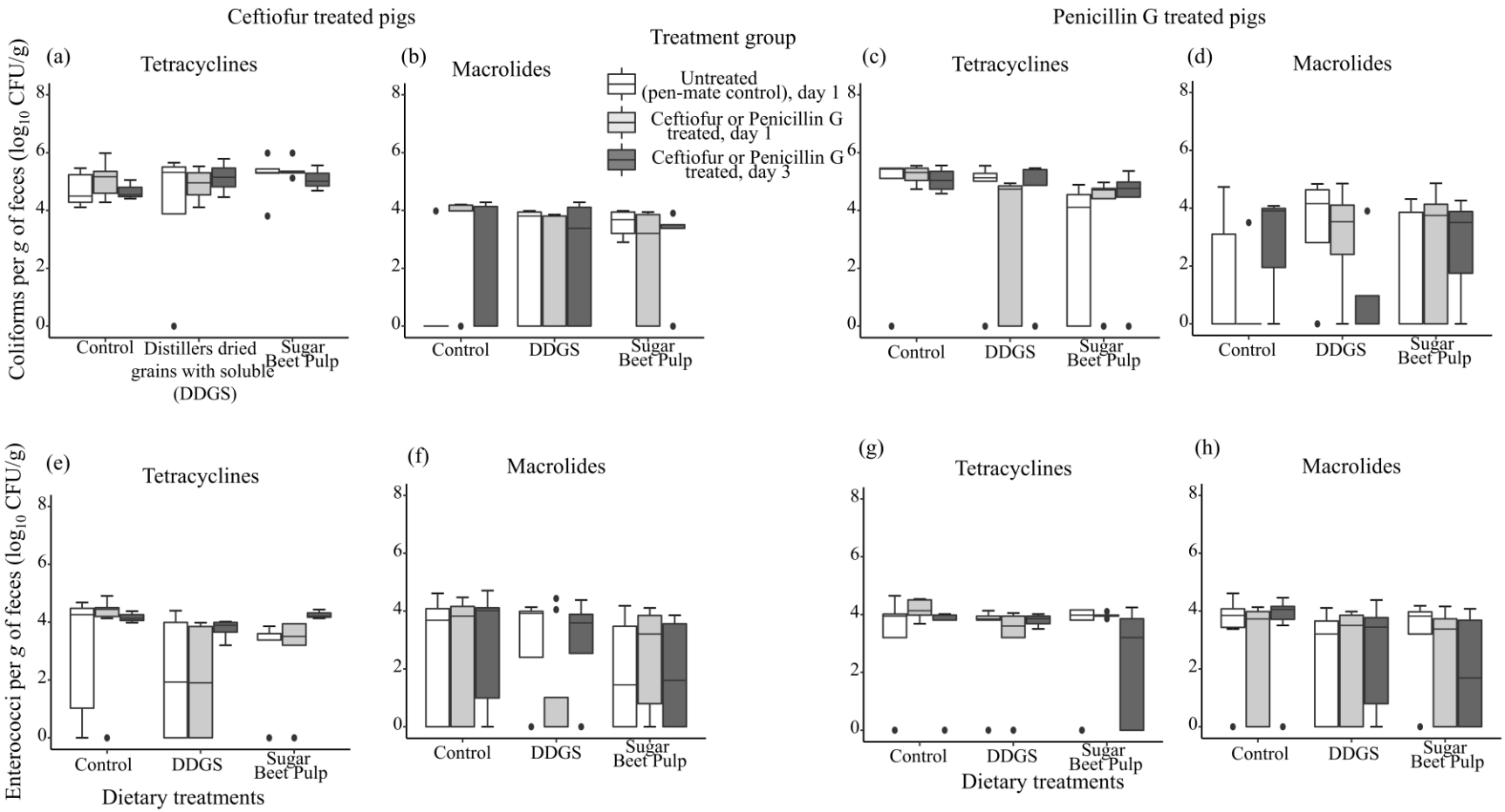

Figure 5.15. Boxplot of the abundance of fecal coliforms (a-d) or enterococci (c-d) (log_{10} CFU/g) growing in the presence of the clinical breakpoint concentration of a drug of that antimicrobial class, for tetracyclines and macrolides. The data are for finisher pigs receiving 1 of 3 dietary treatments and receiving either no antimicrobial treatment or treated by ceftiofur (a, b, e, and f) or penicillin G (c, d, g, and h), for the days 1 and 3 of the treatment regimen. The dietary treatments varied in fiber content and its source: control diet (corn-

soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

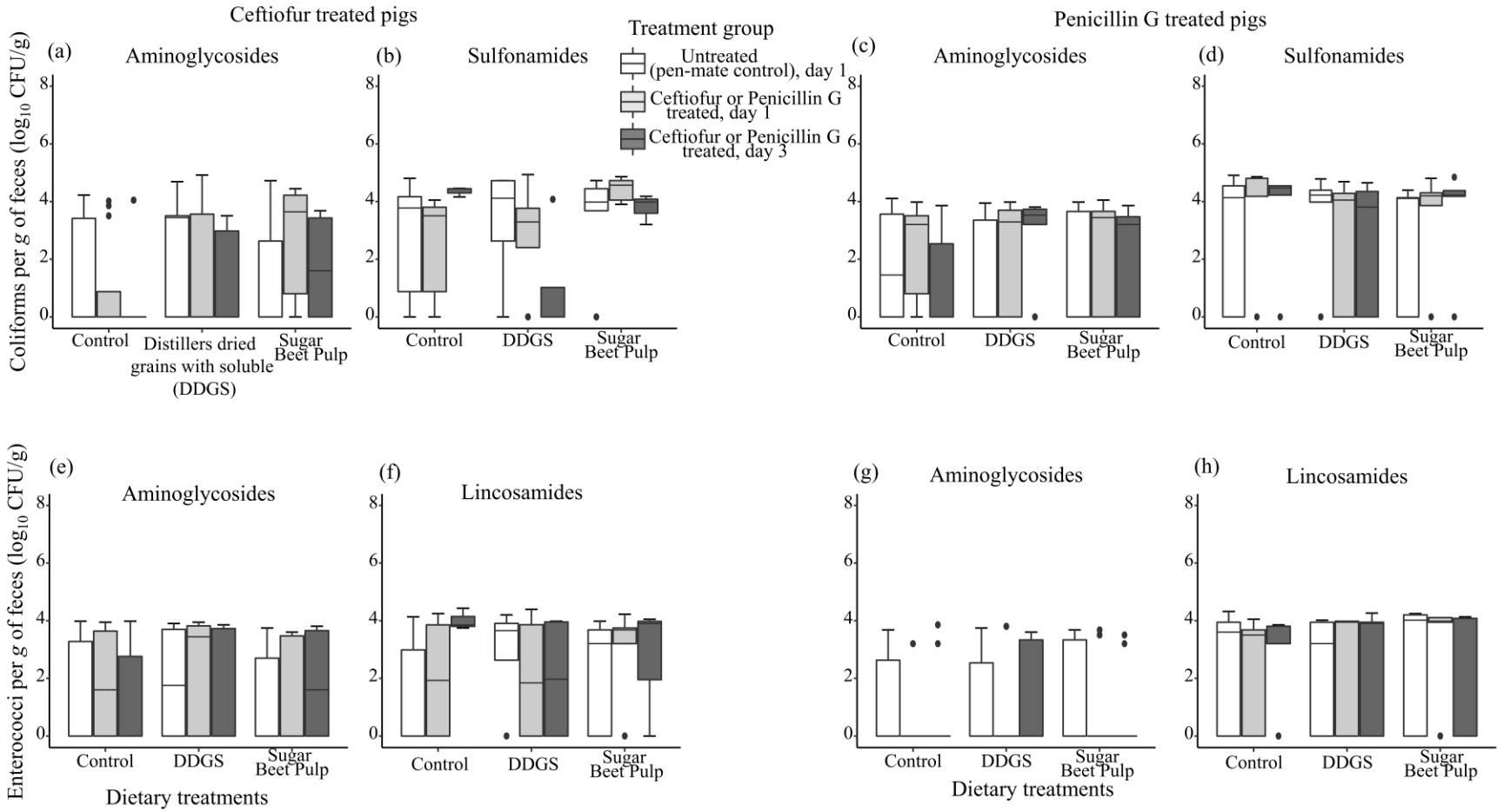

Figure 5.16. Boxplot of the abundance of fecal coliforms (a-d) or enterococci (c-d) (log_{10} CFU/g) growing in the presence of the clinical breakpoint concentration of a drug of that antimicrobial class, for aminoglycosides, sulfonamides, and lincosamides. The data are for finisher pigs receiving 1 of 3 dietary treatments and receiving either no antimicrobial treatment or treated by ceftiofur (a, b, e, and f) or penicillin G (c, d, g, and h), for the days 1 and 3 of the treatment regimen. The dietary treatments varied in fiber content and its source: control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), DDGS—distillers dried grains with

solubles (20% DDGS with 13.6% NDF and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber).

Chapter 6 - Fecal Concentrations of Ceftiofur Equivalents in Pigs Administered with Injectable Ceftiofur: A Pilot Study

Abstract

The objectives of the study were to determine the effects of dietary fiber on antimicrobial active ceftiofur metabolites concentration in feces after intramuscular administration of the cephalosporins ceftiofur drug to finisher pigs. The fecal samples (*n*=40) were collected from ceftiofur treated (*n*=15) and untreated pen-mate control *(n*=15) pigs receiving 1 of 3 dietary treatments: control diet (corn-soybean based with 8.7% neutral detergent fiber and 2.2% crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% neutral detergent fiber and 3.1% crude fiber and primarily contains insoluble fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% neutral detergent fiber and 5.1% crude fiber and primarily contains soluble fiber) in their pens during finisher stage. The amounts of ceftiofur metabolites measured as ceftiofur metabolites, including desfuroylceftiofur were analyzed. Overall, the average concentration of ceftiofur metabolites did not differ significantly between the dietary treatments. In all dietary treatment groups, the average concentration of drug metabolites (µg/mL) was lower on day 3 compared to day 1 of the 3-day treatment regimen by 59% of that on day 1. The decreased in the average ceftiofur concentration on day 3 vs. day 1 of treatment approached marginal statistical significance (ANOVA *p*-value=0.10, *n*=14 treated pigs). This trend was consistent between gender and across three animal diets that varied in fiber content and source. The concentrations of ceftiofur and its active metabolites in the porcine feces can be expected to be affected by which *β*-lactamase producing bacterial strains are present, and the match and potency of the *β*-lactamases to the drug.

Introduction

Antimicrobials have been used in swine production mainly in weaned and finisher pigs, to treat production-related diseases as well as for growth promotion for more than 50 years (Looft et al., 2012a). However, excessive use of antibiotics in the therapeutic setting causes selective pressure that may drive the development of antibiotic resistance as the consequence of horizontal gene transfer or mutation (Martínez, 2008). Thus, with the growing concern over antimicrobial resistance, alternative approaches such as pre/probiotics and dietary interventions have been investigated. The benefits associated with the presence of fiber in the diet have been identified; this includes the change in the gut microbial community composition and promotion of enteric health. Moreover, an earlier study has shown that dietary interventions significantly change both the richness and diversity of the gut resistome in children (Wu et al., 2016). Similarly, studies described the range of modifiable non-antimicrobial factors (including diet) or interventions to mitigate antimicrobial resistance in commensal enteric bacteria in cattle (Murphy et al., 2015). For instance, feeding pigs with fiber-containing diets has shown a change in the gut microbial community composition (Metzler and Mosenthin, 2008). Dietary fiber is comprised of indigestible carbohydrates and has a specific effect on the gut physiology in pigs based on the type and level of fiber in the diet (Yan et al., 2017).

Ceftiofur is a semisynthetic, broad-spectrum third-generation cephalosporin antibiotic (NCBI, 2020). The mechanism of action of cephalosporins involves inhibition of bacterial cell wall synthesis. Ceftiofur belongs to the same class of antibiotics as ceftriaxone and is classified as a critically important antimicrobial by the World Health Organization. Ceftiofur (Excenel®, ceftiofur hydrochloride) has been approved by the U.S. Food and Drug Administration (FDA) for intramuscular injection to treat bacterial respiratory diseases in both beef and dairy cattle and

swine Food and Drug (1988); (FDA, 2006). Following intramuscular administration, ceftiofur is absorbed in its free-acid form and rapidly metabolized to desfuroylceftiofur (DFC) and furoic acid (Beconi-Barker et al., 1996). DFC quickly conjugates with both plasma and tissues or further metabolizes to disulfides, such as DFC- cysteine disulfide, DFC-dimer (Beconi-Barker et al., 1995; Beyer et al., 2015). Free forms of DFC contain an intact β-lactam ring and a major biologically active metabolite, similar in the antimicrobial activity to ceftiofur. Further, carry-over of antimicrobials like ceftiofur and their active metabolites in the pen/farm could enhance the development and or dissemination of AMR due to ingestion by untreated animals. Although many studies has been performed about fiber sources and its importance, there are still lack of reports about role of dietary fiber on drug metabolites on feces from antimicrobial treated pigs. The study aimed to evaluate the effect of dietary fiber on the concentration of the ceftiofur metabolites in feces after intramuscular injection of the ceftiofur hydrochloride in finisher pigs.

Materials and methods

Animal experiment, dietary treatments, and sample collection

The study was performed at the Swine Teaching and Research Center, Kansas State University Manhattan, Kansas. A total of 288 pigs (~36 kg starting body weight) from one finishing group were randomly distributed to 36 pens balanced by gender, and these pens were randomly assigned to 1 of 3 dietary treatments: control diet (corn-soybean based with 8.7% neutral detergent fiber (NDF), 2.2 % crude fiber), distillers dried grains with solubles (DDGS)-based diet (20% DDGS with 13.6% NDF and 3.1% crude fiber and primarily contains insoluble fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% NDF and 5.1% crude fiber and primarily contains soluble fiber). The diet was formulated and prepared at the Kansas State University O.H. Kruse Feed Technology Innovation Center (Manhattan, KS). All the pigs

were followed throughout the finisher stage. When clinical signs of diarrhea, swelling, or other production-related diseases prompted antimicrobial drug use, the animals were treated with either penicillin G or Excenel® (ceftiofur hydrochlorides) by intramuscular (IM) injection 3 mg/kg body weight once per day for 1-3 days under an approved veterinarian-client-patient relationship (VCPR). On each day 1 and 3 of the regimen, per rectum fecal sample (~ 50 g into a Whirl-Pak bag, Nasco, Ft. Atkinson, WI) was collected from the treated animal in ~6 hours (no later than 8 hours) after the injection. However, some of the animals were only treated for 1-2 days when clinical improvement was seen; hence, only day 1 samples were collected.

Of the ceftiofur-treated animals on the control diet, 3 were sampled on day 1 only and 3 animals on both days 1 and 3 of the regimen; on the distillers dried grains with solubles (DDGS) based diet, 4 animals were sampled on both day 1 and 3 of the regimen and on the sugar beet pulp-based diet, 2 were sampled on day 1 only and 3 animals on both day 1 and day 3 of the regimen. The fecal samples were transported on ice to a laboratory at the College of Veterinary Medicine, Kansas State University, Manhattan, KS. Upon arrival to the laboratory, the samples were transferred to Eppendorf® centrifuge tubes (¾ full of feces) and centrifuged for 15 minutes at 15,000 rpm. The supernatant was pipetted from each Eppendorf tube to a 2 mL-0.45 µm filter Spin-X® centrifuge cryotube and again centrifuged at 5,000 rpm for 5 minutes. Fecal supernatants were labeled appropriately and stored at -80°C for the measurement of antibiotic concentrations. The supernatant was not possible to obtain from the sample from one pig treated with ceftiofur and sampled on day 1 only, which was reared on the control diet. The supernatants were submitted to a pharmacological laboratory at the Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, for determination of the ceftiofur metabolite concentrations.

Determination of ceftiofur metabolite concentrations

The amounts of ceftiofur metabolites such as DFC and DFC in fecal samples were analyzed by the liquid chromatography (LC) as previously described (Jaglan et al., 1990). Briefly, fecal supernatants in 0.1 M pH 8.7 phosphate buffer containing dithioerythritol were incubated at 50 °C for 15 minutes. The samples were centrifuged, charged to a C18 cartridge, and then washed with 0.1M ammonium acetate. The desfuroylceftiofur residue in the cartridge was derivatized by adding 0.1M ammonium acetate with iodoacetamide and left in the dark for 30 minutes. The cartridge was rinsed, and desfuroylceftiofur acetamide was eluted with methanol and allowed to evaporate until dry and dissolved in a sodium hydroxide (pH 10.6) and charged to a SAX cartridge. These derivates were eluted with 2% acetic acid and dissolved in the mobile phase for liquid chromatography (LC). To recover the metabolites, the gradient mobile phase (1 mL/minute) was 0.01M ammonium acetate (pH 5) programmed to 29% methanol-water (60 + 40) in 25 minutes for the recoveries.

Statistical analysis

Descriptive statistics of the ceftiofur metabolite concentrations by the dietary treatment and animal age and gender were obtained. Statistical significance of the dietary treatment, age, and gender effects was tested using ANOVA in the R software (R, 2019). The level of significance was set at $p < 0.05$ and marginally significant at $p=0.10$.

Results

Overall, the mean concentration of ceftiofur metabolites (µg/mL) in feces on day 3 of the treatment regimen (0.36±0.39 µg/mL) was 59% of that on day 1 (0.15±0.08 µg/mL) (n=14 animals were sampled on day 1, and 10 of these also on day 3 of the treatment regimen). This decrease in the average ceftiofur concentration on day 3 vs. day 1 of the treatment approached

statistical significance (p=0.1, Figure 6.1, a). This trend was consistent between sex and across three animal diets. For example, the mean concentration of ceftiofur metabolites in the feces on day 3 was lower by 72%, 33%, and 59% from that of day 1 in feces of pigs fed control diet (corn-soybean based with 8.7% neutral detergent fiber, 2.2 % crude fiber), distillers dried grains with solubles (DDGS) (20% DDGS with 13.6% neutral detergent fiber and 3.1% crude fiber and primarily contains insoluble fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% neutral detergent fiber and 5.1% crude fiber and primarily contains soluble fiber). However, the mean concentration of drug metabolites did not differ significantly between the dietary treatments, and there was no significant interaction between the day of treatment and diet (Figure 6.1, b). The mean concentration of drug metabolites present in day 3 were 55% lower than day 1 in male (day 1: 0.44±0.45, day 3: 0.2±0.09) and female (day 1: 0.25±0.19, day 3: 0.11±0.05) pigs. However, gender did not significantly affect the concentrations, and there was no significant interaction between the gender and dietary treatment effects on the metabolite concentration (Figure 6.1, c). At the individual animal level, of 14 ceftiofur-treated pigs, 10 pigs were treated and sampled on both day 1 and day 3, and 4 pigs were treated and sampled only on day 1. Among the 10 pigs, 70% had a lower ceftiofur metabolite concentration in feces on day 3 compared to day 1 of the treatment.

Discussion

We measured the active ceftiofur metabolite concentrations in feces of pigs that were treated with ceftiofur hydrochloride by intramuscular injection and fed one of three diets. The main finding of the study was that the average concentration of drug metabolites (µg/mL) was lower in feces of pigs on day 3 of the 3-day treatment compared to day 1 across all the dietary treatment groups and across animal genders. Furthermore, we observed that the concentration of

the drug metabolites was more predictable (tighter distribution) on day 3 compared to day 1 across the dietary treatments. We have no obvious explanation for the decline in concentration on day 3, but we suspect that this might be due to the expansion of endogenous *β*-lactamases of microbial origin. Ceftiofur's *β*-lactam ring is crucial for its antibacterial activity, but it is also vulnerable to degradation (Dołhań et al., 2014) by *β*-lactamases that are secreted by bacteria and hydrolyze the *β*-lactam ring. Earlier studies have demonstrated that ceftiofur metabolite degradation in animal feces is biotic (Gilbertson et al., 1990), and thus the intestinal microbial community may be contributing to the degradation in the intestine of biliary excreted *β*-lactams or their metabolites (Gilbertson et al., 1990; Hornish and Kotarski, 2002; Stentz et al., 2014; Beyer et al., 2015). The production of beta-lactamases by *Bacteroides,* a member of the gut microbiome, may be protecting other members from *β*-lactams (Stiefel et al., 2015).

The concentrations of ceftiofur active metabolites in porcine feces can be expected to be directly affected by the *β*-lactamases produced by intestinal bacteria in response to the antimicrobial exposure, and the match and potency of the *β*-lactamases to the metabolites. Moreover, the *β*-lactamase production can be expected to be dose-dependent, accelerating in response to an increase in the *β*-lactamase concentration (Livermore, 1995). Thus, lower *β*-lactam concentrations on day 3 of the treatment may be due to the expansion or better match and potency of *β*-lactamases produced by the intestinal microbiome by day 3 of the treatment compared to indigenous *β*-lactamases present on day 1.

Conclusion

The concentration of ceftiofur active metabolites in porcine feces was lower on day 3 compared to day 1 of the 3-day ceftiofur treatment, irrespective of the animal diet or gender.

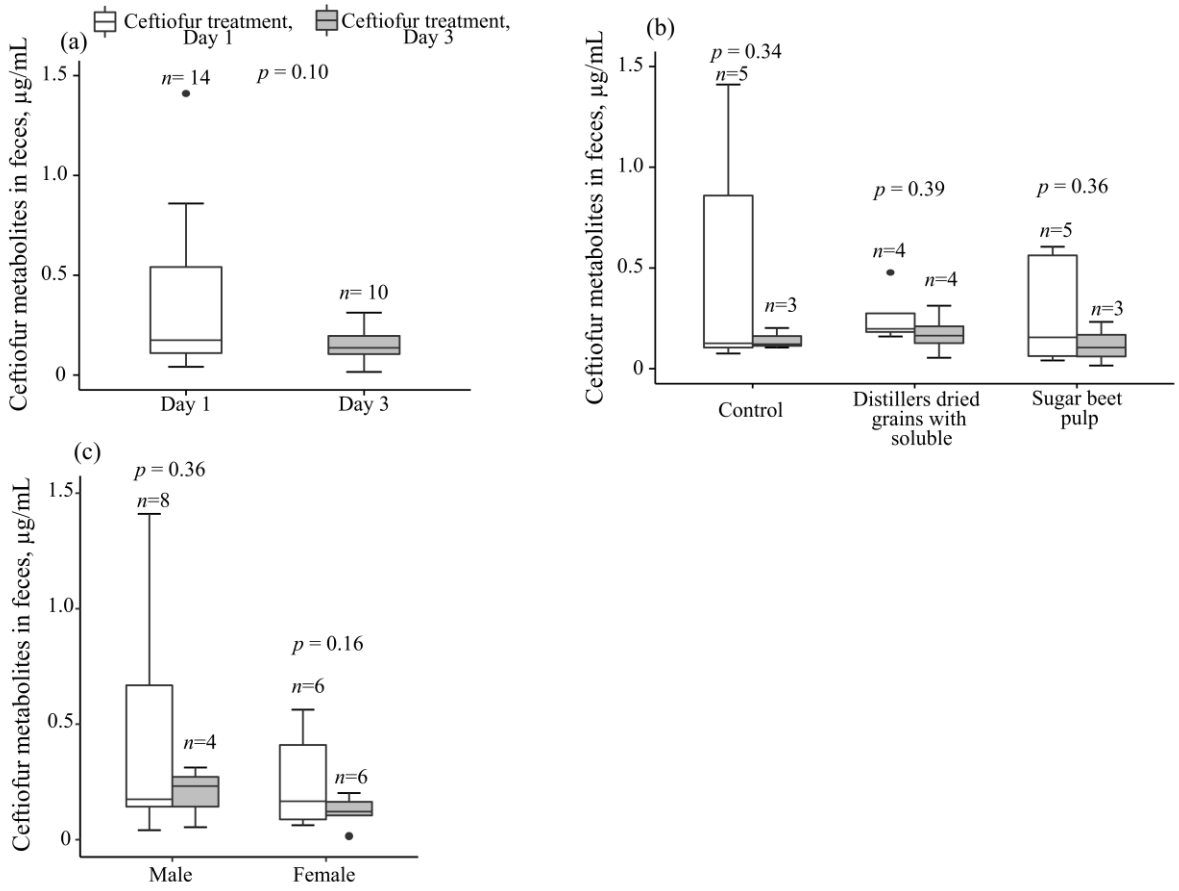

Figure 6.1. Boxplots of concentration of ceftiofur metabolites (µg/mL) in feces of finisher pigs by (a) day of the 3-day ceftiofur treatment; b) animal diet; and c) in male and female pigs across the three diets. Ceftiofur was administered intramuscularly (in the dose 3 mg/kg body weight, by infection at a 24 h interval for a total of three consecutive days). Fecal samples were collected in 6 hours post-injection on days 1 and 3. Some of the animals were only treated for 1-2 days when clinical improvement was seen; hence, only day 1 sample was collected. Fourteen animals were sampled on day 1, and 10 of these also on day 3 of the treatment. *n*, number of pigs. The dietary treatments varied in fiber content: control diet (corn-soybean based with 8.7% neutral detergent fiber, 2.2 % crude fiber), DDGS—distillers dried grains with solubles (20% DDGS with 13.6% neutral detergent fiber and 3.1% crude fiber), and sugar beet pulp-based diet (14.5% sugar beet pulp with 13.6% neutral detergent fiber and 5.1% crude fiber).

Chapter 7 - Distribution of AMR Genes and Taxonomic Composition of Bacteria Associated with Gut Contents and Mucosal Epithelium in Piglets

Abstract

Fecal samples are commonly used to describe the taxonomic composition and antimicrobial resistance (AMR) gene content of the host enteric bacteria. However, it remains unknown how the AMR-genes in the feces are related to the prevalence in different locations of the gastrointestinal (GI) tract. The objective was to describe the bacterial taxonomic composition and AMR genes throughout the GI tract of piglets. Six to seven weeks old, weaned piglets (n=3), clinically healthy and did not receive antimicrobial drugs for 2 weeks prior to being euthanized, were used. Paired luminal and mucosal epithelium samples were collected, within 30-40 minutes post-euthanasia, from the stomach, duodenum, ileum (at two locations), cecum, spiral colon, and rectum of each animal. The bacterial composition was determined using the 16S rRNA gene sequencing and individual AMR genes using the targeted amplicon sequencing methods. The richness, diversity, and evenness of taxa did not change significantly (Kruskal-Wallis ANOVA, p >0.05) across the GI locations nor with sample types (contents vs. mucosa). However, bacterial community composition changed across the GI location (p <0.05). The most abundant genera were *Lactobacillus*, *Prevetoella*, *Campylobacter*, *Roseburia* in the GI location. The relative abundance of *Campylobacter* was significantly (Wilcoxon-rank test, p <0.05) higher in mucosa samples compared to contents samples. A total of 102 AMR genes were detected across all pig samples. Genes encoding bacterial resistance or reduced susceptibility to tetracyclines, β-

lactams, aminoglycosides, and glycopeptides were most abundant in the samples. The AMR-gene content of the luminal contents and mucosa changed between the compartments of the GI tract of animals. The AMR-gene diversity in the contents was higher than in the mucosa in individual GI compartments.

Introduction

The pig gut microbiota has been shown to be linked to feed intake, body weight gain, feed efficiency, immune response, and overall health (Mann et al., 2014; Mach et al., 2015). It is suggested that the microbial colonization in pigs starts at birth (farrowing), and subsequent establishment occurs via contact with their mother's skin, feces, or environment while they are nursing until weaning (Thompson et al., 2008). For instance, within a few days after birth, anaerobic microbes become dominant, and the neonatal microbial community then shifts later to become compositionally distinct after postweaning (Katouli et al., 1997; Thompson et al., 2008). There is little known about the microbiome community established in different locations of the GI tract of pigs and how they differ across mucosa and content samples in these locations.

Several studies have examined fecal microbial community compositions and diversity over time in pigs, but few studies evaluated microbiome profiles in different segments of the gastrointestinal tract (Looft et al., 2014; Yang et al., 2016a; Kelly et al., 2017; De Rodas et al., 2018). An earlier study exhibited that microbial communities vary both longitudinally (proximal to distal) and radially (mucosal to luminal) across the GI tract. Further, the small intestine was characterized as having increased aerobic conditions and increased transit times (Schwarz et al., 2002), and was associated with having a source of antimicrobial peptides in the Paneth cells (specialized cells in the epithelium of the small intestine) (Bevins and Salzman, 2011). However, there is limited knowledge of the AMR gene composition across the GI tract of pigs. Earlier studies indicated that fecal samples do not necessarily represent the bacterial taxonomic composition and antimicrobial resistance (AMR) gene content of the host enteric bacterial community of the GI tract (Hill et al., 2010; Looft et al., 2014). Therefore, it remains unclear how the AMR-gene content of the feces is generated throughout the GI tract.

We investigated and analyzed the microbial communities for the presence of AMR genes and their compositions along the digestive tract of piglets. We evaluated the taxonomic similarities and differences and AMR gene contents of mucosal versus luminal contents (digesta) of healthy pigs fed a diet containing no antibiotics (at least 3 weeks prior to collection of the samples).

Materials and methods

Animals and sampling

A total of three, 6-7 weeks old, weaned piglets were used. The piglets were clinically healthy and had no exposure to antimicrobial drugs for three weeks prior to euthanasia. The feeding and watering conditions were typical for the species and age.

Gastrointestinal (GI) contents and mucosa sample collection

The contents and mucosa samples of the pig GI tract were collected from seven different locations of the GI tract (stomach, duodenum, two locations of ileum, the blind end of the cecum, spiral colon, and rectum) (Figure 7.1). Piglets were humanly euthanized, and the samples were collected aseptically within approximately 30 minutes after euthanasia. The content samples were obtained from each location by pipetting ~ 1 mL of the luminal contents and transferring into a tube containing 10 mL RNAlater® stabilization solution (Thermo Fisher Scientific, Carlsbad, CA, USA). The mucosal samples were collected by cutting the intestinal tube sections. These samples were then transferred to a tube containing 25 mL phosphate buffer saline (PBS) (Thermo Fisher Scientific) and stored at 4 °C for 2.5-4.5 hours. The mucosa samples of each gut section were collected by aseptically scraping with a sterile blade after rising off with PBS. The mucosal samples were placed into sterile plastic tubes containing 10 mL of RNAlater and mixed properly. From the ileum of each animal, two mucosa samples were taken in the region where

there no Payer's patches. One mucosal sample was obtained from each of the other intestinal parts. Each of the contents and mucosal samples were examined, and if it appeared clumped, it was mixed using a sterile plastic bacteriological loop to maximize contact with the stabilizer. The contents and mucosal samples were left to soak in RNAlater for 1 day at 4^{o} C, then frozen at -80^{o}C.

Sample processing, sequencing, and statistical analysis

From each sample, metagenomic DNA was extracted from 0.25 g of the sample using UltraClean 96-well Microbial DNA kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer instructions. The 16S rRNA gene sequences were performed for microbiome taxonomic composition, and AmpliSeq™, target amplicon sequencing protocol, was performed to determine the presence of AMR genes in the samples as previously described (Gardner et al., 2015; Urbaniak et al., 2018). AmpliSeq™ libraries were constructed with the Ion AmpliSeq™ library protocol (2x primer mix, 6 µL of samples, and 16 PCR cycles consisting of a 99° C for 15 seconds: 60° C for 4 minutes). The final libraries were checked using the Agilent BioAnalyzer™ (Agilent, CA, USA) and quantified using the Ion library quantification kit™ (Thermo Fisher Scientific). AmpliSeq™ custom panel was designed to match the presence of antimicrobial resistance determinant microarray (ARDM). Amplicon sequence read quality control and reference mapping were performed as previously described (Gardner et al., 2015; Urbaniak et al., 2018). Alpha diversity indices of the microbiome were captured by taxa richness (Chao1 index), diversity (Shannon index) indices, and the evenness index was analyzed. The difference in the relative abundance of bacterial genera between the contents and mucosal samples for each GI location were analyzed using non-parametric ANOVA. Similarly, non-metric multidimensional scaling (NMDS) ordination plots were made using the vegan package in

R (Jari Oksanen, 2019) based on bacterial taxonomic counts using the Bray-Curtis dissimilarity distances; NMDS ordination plots were visualized using the ggplot2 package (Wickham, 2009). The difference in beta diversity for each GI location (and contents vs. mucosal samples) were evaluated by using the permutational analysis of variance (PERMANOVA) (based on Bray-Curtis distances) using *adonis* in the *vegan* package. The permutational analysis of multivariate dispersion (PERMDISP) was then performed to compare the Bray-Curtis distance to group centroids among samples. Similarly, the richness and diversity of the ARGs (gene encoding AMR, by mechanisms of AMR present genes, and by drug classes) were also evaluated.

Results

The taxonomic composition and AMR-gene repertoire in the content and mucosal samples changed throughout the GI tract in the piglets. Alpha diversity of the bacterial taxa was evaluated across the GI locations using the richness index (Chao1 index), the diversity index (Shannon index), and the evenness (evenness index) (Table 7.1). The (detectable) diversity, richness, and evenness show an *S*-shape pattern, being higher in the contents of the stomach and the large intestine, compared to the small intestine. The richness, diversity, and evenness of taxa did not differ with contents vs. mucosa samples (Kruskal-Wallis ANOVA, $p > 0.05$). Similarly, richness, diversity, and evenness of taxa did not change significantly (Kruskal–Wallis ANOVA, $p > 0.05$) across the GI location (Figure 7.5).

Comparison of the microbial taxonomic composition across the GI tract

The genus-level analyses were performed to compare bacterial community composition associated across GI location and sample type (contents vs. mucosa). Several bacteria genera were detected across the GI location of the pig with some degree of variability (Figures 7.2 and 7.3). The most abundant genera were *Lactobacillus*, *Prevetoella*, *Campylobacter*, and *Roseburia*

across GI sections. The relative abundance of *Campylobacter* was significantly (Wilcoxon-rank test, *p*-value adjusted for multiple testing by FDR at α=0.05, p <0.05) higher in mucosal samples compared to content samples (Figure 7.5). Similarly, across the GI location, the relative abundance of several bacterial genera was significantly changed, including *Prevotella*, *Roseburia*, *Faecalibacterium*, *Succinivibrio*, *Oxalobacter*, *Lactobacillus*, *Mitsuokella*, *Brachyspira*, *Phascolarctobacterium*, *Clostridium* (Table 7.2).

The difference in bacterial taxonomic composition between those located within the mucosa with those in the contents and across the GI location using non-metric multidimensional scaling (NMDS) plot using Bray-Curtis distance were investigated (Figure 7.6, a). These plots demonstrated a shift in the bacterial community across the GI location (stomach, duodenum, two locations of ileum, cecum, colon, and rectum). The PERMANOVA (based on Bray-Curtis distances) results showed were significantly different among the microbial community of the GI tract (p=0.001). However, there is no distinct separation according to sample types (i.e., contents vs. mucosa) (Figure 7.6, b), and multivariate analysis further indicated that there were no significant differences among the microbial community compositions of the contents and mucosal samples across the GI tract (p=0.086).

Distribution of AMR genes between contents and mucosal samples across the GI locations

The presence of AMR genes across the GI location (contents and mucosal samples) were determined. In total, 102 AMR genes (range 3-80) were detected across all pig samples. Similarly, a total of 28 AMR genes encoding specific resistant mechanisms (range 3-28) by drug class were detected across all samples. The several AMR genes by drug class were tetracycline, aminoglycosides, β-lactam, chloramphenicol, glycopeptides, lincosamides, macrolides,

nitroimidazole, quinolones, streptogramins, sulfonamides, trimethoprim (Figures 7.7, 7.8, and 7.9) were detected in the samples. Among these drug classes, tetracycline, macrolides, aminoglycosides, and β-lactam resistance comprised the highest proportions in all samples.

The overall diversity of AMR genes, the gene encoding specific resistant mechanism, and by drug class were higher in contents samples compared to mucosal samples are shown in Figure 7.10. High variability in the diversity of AMR genes in the stomach and small intestinal locations compared to the large intestine was observed. However, AMR diversity did not differ across the GI location (Kruskal–Wallis ANOVA, $p > 0.05$), but significantly higher in luminal samples compared to mucosal samples ($p < 0.05$).

Further, we performed the nonparametric Spearman correlation test between the diversity of AMR genes and the diversity of bacterial genera. The results showed that there was a significant positive relationship between the diversity of AMR genes and the diversity of bacterial genera in luminal samples ($\rho = 0.68$, $p=0.007$) but weak correlation and non-significant correlation ($\rho = 0.13$ $p=0.56$) with mucosal samples across the GI section.

Discussion

This study characterized the bacterial taxonomic composition and AMR genes throughout the GI tract of piglets. Our data suggest that the piglet GI comprises diverse bacterial genera of the piglet luminal and mucosa-associated are influenced by the GI location with a clear separation between proximal and distal GI parts, particularly between the lower small intestine and large intestine.

The identified bacterial diversity, richness, and evenness were higher in the contents in the stomach and large intestinal locations, compared to the small intestine; however, these dynamics were similar between the luminal contents and mucosa. The finding suggests that the

bacterial communities of gastric, duodenum, ileum are more variable than large intestine (cecum, colon, and rectum), which is similar in other production animals (Danzeisen et al., 2013). The lower alpha diversity in the midgut (duodenum, jejunum) and higher alpha diversity of the large intestine (cecum, colon, and rectum) were also found in another study of the mucosal microbiomes of ~28 days-old piglets (Kelly et al., 2017). The higher variability in the stomach, duodenum, and ileum compared to the large intestine could be due to the lower abundance of a bacterial community; the abundance is less stable due to the continuous influx of new bacteria from the feed, and overall shorter transient time, and different aspects of the local environment (Donaldson et al., 2016). Further, this variability could be due to individual piglet genetic factors and the immune response of animals during sampling (Fagarasan et al., 2002). An earlier study also showed that the GI tract contains a dynamic bacterial population with distinct bacteria residing in different sections of the gut. The most diverse bacteria were found in the large intestine, most notably in that of the colon of a pig (Kim et al., 2012; Maradiaga et al., 2018). Meanwhile, De Rodas et al. (2018) found that several bacterial communities (beta diversity) were significantly different when comparing the content-mucosa in the cecum but not in the ileum.

In our study, the *Lactobacillus* and *Prevetolla* genera were the most dominant throughout the studied GI locations in the piglets. Therefore, these genera could be the core microbiome of the GI tract in piglets. Moreover, an earlier study also found that *Lactobacilli* was dominant across all GI sites (duodenum/jejunum, Ileum, cecum, and colon) of piglets (De Rodas et al., 2018). The relative abundance of the *Campylobacter* was higher in mucosal samples than that of the samples of the luminal contents. In the pig GI tract, *Campylobacter* is considered a commensal bacterium and is prevalent at various ages (Alter et al., 2005). Holman et al. (2017b)

performed a meta-analysis to define the core bacteriome of the pig gut. They found that the bacterial composition and structure varied significantly at each GI tract section of the pig. The bacterial genera included *Clostridium, Blautia, Lactobacillus, Prevotella, Ruminococcus, Roseburia*, and the RC9 group comprised 90% of all of the GI samples in their study. Similarly, in our study, we detected several bacterial genera distinct to different GI tract locations, including *Prevotella, Roseburia, Brachyspira, Clostridium, Faecalibacterium*, etc. We believe these genera could represent the core microbiome of the pigs in our study.

Similarly, the study also reported distinct mucosa-associated bacterial communities at different GI locations. Among these, *Helicobacter*, *Prevotella*, *Campylobacter*, and *E. coli* were the most abundant (Mann et al., 2014). Looft et al. (2014) also examined the lumen (digesta) vs. mucosa-associated bacterial communities in both treated (in-feed antibiotics; chlortetracycline, sulfamethazine, and penicillin) and control (non-medicated) pigs. The authors found an overlap in some of the bacterial taxa. The main influencing factors on the bacterial community compositions were GI location rather than sample types (contents vs. mucosal). Similarly, an analysis of multiple colonic mucosal sites and feces from healthy humans indicated significant variability in microbial communities among individuals and between feces and mucosa (Eckburg et al., 2005). The study also showed that the microbial concentration increases along the GI tract, with the lowest concentration in the stomach and the highest in the colon. Another study reported that *Bacteroides* and *Firmicutes* were the dominant mucosa-associated bacteria in the small intestine and colon (Sekirov et al., 2010). In the proximal gut, *Lactobacillus*, *Veillonella*, and *Helicobacter* were the most abundant taxa; *Bacillus, Streptococcus, Actinomyces*, and *Corynebacterium* were the most abundant in the duodenum, jejunum, and ileum; *Firmicutes* and *Bacteroidetes* were dominant in the colon.

The AMR-gene repertoire in the luminal contents and the mucosa changed throughout the GI tract of piglets. The AMR repertoire was more diverse in the luminal contents compared to mucosal samples. The most abundant AMR gene class encoding resistance were tetracyclines, *β*-lactams, aminoglycosides, macrolides, and glycopeptides. Several important AMR genes that are known to impose reduced susceptibility to multiple drug classes in nontyphoidal *Salmonella enterica* subsp. *enterica*, such as those of *β*-lactam resistance (e.g., *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXY}}$), quinolone resistance (e.g. *qnr*), aminoglycoside resistance (e.g. *str*B, *aph*(6)Iia) and tetracycline resistance (e.g. *tet*(A), *tet*(B), *tet*(C)) were detected.

These genes were more abundant in the large intestine than the small intestine for both the luminal contents and mucosal samples. To our knowledge, only a few metagenomic reports that evaluate AMR gene distribution in different gut locations in pigs. A study by Kim et al. (2012) showed bacterial population shifts in the distal gut in response to treatment with tylosin, an antimicrobial growth promoter. Looft et al. (2014) examined specific differences in the microbiome communities of treated and control pigs in regard to the lumen and mucosa-associated samples in different sections of the GI tract (the ileum, the cecum, and mid-colon). They also evaluated discrepancies between the gut contents and freshly voided feces from piglets (~3 months). The study found that mucosa-associated ileal microbiota harbored greater bacterial diversity than the lumen; the ileal contents (control and medicated) showed reduced richness and abundance compared to other parts of the intestinal tract communities.

Interestingly, in our study, the correlation between the AMR gene diversity and the microbial taxa diversity was positive and significant for the luminal samples. This suggests that AMR genes also varied according to the microbial community abundance and diversity. Therefore, the microbiome composition could influence the resistome composition. It is yet to be

determined which bacterial taxa or cluster is linked to the resistome in each GI location of the pig. However, the presence of AMR genes and their source is complex in food animal production (Noyes et al., 2016b) as it is continuously seeded from the external environment. An earlier study also reported a positive and significant correlation between the total resistome and total microbiome in healthy pigs (Joyce et al., 2019). It has been suggested that antimicrobial mediated destruction of the gut microbial communities and consequent loss of colonization resistance are important factors leading to the persistence and spread of AMR bacteria. However, the microbial communities across GI locations that carry the AMR genes are still not apparent. Studies also show that change in the microbiome composition, mainly the elimination of a specific group of anaerobic bacteria, leads to vancomycin-resistant *Enterococcus faecum* (VRE) in the GI tract (Ubeda et al., 2013). Nevertheless, the microbiome of animal samples has been shown to be linked with the antimicrobial resistome.

Conclusions

Several studies have examined the microbiome of fecal samples of pigs. This study provides an additional understanding of the pig microbiome and AMR gene contents across the seven different locations of the GI tract. Several bacterial genera were significantly associated with different locations of the GI tract, suggesting "core" microbial communities across the gastrointestinal tract of the studied animals. The overall diversity of AMR genes was higher in luminal samples compared to mucosal samples. Significant correlations between the diversity and number of AMR genes and microbial taxa were observed, which further suggests that the microbiome composition and diversity could influence the resistome composition.

Table 7.1

Alpha diversity indices of microbial taxa (mean ± sd) across the gastrointestinal (GI) location (contents and mucosa samples) (*n*=3 piglets).

GI location	The richness of taxa (Chao1 index)		Diversity of taxa (Shannon index)		Evenness of taxa (Evenness index)	
	contents	mucosa	contents	mucosa	contents	mucosa
Stomach	26.6±9.71	25.1±19.05	2.5±0.33	1.4±0.53	0.6±0.1	0.4±0.12
Duodenum	18.6±16.51	21.4±15.89	0.2±0.24	1.5±1.3	0.09±0.09	0.4±0.31
Ileum1	5.6±1.52	10 ±4.39	0.1±0.05	0.42±0.43	0.02±0.01	0.1±0.17
Ileum 2	9.9±4.52	13.4±5.61	0.7±1.19	0.9±0.42	0.3±0.04	0.3±0.18
Cecum	22±8.66	25.3±8.9	1.8±1.18	2.8±0.09	0.5±0.34	0.7±0.02
Colon	29.7±15.55	34.8±5.36	2.6±0.22	2.8±0.08	0.7±0.06	0.7±0.03
Rectum	27.3±15.14	20.4±6.95	2.7±0.57	1.8±1.22	0.8±0.06	0.5±0.37

Table 7.2

Taxa (genus level) differing in relative abundance across the GI location of piglets (*n*=3).

Genus	*p*-value (rank test)	Adjusted *p*-value, FDR*	Stomach	Duodenum	Ileum1	Ileum2	Cecum	Colon	Rectum
Prevotella	0.0011	0.022	3.73	1.54	0.18	1.39	5.09	6.19	5.01
Roseburia	0.0016	0.022	2.13	0.87	0.05	0.39	2.67	3.23	2.02
Faecalibacterium	0.0016	0.022	1.32	0.39	0	0.19	1.27	1.63	1.11
Succinivibrio	0.0024	0.022	0.55	0.11	0	0.2	0.85	0.99	0.62
Oxalobacter	0.0025	0.022	0	0	0	0	0	0.13	0.66
Lactobacillus	0.0027	0.022	6.93	6.62	9.72	8.11	4.86	3.3	3.42
Mitsuokella	0.0031	0.022	0.8	0.09	0	0	0.2	0.34	0.25
Brachyspira	0.0046	0.029	0.85	0.07	0.01	0.5	1.95	0.76	0.52
Phascolarctobacterium	0.0066	0.037	0.66	0.27	0	0.72	1.5	1.87	1.88
Clostridium	0.0077	0.038	0.5	0.28	0.06	0.45	0.83	1.13	1.12
Ruminococcus	0.013	0.054	0.54	0.19	0.02	0.03	0.73	1.05	0.57
Gemmiger	0.013	0.054	0.49	0.15	0	0.05	0.51	0.66	0.34
Megasphaera	0.015	0.058	0.89	0.44	0.02	0.01	1.12	1.23	1.19
Anaerobiospirillum	0.021	0.075	0	0.03	0	0	0.07	0.51	0.04
Dorea	0.027	0.081	0.31	0.16	0	0	0.24	0.42	0.47
Acidaminococcus	0.027	0.081	0.34	0.04	0	0	0.15	0.29	0.33
Bulleidia	0.028	0.081	0.25	0.06	0	0.018	0.3	0.36	0.29
Collinsella	0.029	0.081	0.38	0.01	0	0	0.1	0.21	0.11
Pseudomonas	0.04	0.1	0.7	0.35	0.1	0.09	0	0	0.04
Blautia	0.045	0.1	0.28	0.12	0	0.06	0.21	0.43	0.25
Anaerovibrio	0.047	0.1	0.19	0	0	0.71	0.72	0.72	0.12

*FDR, False discovery rate (α=0.05)

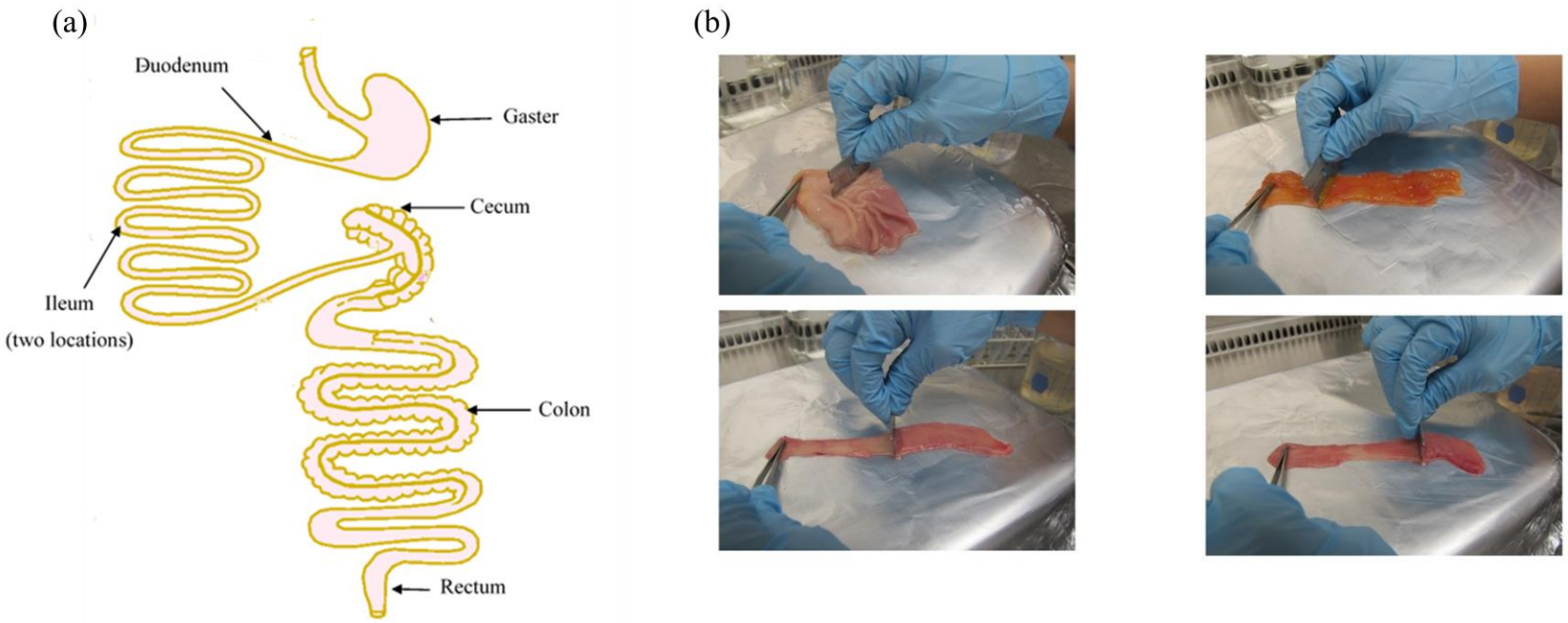

Figure 7.1. The gastrointestinal (GI) tract of pigs from which a) paired (luminal contents and mucosal samples) were collected from each location, b) mucosa samples collected from the gastrointestinal tract of piglets.

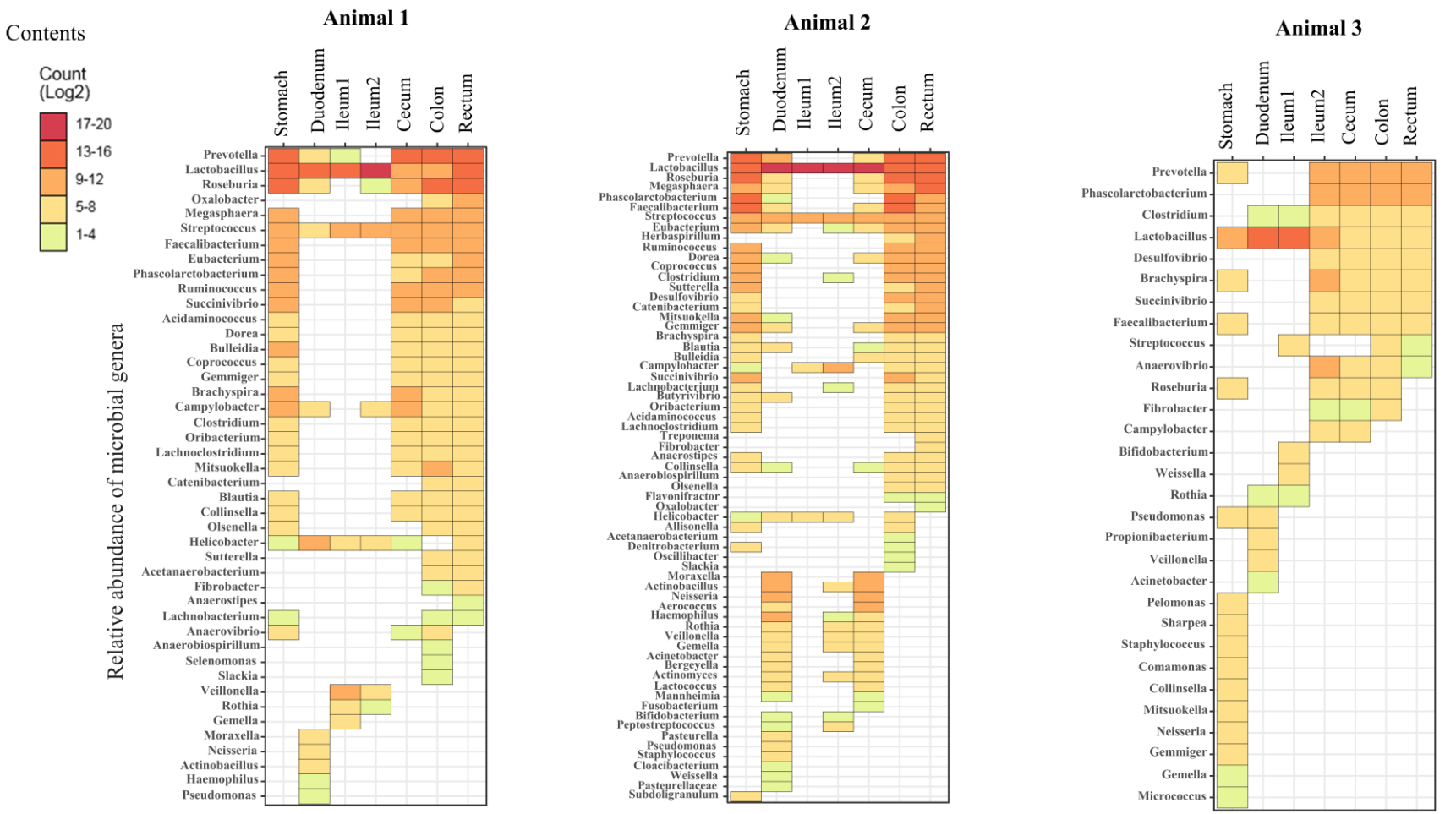

Figure 7.2. Heatmaps of the relative abundance of microbial genera present in the pig mucosa sample across the gastrointestinal tract.

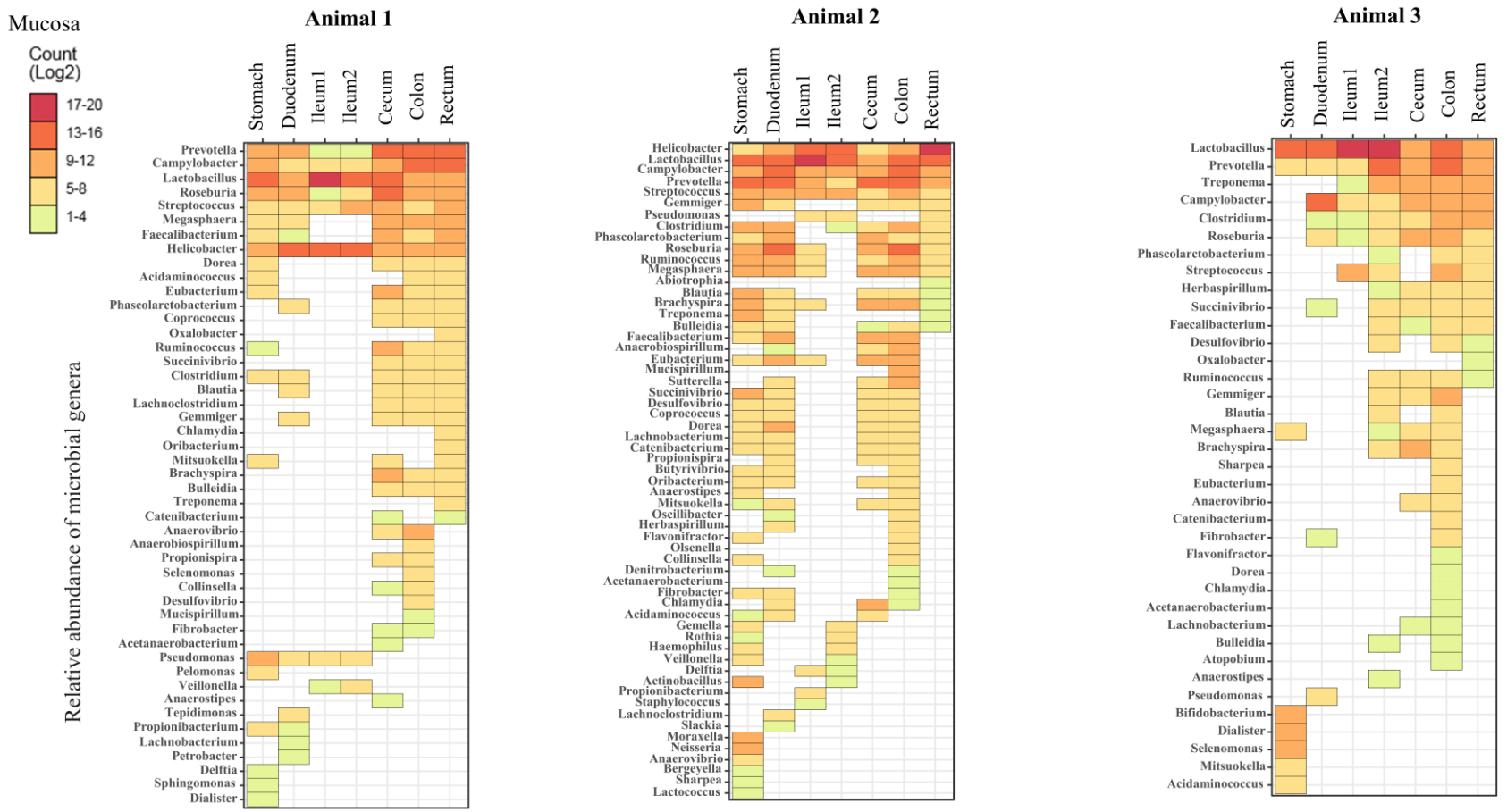

Figure 7.3 Heatmaps of the relative abundance of microbial genera present in the pig mucosa sample across the gastrointestinal tract.

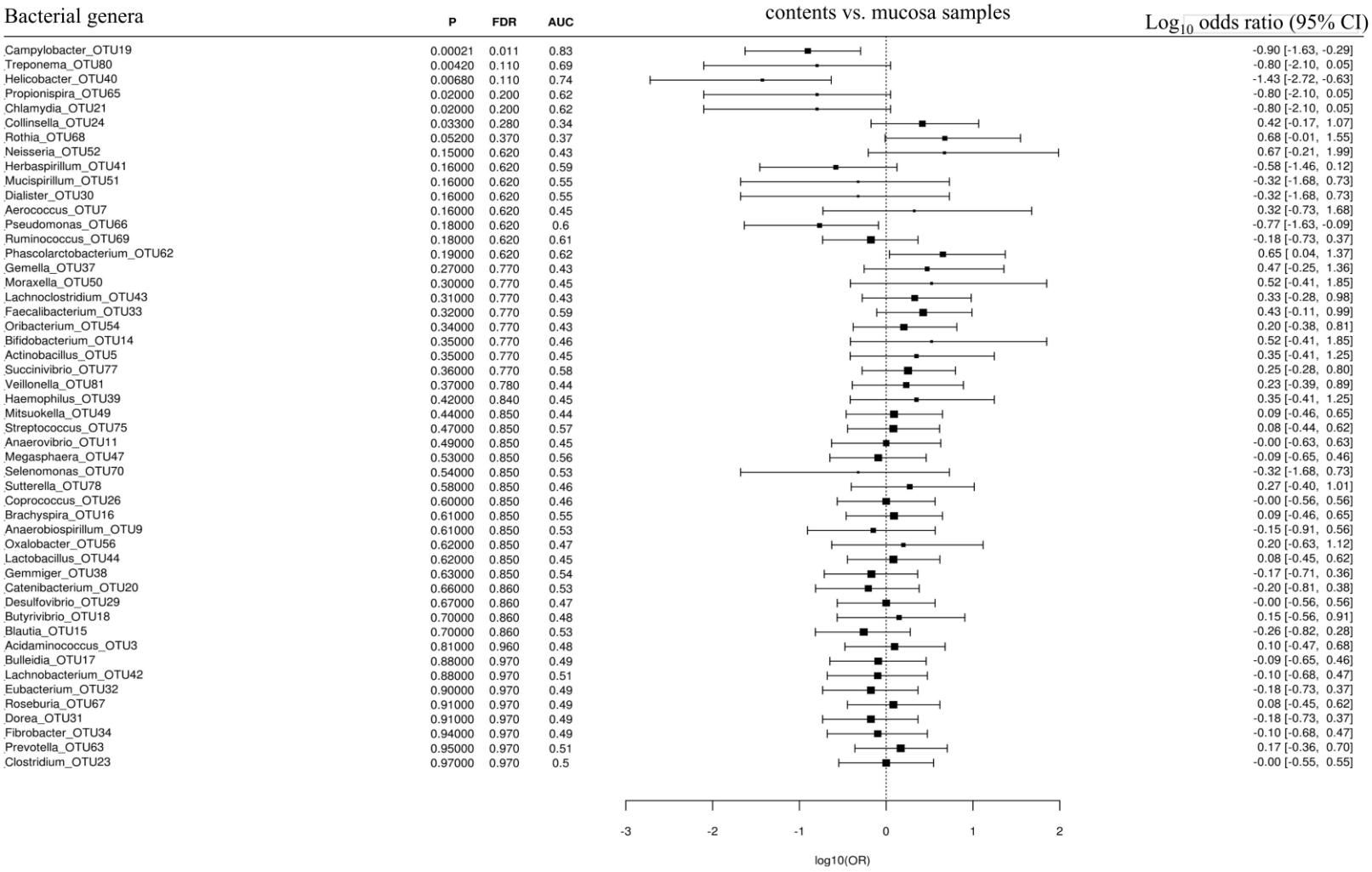

Figure 7.4. Taxa (genus level) differing in the relative abundance (log_{10} odds ratio) between contents and mucosa samples of piglets (*n*=3).

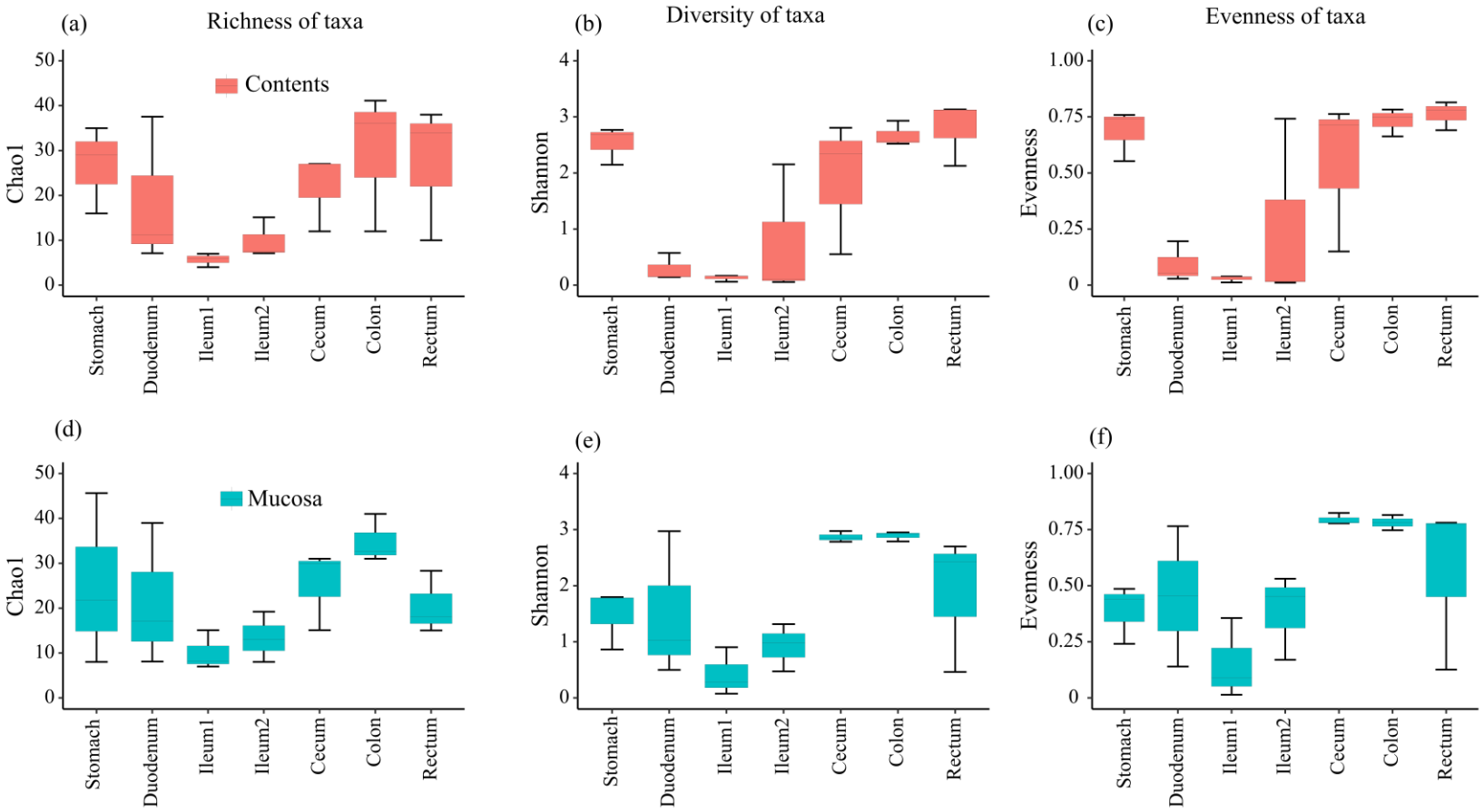

Figure 7.5. The richness, diversity, and evenness of taxa in contents (a-c) and mucosa samples (d-f) across the GI location of piglets (*n*=3).

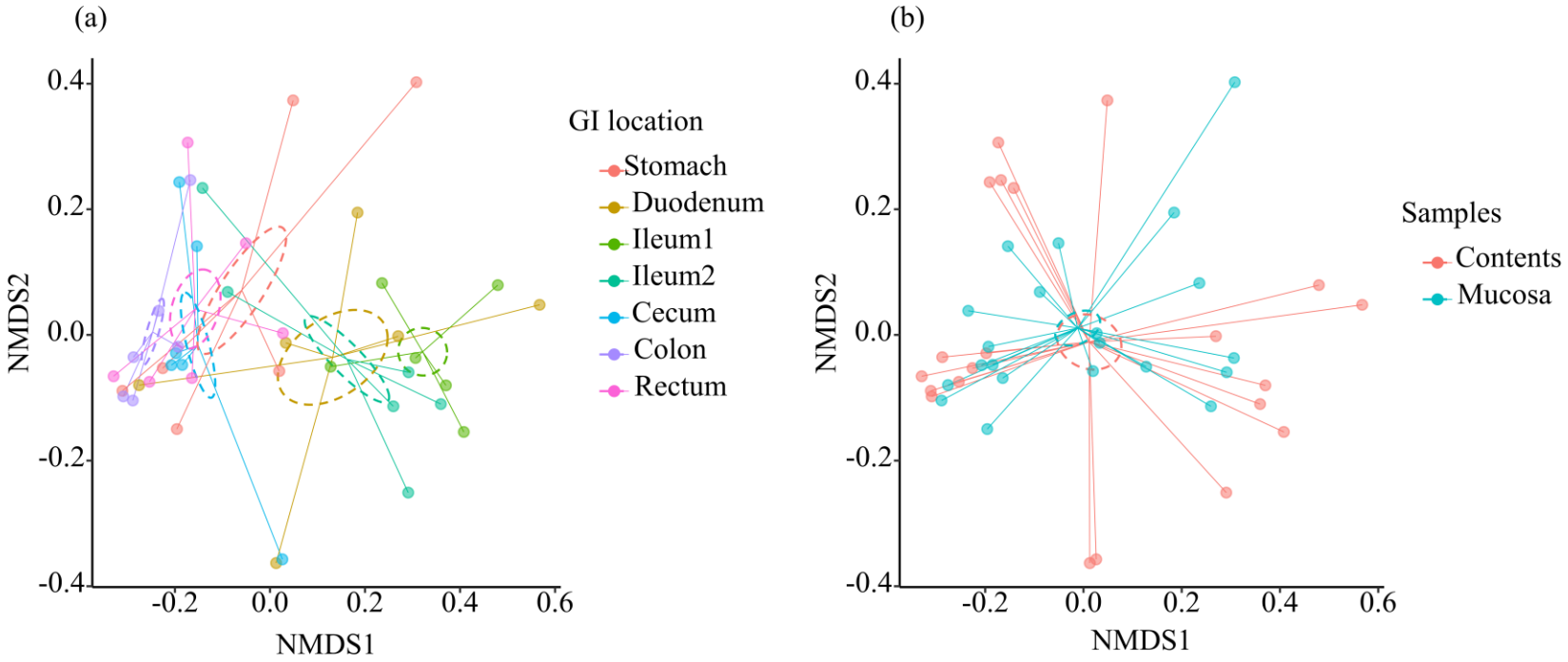

Figure 7.6. Two-dimensional non-metric multidimensional scaling (NMDS) plots of beta diversity on Bray-Curtis dissimilarities (stress=0.14) in the a) seven different GI location b) contents versus mucosal samples in the piglet across the GI section. Ellipses indicate one standard error from the centroid.

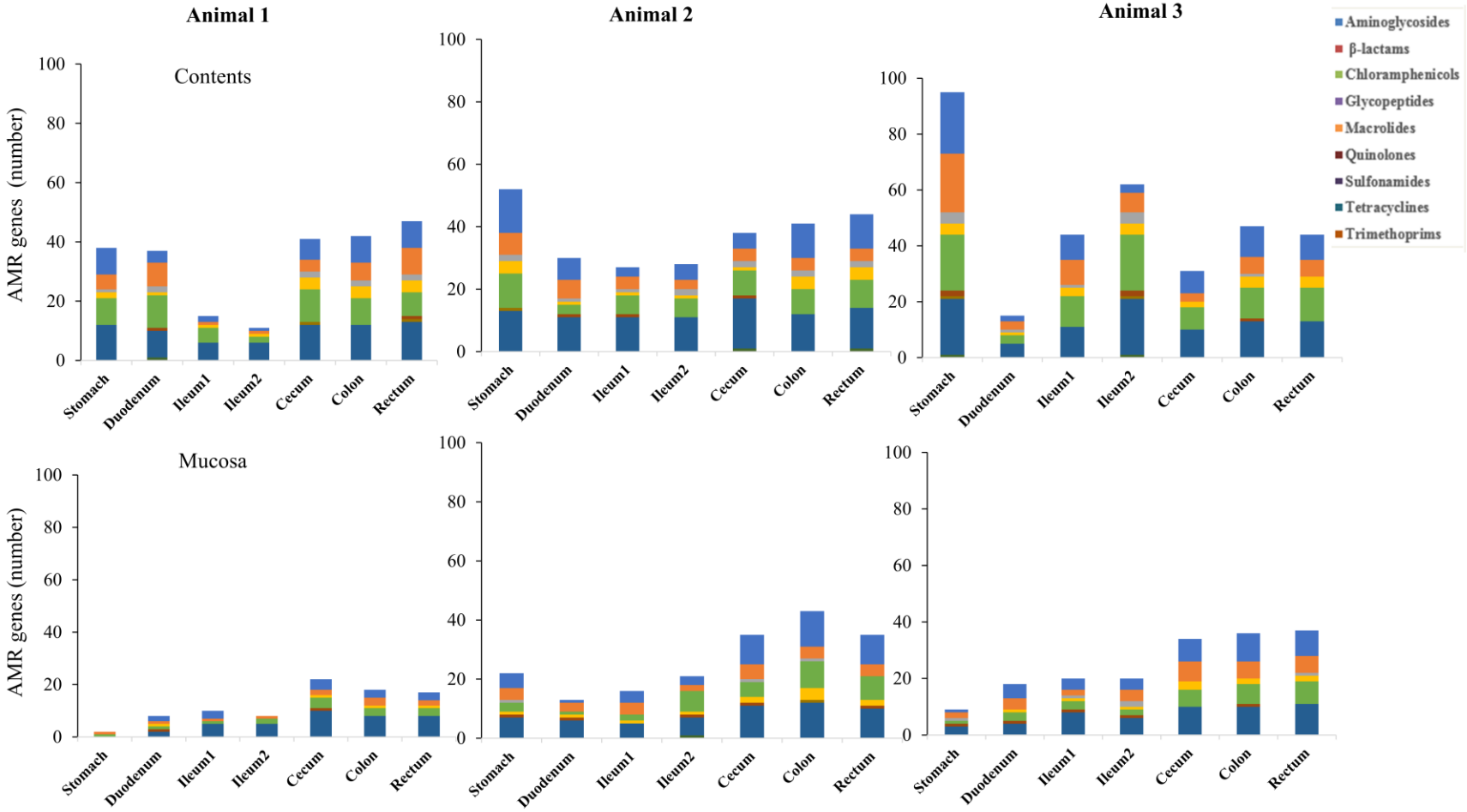

Figure 7.7. The number of AMR genes present between contents and mucosal samples across the GI tract. The different colors represent the AMR genes by drug class.

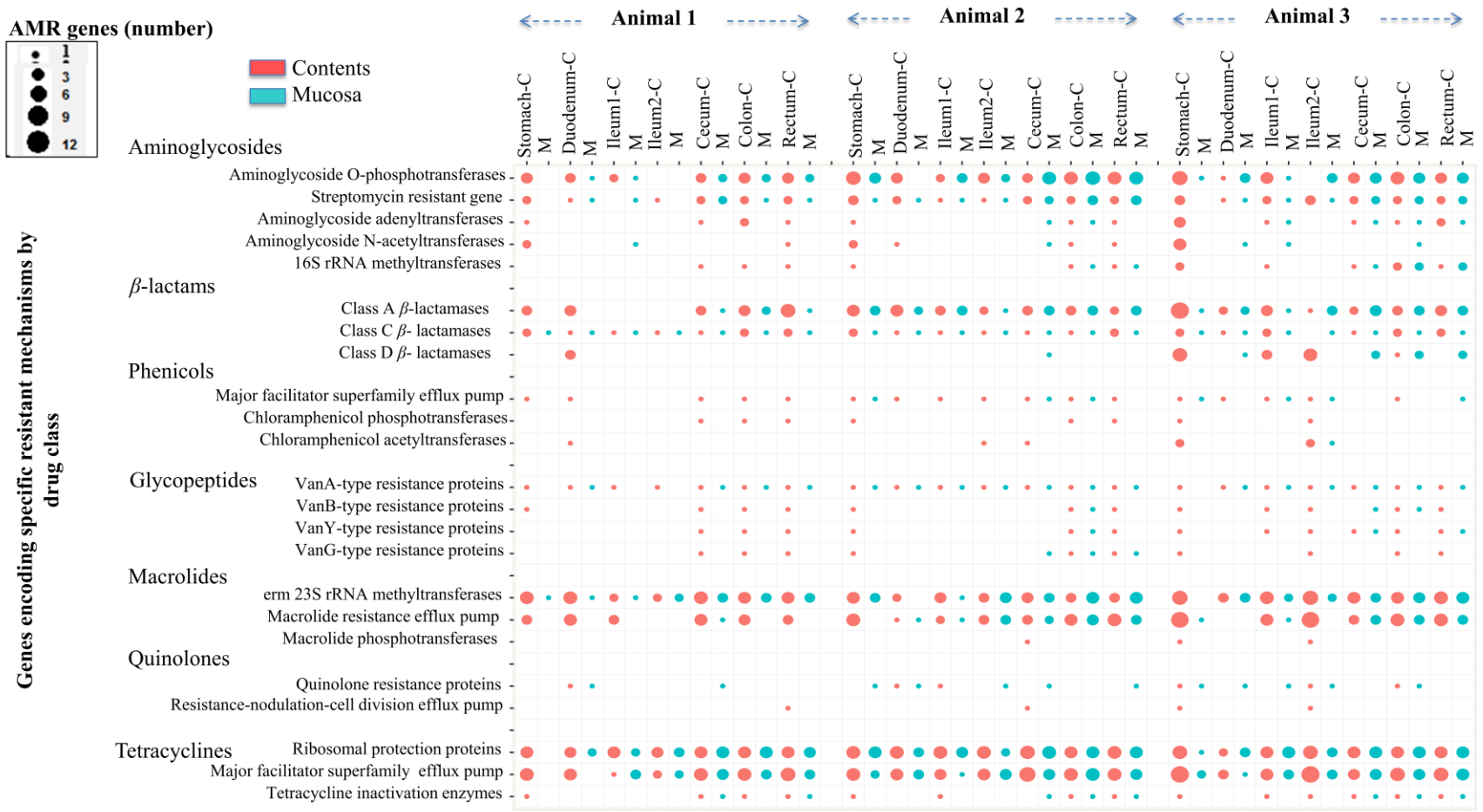

Figure 7.8. The AMR genes encoding specific resistant mechanisms by drug class across the GI location of piglets. The M-represent the mucosa samples matched with corresponding contents (-C) samples in each compartment of the GI location.

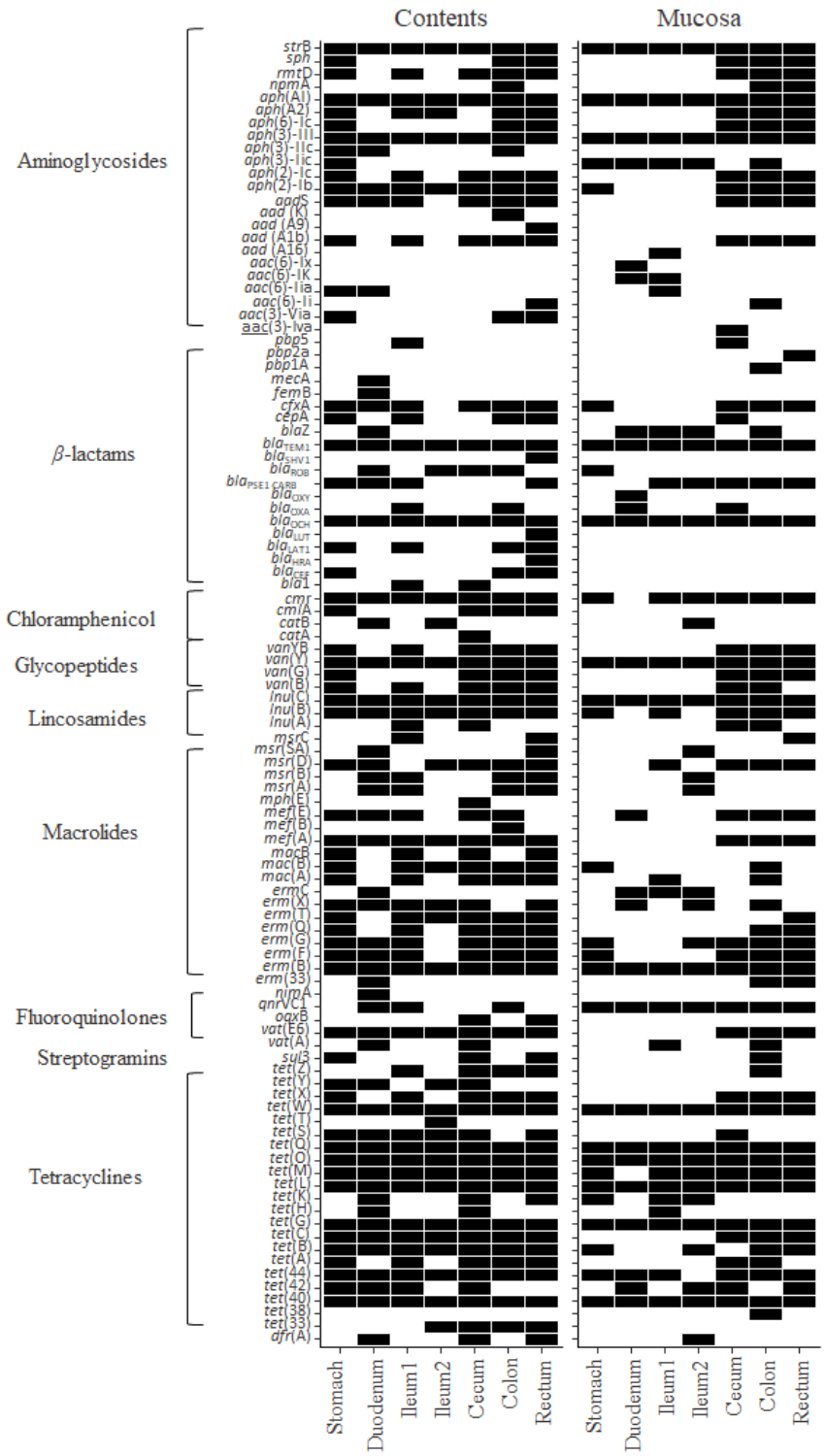

Figure 7.9. Presence of AMR genes between contents and mucosal samples across the GI tract of piglets (*n*=3). Black color represents the presence of AMR genes, while the white color represents the absence of AMR genes in the samples.

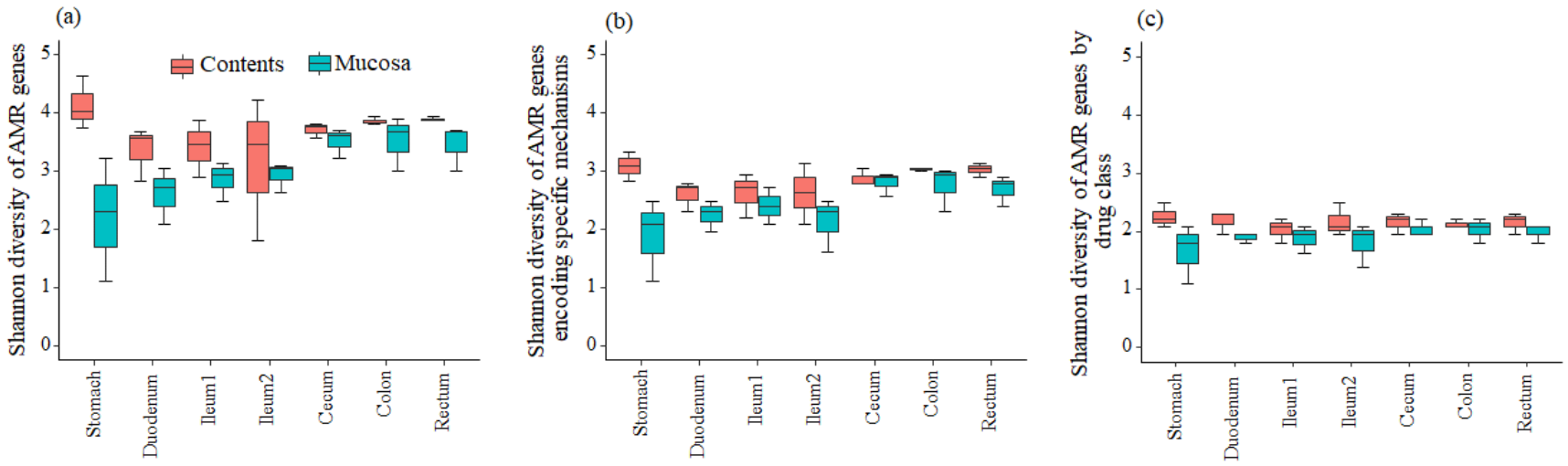

Figure 7.10. The Shannon diversity of a) antimicrobial resistance (AMR) genes b) AMR genes encoding specific mechanism c) AMR genes by drug classes between contents and mucosal samples across the GI location.

Chapter 8 - Summary and Conclusions

The studies reported in this dissertation describe the dynamics of microbial diversity and antimicrobial resistance (AMR) in gut and fecal microbial communities influenced by age and diet in swine. A scoping review of the age-dependent AMR in fecal bacteria of food animals summarized the literature published since the early 1970s on the association between host age and AMR in fecal bacteria in animals raised in different geographical locations. Two-thirds of the studies indicated a decline in the prevalence and abundance of AMR in fecal bacteria with age for production pigs, beef, and dairy cattle.

Most research on AMR in food animals have focused on one or more bacterial species (e.g., *E. coli*, *Enterococcus* spp.), but the results do not fully explain the AMR dynamics of diverse microbial communities carried by the host populations. Therefore, longitudinal studies to elucidate and compare the dynamics of fecal microbiome and mycobiome taxonomic compositions and AMR in cohorts of production pigs (from 2 days to 6 months old) and breeding sows (from 3 weeks old to first farrowing/weaning) were conducted. Culture-dependent and metagenomic methods were used to infer the relationships between phenotypic AMR and AMR gene occurrences in relation to age and diet of the pigs. The fecal microbiome and mycobiome taxonomic structure and AMR dynamics from the first weeks of life to a young age were similar between production pigs and breeding sow cohorts. On average, in each cohort, abundances of fecal coliforms or enterococci with AMR were the highest at the earliest age-points sampled, then decreased within the first weeks of life. Our data suggested age had a strong influence on the fecal microbiome and AMR dynamics compared to production practices.

We found that the age of animals is one factor affecting fecal microbial community composition and phenotypic and genotypic AMR; however, other management factors such as

diet or use of antimicrobials may influence the overall findings. Therefore, age-related dynamics and the effects of diet (different levels and sources of fiber contents) and antimicrobial treatments (injectable ceftiofur hydrochloride or penicillin G) on the fecal microbiome and AMR were investigated. The data suggested a strong age-dependent, but diet-independent, effects on the fecal microbiome composition and AMR. The concentrations of ceftiofur active metabolites in pig feces were lower on day 3 than on day 1 of the 3-day ceftiofur treatment regimen, irrespective of the animal diet or gender.

Because bacterial communities are not uniformly distributed throughout the gastrointestinal tract and fecal samples do not necessarily represent the complete bacterial taxonomic composition and AMR genes of the entire GI tract, a study examined differences in the bacterial community and AMR genes occurrence associated with luminal contents and mucosal epithelium in different location of the GI tract of piglets. The luminal contents and mucosa were collected from euthanized piglets from the stomach, duodenum, ileum (at two locations), cecum, spiral colon, and rectum. Our data showed that the bacterial taxonomic compositions and AMR gene repertoire change throughout the gastrointestinal tract of piglets. Several bacterial genera were significantly associated with different locations of the GI tract, suggesting that “core” microbial communities exist across the gastrointestinal tract of piglets. In summary, the results of this study indicated that age and diet, in addition to use of antimicrobials and location of the GI tract, contribute to the taxonomic composition of the gut microbial communities, which in turn influence the fecal AMR in pigs.

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