CHARACTERIZATION OF THE BOVINE GUT MICROBIOME, RESISTOME, AND METABOLOME ASSOCIATED WITH SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* SHEDDING AND THIRD-GENERATION CEPHALOSPORIN APPLICATION

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ABSTRACT

Bovines are the most important source of protein, and their production is expected to intensify with the increased human population. Despite the benefits of cattle, their intensification can result in increased negative footprints such as the transmission of foodborne pathogens and antibiotic-resistant (AR) bacteria. Cattle are the primary carriers of Shiga toxin-producing *Escherichia coli* (STEC) which causes over 250,000 human infections in the U.S. every year. Furthermore, about half of the mass of antibiotics sold in the U.S. are used in bovines. Third-generation cephalosporins are commonly used in cattle (i.e., ceftiofur); however, these antibiotics are considered of last resort. Globally, Enterobacteriaceae resistant to third-generation cephalosporins are the main cause of death by AR infections. In this dissertation three aims were addressed: 1) identifying microbiome diversity and composition that may favor the colonization of STEC in the hindgut of cattle; 2) determining the impacts of intramammary (IMM) ceftiofur in dairy cattle in the abundance of AR bacteria in feces; and 3) characterizing changes in the functional microbiome and metabolome associated with the IMM ceftiofur application.

In Chapter 2, we analyzed 660 fecal samples from beef and dairy cattle from 5 farms with varying prevalences of STEC. The microbiome composition analyzed with 16S *rRNA* gene sequencing revealed that the microbiota of animals from farms with a high-STEC prevalence (HSP) had greater richness compared to those of farms with a low-STEC prevalence (LSP). Higher microbiome diversity was also identified in STEC-shedders from LSP farms but not in animals from HSP farms. Finally, we evidenced that bacterial taxa associated with STEC shedding in dairy farms were also correlated with differences in the diet and risk factors of STEC carriage such as days in milk, number of lactations, and warm temperatures.

In Chapter 3, we evaluated the effects of intramammary (IMM) ceftiofur application on the abundance of resistant bacteria in cattle feces. Twenty dairy cows were treated with IMM ceftiofur and a non-antibiotic internal teat sealant and another group of 20 cows (controls) received only the non-antibiotic sealant. Feces were collected the day before the treatment and in weeks 1, 2, 3, 5, 7, and 9 after the treatment (n = 278). Through culture-based methods, no differences were observed in the number of β-lactam resistant bacteria (i.e., ampicillin and ceftiofur) between treatment groups. However, metagenomic sequencing revealed a greater abundance of genes encoding extended-spectrum β-lactamases (ESBL) that confer resistance to third-generation cephalosporins. Furthermore, an increased number of correlations between β-lactam resistance genes, mobile genetic elements, and bacterial genera were observed a week after IMM ceftiofur treatment.

Finally, in Chapter 4 we analyzed the effects of IMM ceftiofur in the functional microbiome and metabolome of feces collected in the prior chapter. The IMM antibiotic treatment had minor effects on the functional microbiome, while no differences were observed in the metabolome between treatment groups. Multi-omics analyses identified correlations between natural antimicrobial compounds, pesticides, bacteriophages from enterobacteria, and ESBL genes. This suggests the role of natural bacterial stressors in the abundance of MGEs and ARGs.

This dissertation aims to provide information for reducing the prevalence of STEC and antibiotic resistance in cattle farms, which is critical to prevent infections in humans and ensure the effectiveness of last-resort treatments.

To my sweet daughter Jazmin Hamel,
for showing me that everything is possible
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LIST OF ABBREVIATIONS

A/E	Attaching and effacing
ACC	Antibiotic-resistant gene carrying contig
ADAP	Automated Data Analysis Pipeline
AHLs	Acyl-homoserine lactones
Amp^R	Ampicillin resistant
Amp^S	Ampicillin susceptible
ANCOM-BC	Analysis of compositions of microbiomes with bias correction
AR	Antibiotic resistance
ARG	Antibiotic resistance gene
ATCC	American Type Culture Collection
BHT	Butylated Hydroxytoluene
BLASTP	Protein Basic Local Alignment Search Tool
BLV	Bovine leukemia virus
BMR	Brown midrib
BVD	Bovine viral diarrhea
CAT	Contig Annotation Tool
CCFA	Ceftiofur crystalline-free acid
CDC	Centers for Disease Control and Prevention
Cef^R	Ceftiofur resistant
Cef^S	Ceftiofur susceptible
CFB	Cytophaga, Fusobacterium, and Bacteroides
CFU	Colony forming unit

CHCL	Ceftiofur hydrochloride
CLSI	Clinical & Laboratory Standards Institute
CNA	Columbia Nalidixic Acid Agar
CP	Crude Protein
CS	Corn silage
DALYs	Healthy life years
DFMs	Direct-fed microbes
DHIA	Dairy Herd Improvement Association test
DIM	Days in milk
DM	Dry matter
DNA	Deoxyribonucleic acid
EHEC	Enterohemorrhagic *E. coli*
ELISA	Enzyme-linked immunosorbent assays
ESBL	Extended spectrum β-lactamase
FAO	Food and Agriculture Organization of the United Nations
FBMN	Feature-Based Molecular Networking
FC	Fold-Change
FG	Fecal grab
GALT	Gut-associated lymphoid tissue
Gb3	Glycolipid globotriaosylceramide receptor
GC/MS	Gas Chromatography/Mass Spectrometry
GDP	Gross domestic product
GIT	Gastrointestinal tract

GNPS	Global Natural Product Social Molecular Networking
HGT	Horizontal Gene Transfer
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-Performance Liquid Chromatography
HSP	High-STEC prevalence
HUMAnN	HMP Unified Metabolic Analysis Network
HUS	Hemolytic Uremic Syndrome
ICE	Integrative and Conjugative Element
IMM	Intramammary
JD	Johne's disease
LC–MS	Liquid chromatography–mass spectrometry
LDA	Linear Discriminant Analysis
LEE	Locus of enterocyte effacement
LEfSe	Linear Discriminant Analysis Effect Size
LNDF	Lignin as a percent of the NDF
LPS	Lipopolysaccharide
LSP	Low-STEC prevalence
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MDA	Mean decrease accuracy
MDR	Multidrug Resistance
MGE	Mobile Genetic Element
MLS	Macrolide, Lincosamide, Streptogramin
MRGs	Metal resistance genes

MS/MS	Tandem mass spectrometry fragmentation
MS^2	Tandem Mass Spectrometry
NARMS	National Antibiotic Resistance Monitoring System for Enteric Bacteria
NASS	National Agricultural Statistics Service
NDF	Neutral Detergent Fiber
OIE	World Organization for Animal Health
OOB	Out-of-bag error
OTUs	Operational taxonomic units
PAI	Pathogenicity Island
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
PWY	Pathway
QS	Quorum-sensing
RAJ	Recto-anal junction
RF	Random Forest
RPK	Reads-per-kilobase
RT	Retention time
RWA	Raised without antibiotic
SARA	Subacute ruminal acidosis
SC	Subcutaneous
SCC	Somatic cell count
SCFA	Short-Chain Fatty Acid
STEC	Shiga toxin-producing *Escherichia coli*

T3SS	Type III secretion system
TLRs	Toll-like receptors
UHPLC	Ultra High-Performance Liquid Chromatography
USDA	U.S. Department of Agriculture
VCPR	Veterinarian-client-patient relationship
VFAs	Volatile fatty acids
WHO	World Health Organization

CHAPTER 1

Literature review: Shiga toxin-producing *Escherichia coli* (STEC) and antibiotic resistance in bovines; insights into their epidemiology, molecular mechanisms, and ecological associations with the gut microbiome and resistome.

CATTLE'S IMPORTANCE AS A PROTEIN SUPPLY

The United Nations projects a world population of 9.7 billion in 2050 [1]. Hence, the demand for meat and dairy products is estimated to rise by 73% and 58%, respectively, by that year as compared to the global production in 2010 [2]. To fulfill the increased nutritional demands of the continued growing population, industrial farming will dominate dairy and meat production [2]. Indeed, the adoption of intensive production systems in low- and middle-income countries has increased meat production by 68% in Africa, 64% in Asia, and 40% in South America since 2000 [3]. Industrial practices to improve growth and feed efficiency include the use of feeds with high energy content, high-density farms, and the use of growth promoters [4,5]. Nevertheless, increased densities can intensify the spread of foodborne pathogens in farm environments [4].

Cattle have been the most stable supply of protein for human societies since their domestication approximately 10,000 years ago [6]. The supply of cattle meat is positively associated with the average gross domestic product (GDP) per capita [7]. Cattle are highly efficient in protein production and are about 3.2 times greater than monogastric animals [8]. About 45% of the global animal protein comes from ruminants, while 31% is derived from poultry and 20% from pigs [9]. Globally, there are an estimated 3.9 billion ruminants. In the United States, however, the U.S. Department of Agriculture (USDA) National Agricultural Statistics Service (NASS) reported over 98.8 million head of cattle, including 30.35 million beef, 9.45 million dairy, and 59 million other cattle and calves in 2022 [10]. Despite the benefits of livestock production, there are increasing concerns about negative environmental footprints such as greenhouse gas emissions [11], manure management [12], judicious use of antibiotics, and zoonotic disease outbreaks that have been linked to cattle [13–15].

THE GASTROINTESTINAL MICROBIOME OF CATTLE

Obligatory symbionts

Ruminants depend on their gut microbiota for productivity and health. Complex microbial communities residing in the gastrointestinal tract (GIT) are fundamental to produce vitamins, 80% of protein [16], and nearly three-quarters of the energy necessary for the host [17]. Furthermore, the gut microbiota is critical to keep intestinal homeostasis, stimulating the immune responses, preventing pathogen colonization, and for mucosal and lymphoid structure development [18]. Given this obligatory symbiotic relationship between ruminants and their gut microbiota, several studies have explored associations between microbial communities with feed efficiency [19–21]. The implementation of high-throughput sequencing enabled the study of the gut microbiome, which has been largely studied in the rumen where most nutrient components are synthesized [22–24]. However, little research has been conducted to define the role that the microbiota residing in the lower GIT plays in production, health, or pathogen carriage [25,26]. Elucidating the interactions between the gut microbiota, host, and environment is key to increasing feed efficiency and preventing negative impacts associated with cattle.

Role of the gastrointestinal microbiota of cattle

The cattle GIT includes mouth, esophagus, a four-compartment stomach (rumen, reticulum, omasum, and abomasum), small intestine (duodenum, jejunum, and ileum), and large intestine (cecum, colon, and rectum). During the first two weeks of life, calves are in a monogastric stage where the abomasum is the only stomach compartment used to digest milk. The first bacterial colonizers of the calf GIT are facultative anaerobes, such as *Lactobacillus*, *E. coli*, and other Enterobacteriaceae [27–32]. A few days after birth strictly anaerobic bacteria colonize the GIT, with *Clostridium*, *Bifidobacterium*, *Eubacterium*, and *Bacteroides*

predominating [27–32] followed by an increase in microbiome diversity and composition with age [27–30,32]. In adult cattle, transitioning microbial communities dominated by Firmicutes, Bacteroidetes, and Proteobacteria are observed in each GIT compartment, which varies in functionality and environment [33].

The rumen microbiota, dominated by Firmicutes and Bacteroidetes [33], degrades and ferments diet compounds for the biosynthesis of volatile fatty acids (VFAs), amino acids, and vitamins [34]. The characteristic papillae present in the rumen and high vascularity allow efficient absorption of these nutrients by the host [35]. The reticulum allows particle separation to selectively pass small particles to the abomasum [35]. Water and other nutrients are absorbed in the omasum; while the abomasum has a similar role as nonruminant stomachs where hydrochloric acid and digestive enzymes are released to break down nutrients [35]. The low pH in the abomasum (pH < 4) as compared to the other GIT regions (pH 6 to 8), significantly decreases the number of bacteria which gradually increases in the hindgut [33]. While along the large and small intestines Firmicutes and Bacteroidetes increase, Proteobacteria decrease their abundance [33]. The small intestine receives compounds from the pancreas and gallbladder for further digestion and nutrient absorption [35]. Finally, resident microbiota of the large intestine digest undigested feed such as cellulose and hemicellulose [36,37], and biosynthesize VFAs, vitamins, and amino acids [42]. However, the central function of the last section of the GIT is water absorption.

The hindgut microbiota is crucial for gut homeostasis and immune system stimulation [18,38]. Immune mechanisms induced by the microbiota residing in the lower GIT include the generation of antibiotic peptides, mucus production, and the stimulation of immune cells for the production of immunoglobulin A and pattern-recognition receptors such as toll-like receptors

(TLRs) [18,39]. In fact, the development of innate and adaptative immune responses in calves depends on *Lactobacillus*-dominant bacteria that trigger the expression of genes involved in the chemotaxis of leucocytes and lymphocytes and cytokine signaling pathways [38].

Microbiome dysbiosis in cattle

Dysbiosis is generally outlined as an imbalance in the gut microbial communities associated with disease [40]. This imbalance is characterized by changes in the abundance of some community members reflected in the microbiome diversity and composition [40]. Bovines facing dysbiosis can decrease their productivity and present multiple disorders [41]. Such is the case of the microbiome imbalance caused by high-grain feed occasioning subacute ruminal acidosis (SARA) that has been broadly studied [42–44]. The rapid fermentation of carbohydrate feeds by the rumen microbiota can result in the excessive production and accumulation of acids [55]. During SARA, the ruminal microbiome is characterized by an increase in the Firmicutes to Bacteroidetes ratio and a reduction in microbial richness [42]. High concentrations of SCFA, ethanol, biogenic amines, and endotoxins affect the barrier function of the rumen epithelium resulting in the leakage of endotoxins and bacteria into the portal circulation causing systemic inflammation and in some cases liver abscesses [45–47]. In addition, increased amounts of histamine, LPS, and lactic acid produced during SARA can predispose the animals to lameness [48]. SARA-induced dysbiosis activates the gut-associated lymphoid tissue (GALT) [49] which includes the intestinal Peyer's patches and leukocytes present in reticulo-ruminal digesta [50,51]. Because GALT prevents pathogen colonization, dysbiosis of the GIT caused by SARA may reduce the cow's ability to fight pathogen invasion and regulate homeostasis.

Other causes of GIT dysbiosis in cattle include infectious illnesses such as Johne's disease (JD), which is comparable to Crohn’s disease in humans [52]. JD is a chronic intestinal

infection caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) that can be fatal for bovines [52]. During JD, the intestinal GIT has a lower abundance of Bacteroidetes and Firmicutes and higher levels of Actinobacteria and Proteobacteria [52]. Bovine Viral Diarrhea (BVD) and acute Salmonellosis are also implicated in enteric and systemic infections in cattle [53], though no studies have analyzed their role in GIT dysbiosis.

FOODBORNE INFECTIONS LINKED TO LIVESTOCK

The World Health Organization estimates that almost 600 million cases of foodborne illnesses occur every year contributing to ~420,000 deaths and the loss of 33 million healthy life years (DALYs) [15]. Roughly 350 million foodborne infections are attributed to pathogenic bacteria [15], with *Campylobacter* spp., *Salmonella enterica*, *Listeria monocytogenes*, and diarrheagenic *Escherichia coli* (e.g., Shiga toxin-producing *E. coli* (STEC)), predominating in advanced and developing countries [15]. Livestock, including cattle, poultry, and pigs, are important carriers of these pathogenic bacteria [15]. Cattle, for instance, have a prevalence of 39-99% of *Campylobacter jejuni* in dairy farms [54], 6-54% of Shiga-toxin-producing *Escherichia coli* in beef and dairy farms [55], and 7-11% of *Salmonella enterica* in beef, dairy, and mixed farms [56]. Considerable variation, however, has been observed across geographic locations. Factors that influence the prevalence of foodborne pathogens in cattle farms include high-grain diets, housing, seasonal variations, breed, housing, and production intensification [57].

The USDA estimated that 10–83 billion USD is spent on all aspects of foodborne-associated disease each year, including pathogen identification, outpatient and inpatient expenditure, lost wages, and disease prevention. Bacterial enteric pathogens causing 9.4 million cases, 55,961 hospitalizations, and 1,351 deaths annually [13]. Nevertheless, the actual incidence of foodborne diseases was estimated to be at least 10 times higher given the number of

underreported cases [13]. The underreporting of foodborne diseases is associated with the lack of appropriate testing, no access to medical care, or failure to seek medical care given that these infections are self-limiting.

STEC INSIGHTS IN HUMANS AND CATTLE

STEC infection in humans

One of the most dangerous foodborne pathogens is STEC, which causes approximately 2.8 million cases of acute enteric disease and 230 deaths annually worldwide [58]. The primary reservoir of STEC is bovines [59,60], although other ruminants including sheep, goats, and deer have been shown to carry STEC. Pigs, pigeons, cats, dogs, rats, and rabbits are among the other species [61] that have also been found to carry this pathogen. Sources for human infections of STEC include undercooked ground beef, unpasteurized milk, and juices, as well as contaminated fruits and vegetables [62]. STEC has a low infectious dose that ranges from 10 to 100 cells [63] with an enhanced ability to survive the acidity of the stomach [64]. In humans, it can cause mild to severe symptoms including gastroenteritis, hemorrhagic colitis, hemolytic uremic syndrome (HUS), acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia in 5 to 10% of the cases [60]. Infections can affect multiple organs including kidneys, heart, lungs, pancreas, and the central nervous system [60], and children and the elderly are the most susceptible to infection.

Particular attention has been paid to STEC serotype O157:H7, which causes the highest morbidity and mortality rates in the U.S. [13]. In 2017, the Centers for Disease Control and Prevention (CDC) reported 6,034 cases of STEC including 2,363 O157 and 3,646 non-O157. However, it is estimated that for every STEC O157:H7 case reported 26 cases are undiagnosed, while that number reaches 106.8 cases for non-O157 STEC [13].

Despite the high frequency of STEC infections, antibiotic treatment is not recommended because they have been linked to enhanced Shiga toxin (Stx) production, which can facilitate the development of hemolytic uremic syndrome (HUS) and fatalities in some [65]. Because cattle are important reservoirs of STEC, reducing the carriage of this pathogen in livestock and preventing dissemination through food and the environment are priorities for preventing human infections [66]. In fact, model predictions determined that a 50% reduction in STEC shedding by cattle could diminish 80% of human cases [67].

STEC virulence factors

There are more than 470 STEC serotypes, which are classified based on the combination of their O- and H- antigens; over half have been linked to serious enteric disease [60,68,69]. The most studied serogroup is O157 [70,71], though ~50 % of infections are caused by other STEC serogroups, such as O26, O111, O103, O121, O45, and O145 [72,73] worldwide. These six serogroups vary in frequency across geographic locations and have been linked to varying degrees of illness, with strains ranging from avirulent to hypervirulent [74,75]. Because STEC is defined by the presence of the Stx genes, however, all STEC can be pathogenic to humans [76,77].

STEC carry genes encoding several virulence factors, which are located on multiple mobile genetic elements (MGEs) such as prophages, plasmids, and pathogenicity islands (PAIs); thus, different strains can possess different virulence genes [78]. STEC are defined by their ability to produce the Shiga toxin (Stx), which is encoded by genes (*stx*) found in lambdoid bacteriophages [79]. There are two antigenic forms of Stx, Stx1 and Stx2, with multiple variants of each including four Stx1 (Stx1a, Stx1c, Stx1d, and Stx1e) and twelve Stx2 (Stx2a–Stx2l) subtypes [77,80]. While Stx1 can contribute to disease in humans, STEC strains that produce

Stx2 have enhanced pathogenicity and are associated with HUS [77,81]. Stx production is induced upon entry into the lytic phase of the bacteriophages [81]. Once released, the Stx binds to the glycolipid globotriaosylceramide (Gb3) receptor, which is present in enterocytes, glomerular endothelial cells, and other cells. This binding induces changes in protein expression and cell death [79]. Shiga toxin-encoding phages are highly mobile and are involved in the HGT of *stx* genes between *E. coli* resulting in the emergence of new strain types [82]. Furthermore, transduction of Stx-phages has been also identified between *Shigella* and *E. coli* rough types [83].

A group of STEC, which are also classified as enterohemorrhagic *E. coli* (EHEC), possesses unique virulence genes found on the locus of enterocyte effacement (LEE) pathogenicity island (PAI) [84]. The LEE has genes encoding the Type III secretion system (T3SS), the intimin (*eae*) and its translocated intimin receptor (Tir), and effector molecules. These virulence factors induce attaching and effacing (A/E) lesions that promote bacterial attachment to the intestinal epithelium [76,77,85]. Other virulence agents include the non-locus of enterocyte effacement (Nle) effectors [86], which are important for immunomodulation, bacterial colonization, and prolonging infection.

Cattle are asymptomatic carriers of STEC

Adult cattle are generally asymptomatic carriers that can shed STEC in their feces [58]. The lack of symptoms is due to the absence of Gb3 in ruminants which represent the vascular receptors for the Shiga toxins [87]. It has also been suggested that some STEC virulence factors are necessary to promote a symbiotic relationship in the GIT of bovines [88,89]. Some hypotheses, for instance, suggest that STEC can induce immunomodulation and immunotolerance because the Stx can alter the T and B lymphocytes [90] and some STEC

antigens cannot reach their target cells [91]. Prior studies, however, have demonstrated that cattle have partially protective innate and acquired immune responses against STEC [92,93]. Furthermore, grazing protozoa present in the bovine GIT are believed to increase the rate of survival of STEC O157:H7; nevertheless, contradictory findings compel further investigation [94,95].

Interestingly, quorum-sensing (QS) signals from the native microbiota such as acyl-homoserine lactones (AHLs) were shown to activate the acid-resistant mechanisms of STEC through the SdiA protein, which is a QS transcription factor of AHLs [96]. This activation inhibits the expression of the LEE PAI genes [96] that are important for A/E lesions and other key symptoms. Despite the lack of symptoms observed in adult bovines, infected calves have been shown to be susceptible to A/E lesion formation on the apical epithelial surfaces of the rectoanal junction (RAJ) where the bacteria colonize [97].

STEC survival in cattle GIT

STEC strains are able to survive the acid stress of the foregut environment [98,99] because of a specialized glutamate-dependent acid resistance system encoded by *gadABC*; other systems like the Arg have also been implicated, though these require further study [100,101]. In addition, some STEC strains such as those belonging to O157:H7, can resist the bile in the small intestine [102]. STEC colonizes and multiplies in the hindgut [103–105], particularly the RAJ mucosa [105]. Although it is rare, microcolonies of STEC O157:H7 have been observed in the RAJ, where they induce A/E lesions through the expression of multiple virulence factors encoded by the LEE operon [106,107]. Similarly, A/E lesions have been observed in the mucosa of the ileum and colon suggesting the expression of the injectosome in these regions, though no symptoms of infection have been reported [108,109].

Certain types of flagella, which are often referred to as the H-antigen, are able to promote adherence better than others. Flagellar types H7 and H6, for instance, could initiate bacterial adhesion to the intestinal epithelium [110] and bind to bovine mucins I and II [111]. Once the bacteria adhere to the epithelium, the expression of flagella decreases, and increased expression of other factors occurs to promote attachment and facilitate colonization of STEC [110]. Different variants of pili [112–115] and monomeric adhesins [106,112,116] can also be involved in STEC adherence in the hindgut, though few studies have been conducted to elucidate their role in the colonization of the bovine reservoir.

Prevalence of STEC in cattle

E. coli populations, including STEC, are a minority in the GIT of bovines, which corresponds to approximately 0.1-1% in feces [117] and about 10X less in the rumen [118]. Transmission routes for the acquisition of STEC in cattle include direct animal contact, consumption of contaminated feed or water, and flies [119]. STEC can be transmitted by flies belonging to *Muscidae* and *Calliphoridae*, which are important sources of crop contamination [120] and can cause human infections [119]. Fomites can also be an important transmission source in farm environments since viable STEC were found to persist for up to 4 months in manure and pen floors [121–123]. Furthermore, the persistence of STEC in cattle farms has been attributable to its ability to grow well in open ecosystems [95,124] and its potential for forming biofilms [125].

It is estimated that the global prevalence of STEC O157:H7 in cattle is 5.68%, with the USA among the countries reporting the highest prevalence [126]. Weather conditions have been suggested to play a crucial role in the prevalence of diverse STEC serotypes. Whereas STEC O157:H7 prevalence increases in the summer and early fall [127–129], other serogroups such as

O111 and O145, were found to peak during the winter [129]. Similarly, temperatures higher than 28.9ºC have been associated with increased STEC prevalence in dairy farms [55], which is not surprising in that higher temperatures support bacterial growth. Diet was also shown to impact STEC colonization in cattle, as increased STEC shedding was observed with high-grain feeds [88,130] or fasting [118], which contribute to alterations in the ruminal pH. Although beef cattle shed more STEC than dairy breeds, considerable variation has been identified among farms [55]. Geographic locations also play a role in the prevalence and distribution of strain serogroups [131] demonstrating the presence of environmental factors affecting the agroecosystems, though more studies are needed to confirm this hypothesis. Another study of dairy cattle also showed that cows during the first lactation and those with less than 30 days in milk (DIM) have a higher prevalence of the pathogen [55]. Finally, farm practices such as anthelmintic treatment [55] was associated with a lower prevalence of STEC.

STEC shedding levels are highly variable not only between animals but within the same individual. Prior studies have classified some bovines as super-shedders when the number of STEC colony-forming units (CFU) per gram of feces is greater than 10^4 [59,134]. Within a farm, the number of super-shedders ranges from about 3.5% to 5% of the animals [135]. Identifying STEC super-shedders was suggested to be more important than the farm prevalence since models indicate that 20% of the animals shed about 80% of the bacteria [136]. However, other studies have shown that animals have brief periods of increased intensity of STEC shedding, resulting in higher variations within than between individuals [137]. Consequently, targeting super-shedders could be challenging and does not account for differences over time within the same animal [137].

Several strategies have been proposed to decrease the shedding of STEC in cattle farms including vaccination, phage therapy, feed additives, and probiotics or direct-fed microbes (DFMs) [138,139]. DFMs offer multiple benefits in the cattle industry by preventing STEC colonization [138,139], controlling ruminal acidosis, and activating the immune system [140,141]. Nevertheless, identifying efficient DFMs to reduce the carriage of STEC is still in the early stages of study [136], and little is known about their impact on the gut microbiome.

Cattle GIT microbiota associations with STEC

Cattle have a diverse GIT symbiotic microbiota that can interact directly or indirectly with STEC to impact its ability to colonize and survive. Commensal indigenous microbes have been shown to mitigate the proliferation of invading pathogens through predation, nutrient competition, and the excretion of antibiotic compounds [143–145]. Inhibition of STEC has been induced by lactic acid bacteria [139]. Such is the case of lactobacilli and streptococci whose abundance in the rumen is higher in calves and animals with high-grain diets [146,147] as compared to forage-fed animals [146]. Another group of microorganisms that can alter the presence of STEC in the rumen is ciliate protozoa such as *Epidinium*, *Polyplastron,* and *Entodinium* [148], which help stabilize the ruminal pH and have predatory effects on STEC [149,150]. Bacteriophages can also regulate bacterial populations in the GIT and promote the horizontal transfer of genes between bacteria [151]. Associations between bacteriophages from the order Caudovirales and dominant bacteria such as phylum Proteobacteria, which includes STEC, have been identified in the rumen [148,152]. Bacteriophage families representing *Myoviridae* and *Siphoviridae* have also been identified in STEC where they can induce bacterial cell lysis [152].

Factors that have been implicated in STEC shedding in cattle farms can also have profound effects on the GIT microbiota; however, no prior studies have analyzed how changes in the microbiome diversity attributable to these factors are associated with STEC prevalence. Indeed, one of the most important factors known to alter the GIT microbiome of cattle is the diet [24,46,98,118,153]. Forage-fed bovines, for example, were found to have a higher abundance of Bacteroidales, Clostridiales, Ruminococcacea, and *Fibrobacter* [22]. By contrast, cattle fed high-grain diets have been linked to a greater abundance of lactic acid bacteria, lactic acid-utilizing taxa like *Megasphaera elsdenii* and *Selenomonas* [140,154], and the protozoa *Entodinium*, which has been linked to lower levels of STEC shedding [155]. The associations between diet and STEC shedding were also suggested to be related to the ability of *E. coli* to survive low pH levels in the GIT hindgut [156].

ANTIBIOTIC RESISTANCE EPIDEMIOLOGY

Antibiotic-resistant (AR) bacteria are major threats to human and animal health. It was estimated that infections caused by multidrug-resistant (MDR) bacteria caused 4.95 (3.62–6.57) million human deaths in 2019 alone [157]. The CDC reported that approximately 2.8 million AR infections occur in the U.S. every year, resulting in 35 thousand deaths [158]. MDR *E. coli* is the leading cause of global deaths by AR pathogens, particularly those strains resistant to third-generation cephalosporins and fluoroquinolones [157]. Enterobacteriaceae with resistance to third-generation cephalosporins can carry genes encoding extended-spectrum β-lactamases (ESBL) that also provide resistance to penicillins and monobactams [158]. Among all the AR threats, ESBL-producing Enterobacteriaceae are the primary cause of hospitalizations and the most economically impactful group of pathogens in the U.S. [158]. In 2017, a total of 197,400

hospitalizations, 9,100 deaths, and $1.2 billion in costs were attributed to infections caused by ESBL-producing Enterobacteriaceae in the U.S. [158].

Globally, it was estimated that 70% of the total mass of antibiotics is used to treat and prevent infections in animals [3]. This extensive use of antibiotics in both animals and humans has contributed to the emergence of AR threats. The transfer of AR bacteria between humans, animals, and the environment can occur through several routes [159] including water sanitation systems, use of manure on crops, ingestion through the food chain, wildlife, and insects. Moreover, tourism, migrations, and trade are important factors that impact the rapid dissemination of resistant bacteria across borders [160].

The World Health Organization (WHO) classified medically important antibiotics into three categories: critically important, highly important, and important antibiotics [161]. Critically important antibiotics are those that are used to treat infections caused by bacteria transmitted from non-human sources and for which limited therapies are available [161]. This category of antibiotics addresses the concern of increased severity of infections caused by resistant zoonotic bacteria. Among these critically important antibiotics, the third-generation cephalosporins, aminoglycosides, macrolides, sulfonamides, fluoroquinolones, and aminopenicillins are approved for use in cattle farms in the U.S. [162,163]. In this group, third-generation cephalosporins are classified as the drugs with “highest priority” [161]. Ceftiofur is a third-generation cephalosporin approved for use in bovines to treat diverse infections [164–166]. Parenteral application of ceftiofur, for instance, is applied to treat respiratory disease, foot rot, and metritis in beef and dairy cattle [164]. While intramammary ceftiofur is used to treat clinical and subclinical mastitis in dairy breeds caused by *Streptococcus* spp., *Staphylococcus aureus*, and *E. coli* due to its broad-spectrum effects [165].

Preventing the dissemination of antibiotic resistance among animals, humans and the environment compels One Health approaches. Examples include disease prevention in humans and animals, animal husbandry, antibiotic use regulation and policy, stewardship, surveillance, sanitation, and the discovery of alternatives to antibiotics [167]. Globally, the Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (OIE), and the WHO combine efforts to promote best practices for the use of antibiotics in humans and animals. In the U.S., the National Antibiotic Resistance Monitoring System for Enteric Bacteria (NARMS) integrates data from humans, retail food products, and animals through a collaboration among state and local public health departments, the CDC, the U.S. Food and Drug Administration (FDA), and the USDA [163].

Antibiotic usage in animals

Globally, it is estimated that livestock consumed about 63,000 tons of antibiotics in 2010, which is expected to increase by 67% by 2030 [3]. The FDA reported in 2020 that 4 of every 5 pounds of antibiotics sold in the U.S. (81%) are used in animals and the rest are used in humans [162]. The highest mass of antibiotics used in food-producing animals in the U.S. is attributed to tetracyclines and ionophores which are mainly given by feed (**Table 1.1**) [162]. Even though antibiotics have been used in livestock since the 1950s, the FDA did not begin monitoring use until 2008 given the global concerns about the increase in AR infections in humans. In fact, in 2017, the WHO claimed the need to reduce the use of veterinary antibiotics used for growth promotion and disease prevention [161,168].

Remarkably, the highest mass of antibiotics is sold for use by cattle, which accounts for 51% of the total kilograms of antibiotics used in animals in the U.S. [162]. The FDA estimates that about 5.2 million kilograms of antibiotics are intended for use in bovines (**Table 1.2**) [162].

The most common antibiotics used in cattle farms are ionophores (53%), tetracyclines (32.7%), macrolides (4.8%), sulfonamides (3.1%), aminoglycosides (3.3%), β-lactams (2%), and in lower abundance amphenicols, fluoroquinolones, and other drug classes (**Table 1.2**) [162]. However, beef breeds and non-lactating cattle use a greater mass of antibiotics as compared to lactating cows where only certain antibiotic classes and routes of administrations are approved (**Table 1.2**).

Table 1.1. Antimicrobial drugs approved for use in food-producing animals in 2020 in the U.S. reported by route of administration [162].

Route	Drug Class	Annual total (kg)	% total
Feed	Sulfonamides	20,915	0.20%
	Tetracyclines	3,256,519	31.16%
	Ionophores	4,447,420	42.56%
	Other drugs	458,831	4.39%
Water	Aminoglycosides	254,448	2.44%
	Lincosamides	62,297	0.60%
	Penicillins	655,060	6.27%
	Sulfonamides	176,062	1.68%
	Tetracyclines	591,722	5.66%
	Other drugs	90,307	0.86%
Other routes	Amphenicols	48,626	0.47%
	Cephalosporins	26,262	0.25%
	Tetracyclines	100,505	0.96%
	Other drugs	260,502	2.49%
	Total	***10,449,476***	***100.00%***

Table 1.2. Amount of antibiotics by drug class sold for use in cattle in 2020 and approved administration routes in dairy cattle [162].

	Estimated total		Approved routes of administration in cattle	
Antibiotic class	Kilograms	Percentage	Non-lactating cattle**	Lactating cows
Lincosamides*	11,165	0.2	None	Intramammary
Fluoroquinolones	12,446	0.2	Injection	None
Cephalosporins	21,007	0.4	Injection, intramammary	Injection, intramammary
Amphenicols	47,609	0.9	Injection	None
Penicillins	82,008	1.6	Injection, intramammary	Injection, intramammary
Sulfonamides	161,220	3.1	Injection, oral	Injection, oral
Aminoglycosides	174,132	3.3	Injection	None
Macrolides	247,581	4.8	Injection	None
Tetracyclines	1,703,391	32.7	Injection, oral, topical, feed additive	Injection, topical
Ionophore	2,758,786	53.0	Oral, feed additive	Feed additive
Total	***5,208,227***	***100***		

*Reported for cattle and other food-producing animals

**The term non-lactating cattle is defined as dairy bulls, dairy calves, and replacement heifers.

The amount of antibiotics reported by the FDA, however, should be carefully considered given the differences in how antibiotics are used in human and veterinary medicine. Some considerations include: 1) larger animal vs. human populations; 2) some animal species, such as cattle, require higher volumes of antibiotics given their live weight; 3) differences in physiology require adjustments in duration and dosage of the antibiotics; 4) the amounts of antibiotics sold do not show indication use, and 5) some antibiotics used in humans are prescribed to companion animals making a portion of them underrepresented in the FDA report [162].

Despite these concerns, a group of medically important antibiotics, which are readily used in human medicine, have also been approved for use in animals. Examples include tetracyclines, penicillins, macrolides, sulfonamides, aminoglycosides, lincosamides, cephalosporins, and fluoroquinolones [162,163]. These antibiotic classes are mainly intended for use in cattle (41%) and pigs (41%), but they are also commonly used in turkeys (12%), chickens (2%), and other animal species (4%) [162]. Despite the high percentage of medically important antibiotics used in cattle, only few classes of antibiotics are approved for use in lactating cattle (**Table 1.3**). Hence, it is necessary to study the impact that medically important antibiotics used in livestock play in the emergence of resistant bacteria of public health interest.

Table 1.3. FDA approved antibiotics for use in lactating cattle and importance in human health by drug class.

Administration	Class	Antibiotic	WHO importance (antibiotic class)
Injectable	Cephalosporin (third generation)	Ceftiofur crystalline free acid	Critically important of highest priority
	Cephalosporin (third generation)	Ceftiofur hydrochloride	
	Cephalosporin (third generation)	Ceftiofur sodium	
	Penicillin (aminopenicillins)	Ampicillin trihydrate	Critically important of high priority
	Penicillin (narrow spectrum)	Penicillin G (procaine)	Highly important
	Sulfonamide	Sulfadimethoxine	Highly important
	Tetracycline	Oxytetracycline	Highly important
Intramammary	Cephalosporin (first generation)	Cephapirin (sodium)	Highly important

Table 1.3 (cont'd)

Administration	Class	Antibiotic	WHO importance (antibiotic class)
Intramammary	Cephalosporin (third generation)	Ceftiofur hydrochloride	Critically important of highest priority
	Lincosamide	Pirlimycin	Highly important
	Penicillin (aminopenicillins)	Amoxicillin trihydrate	Critically important of high priority
	Penicillin (anti-staphylococcal)	Cloxacillin (sodium)	Highly important
	Penicillin (aminopenicillins)	Hetacillin (potassium)	Highly important
	Penicillin (narrow spectrum)	Penicillin G (procaine)	Highly important
Oral	Sulfonamide	Sulfadimethoxine	Highly important
Feed additive	Ionophore	Monensin (sodium)	Not medically important
Topical	Tetracycline/polymyxin	Oxytetracycline hydrochloride/Polymyxin B sulfate	Highly/critically important of highest priority

Role of antibiotics used in livestock in the emergence of AR bacteria

Antibiotics have different mechanisms of action against bacteria including the disruption of the bacterial synthesis of proteins (tetracyclines, aminoglycosides, macrolides), nucleic acids (quinolones, sulfonamides), or the cell wall (β-lactams, vancomycin, polymyxins) [169]. However, bacteria can evade the effect of antibiotics through several mechanisms such as efflux pumps to remove the antibiotic, modifying the cell membrane porin channels to reduce the uptake of certain drugs, modifying target sites to avoid recognition, or through the presence of inactivating enzymes [169–171]. Numerous genetic events in bacteria allow the development of resistance, such as the overexpression or duplication of existing genes, point mutations, or the acquisition of antibiotic resistance genes (ARGs) through horizontal gene transfer (HGT) [172,173].

The use of antibiotics in animals could result in the selection of resistant pathogens colonizing the GIT that can disseminate into the environment [3,159]. Mathematical modeling identified that the use of antibiotics in food-producing animals can increase the emergence of resistant bacteria in humans [174]. Zoonotic transmission of resistant bacteria has been

evidenced through whole-genome sequencing of *Salmonella* [175], *Campylobacter* [176], and *Staphylococcus aureus* MRSA [177]. Furthermore, active metabolites of antibiotics excreted by urine and feces play a role in the selection and subsequent evolution of bacterial resistance in the environment. Such is the case of hospital wastewater where MDR *E. coli* is strongly selected even when no treatment is applied [178]. Similarly, manure from animals treated with antibiotics carries a higher number of ARGs and MGEs when compared to manure from untreated animals [12,179–181].

The administration of antibiotics can also enhance the spread of drug resistance in the GIT microbiota through the HGT of ARGs [182]. Plasmids are one of the most important mechanisms of spreading ARGs between bacteria [183], though other MGEs can allow the movement of ARGs within bacterial genomes via transposons, integrons, or gene cassettes [172,173]. Thus, within a community or farm, a common pool of ARGs could co-circulate on different plasmids among diverse strains [184] complicating epidemiological efforts to track sources of antibiotic resistance [185,186].

Abundance and composition of antibiotic-resistant determinants in cattle feces

Methods used to analyze the resistome in cattle feces

Traditionally, the evaluation of antibiotic resistance in feces has been performed using culture-based techniques that require bacterial isolation followed by antibiotic susceptibility testing via disk diffusion or minimum inhibitory concentration tests [187]. *E. coli* is the most common bacterial indicator of resistance levels in cattle given that: 1) it is used as a fecal marker in environmental and food samples; 2) it is easy to isolate in laboratory settings; 3) it has an enhanced ability to colonize other animal species and humans; and 4) its gene plasticity promotes the acquisition of ARGs [188]. However, *E. coli* is a minority population in cattle feces and does

not represent the complexity of the resistome, which is defined as the group of genes encoding antibiotic resistance within a microbial community. The use of culture-independent techniques such as RT-PCR, 16S *rRNA* sequencing and shot-gun metagenomic sequencing have enabled a better understanding of the ecology and abundance of microbial populations and ARGs in cattle feces [171].

Metagenomic sequencing provides a broad identification of ARGs compared to RT-PCR and facilitates the discovery of potentially novel ARGs [171]. Nevertheless, *in-silico* detection of antibiotic resistance has several limitations since the detection of a gene is not necessarily indicative of phenotypic resistance. Although a prior study of STEC genomes demonstrated concordance between gene presence and resistance phenotypes, many more ARGs were identified that could not be validated via susceptibility testing assays [238]. Furthermore, current databases often lack unknown antibiotic resistance determinants or intrinsic resistances [171], and hence, a broad understanding of the resistome of cattle feces should combine culture-based and molecular techniques [189]. Recent studies suggest antibiotic enrichment of environmental samples to increase the number of resistant bacteria before sequencing to increase sensitivity and resolution of the molecular analysis, particularly long-read sequencing [190,191]. This method, however, will only select for specific resistant bacterial populations and their ARGs.

Because HGT can occur between different bacterial species, including commensal and pathogenic bacteria, classifying ARGs and identifying ARG mobility and frequencies within members of a microbiome is key to identifying risks in animal and public health [191]. Different bioinformatic and statistical approaches allow for the identification of bacterial taxa and MGEs associated with their carriage of ARGs, including correlation networks and contig analysis [192]. The analysis of long-read sequences also enables the identification of complete ARGs and their

localization in the bacterial genome, providing information on bacterial hosts and linkage with MGEs or other ARGs that can be co-selected [191].

Resistome composition of cattle feces

The fecal resistome composition of cattle shows a similar pattern across studies, with tetracycline as the dominant drug resistance class [193–202]. Genes with resistance to macrolide, lincosamide, streptogramin (MLS), aminoglycosides, and β-lactams were also commonly detected in the bovine resistome. Despite the extensive use of antibiotics in livestock, relatively few studies have assessed their effect on the fecal resistome composition in cattle [203]. While some antibiotics do not affect the resistome composition, others selectively enrich some ARGs by altering the bacterial populations directly. For instance, the use of the feed additives monensin and tylosin did not affect the functional microbiome or resistome [193]. Moreover, no difference was observed in the profiles of fecal resistome of cattle injected with or without tulathromycin, a macrolide used to prevent and treat bovine respiratory disease, but the application of this antibiotic changed the composition of ICE and plasmids [204,205]. The use of third-generation cephalosporins to treat respiratory disease and foot rot [166], augmented the abundance of β-lactam ARGs [206,207]. Similarly, cattle fed chlortetracycline exhibited an enrichment of tetracycline ARGs in feces [207].

Furthermore, beef and dairy breeds face different antibiotic selective pressures since varying bacterial infections in both breeds demand certain antibiotic classes [208]. For instance, β-lactams such as penicillins and cephalosporins, are commonly used in dairy cows to control mastitis [208–210]. Beef cattle farms commonly administer tylosin, chlortetracycline, oxytetracycline, and virginiamycin to prevent liver abscesses, as well as macrolides and tetracyclines to treat bovine respiratory disease [211]. Comparison of the resistome composition

between cattle breeds shows consistent trends across studies. For instance, the fecal resistome of beef breeds has been linked with greater levels of tetracycline resistance compared to dairy cows [195,198]. Whereas dairy cattle carry higher levels of β-lactam ARGs than feedlot animals [195,198]. Other classes of ARGs such as aminoglycoside and MLS, however, showed inconsistent results across studies between both breeds [195,198].

Additionally, variations in diets impact the GIT microbiome composition; therefore, changes in resistome associated with different community members have been observed in the rumen [212,213]. Selection of ARGs can also occur with the ingestion of heavy metals and minerals present in the diet such as zinc, copper, chromium, arsenic, cadmium, and lead [212,213]. For instance, in swine and poultry, zinc is supplemented in diets to improve the immune response and feed efficiency [214], but they have been linked with the selection of ARGs [215,216]. Since ARGs and metal resistance genes (MRGs) can be collocated in MGEs, such as plasmids, the use of metals can co-select both kinds of resistances [217,218]. In beef cattle, zinc supplementation increased the abundance of enterococci resistant to macrolides [219]. Similarly, copper also has shown associations with increased levels of macrolide ARGs [220].

Finally, the exposure to antibiotics at the farm level has been analyzed through a comparison of conventional and raised without antibiotic (RWA) farms. Some studies identified that RWA farms showed a lower abundance of tetracycline, macrolide, and aminoglycoside ARGs as compared to conventional farms [195,198,201,221]. Similarly, through RT-PCR and culture-based methods, feces from animals at conventional farms carried a higher number of *E. coli* resistant to tetracycline and third-generation cephalosporins, as well as a higher abundance of the *erm(B)* gene (MLS class) as compared to RWA cattle [221,222]. However, other studies

did not identify differences in the fecal resistome profile in feedlot cattle under conventional vs. RWA production settings [223]. Based on these findings, withdrawing the administration of antibiotics may not necessarily impact the profiles of resistome in the fecal microbiota of ruminants. Thus, the complexity of the resistome in bovines is multifactorial and includes not only antibiotic exposure but also farm practices, physiology, breed, diet, transmission between animals, and other factors that need further study.

Insights into the limitation of antibiotic use in bovines

Antibiotics are fundamental to treating infections in cattle farms. Particularly, when infections are caused by bacterial pathogens for which there are no vaccines or alternative therapies available. However, the use of non-therapeutic antibiotics in livestock is controversial given the global concern of antibiotic resistance [3,224]. A meta-analysis identified that restrictions on the use of antibiotics in food-producing animals could reduce their levels of resistance by up to 39% [168]. Based on that study, the WHO recommended stopping the use of antibiotics used to prevent diseases and growth promotion in 2017 [161], while enacting judicious use of medically important antibiotics in food-producing animals to maximize therapeutic efficacy in animals and humans.

The American Veterinary Medical Association and the FDA include prophylaxis and metaphylaxis in the category of therapeutic uses of antibiotics [162,225]. However, these practices are considered ‘sub-therapeutic’, ‘non-therapeutic’, or ‘production usage’ in other countries. Despite concerns related to the use of growth promoters, studies showed the lack of effect of major feed additives used in cattle (i.e., tylosin and monensin) in the fecal resistome [5,226]. Similarly, cattle from RWA farms have a high diversity of ARGs whose composition has little or no difference when compared to cattle feces from conventional farms [195,198].

However, studies focused on the effect of β-lactams and tetracyclines have shown their effect on the selection of resistant bacteria in cattle feces [227,228].

Undoubtedly, the misuse and overuse of antibiotics in humans and animals contribute to the emergence of antibiotic-resistant threats [229]. Limiting the use of medically important antibiotics in food-producing animals can potentially decrease the risk of resistance to last-resort drugs, also classified as critically important antibiotics of highest priority [161,229]. For instance, colistin is the last option to treat MDR Gram-negative such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and *Acinetobacter baumannii* [230]. Nevertheless, the broad use of colistin in livestock threatens the spread of resistance to this drug class. Pigs, for instance, are considered amplifiers of the plasmid-associated *mcr-1* gene providing resistance to colistin [231]. Moreover, colocalization of the *mcr-1* gene and ESBL genes identified in plasmids increases the risk of co-selection of important resistant threats [230]. Authorities worldwide banned the use of colistin in livestock; nevertheless, several developing countries are still using this antibiotic in food production [232].

In the U.S., three classes of antibiotics of highest priority are intended to use in cattle farms, including third-generation cephalosporins, macrolides, and quinolones [162]. Third-generation cephalosporins are broad-spectrum antibiotics used in a high number of people, particularly children and pregnant women given their low toxicity [161]. However, the transmission of Enterobacteriaceae resistant to third-generation cephalosporins through the production of ESBLs has been linked to non-human sources [161]. ESBL Enterobacteriaceae of zoonotic origin include *E. coli* and *Salmonella* spp. [158]. In the U.S. 47% of human infections with ESBL Enterobacteriaceae are community-associated [158]. An increased number of infections in the community rises the risk of dispersion of this threat complicating their control

and containment [158]. There is no clear evidence linking the use of cephalosporins in cattle with the rising human cases of ESBL Enterobacteriaceae. Nevertheless, the number of hospitalizations caused by infections with ESBL Enterobacteriaceae increased by 52% between 2012-2017 [158]; whereas the amount of cephalosporins used in livestock increased by 56% between 2009 and 2018 [162].

Macrolides used in beef and non-lactating cattle (i.e., tulathromycin and tylosin) are of highest priority given their frequent use in human medicine and reports of transmission of macrolide-resistant *Campylobacter* spp. from non-human sources [162]. While, quinolones, including danofloxacin and enrofloxacin, are approved to treat respiratory infections in beef and non-lactating cattle. The identification of fluoroquinolone-resistant *Campylobacter* spp. and Enterobacteriaceae, including *E. coli* and *Salmonella* spp., from non-human sources, is an emergent problem in human populations worldwide [158,162,233].

Restrictions of these therapeutic options in cattle are controversial given the limited number of antibiotics approved to use in bovines in the U.S. [163]. The FDA controls the use of drugs following the approved labeling that includes species for which they are approved, indications (disease or other conditions), dosage levels, frequencies, and routes of administration. Extra labeling uses of an antibiotic are possible under a valid veterinarian-client-patient relationship (VCPR), and when the drug is neither administered via feed nor results in a residue that constitutes a risk to public health. However, extra labeled uses are prohibited for fluoroquinolones and cephalosporins to control the misuse of antibiotics of highest priority [234].

In the U.S., cephapirin and ceftiofur are the only cephalosporins currently approved for use in food-producing animals including cattle, swine, sheep, goats, chicken, and turkeys [234].

Approved use of ceftiofur products in cattle includes their parenteral application for the treatment and control of respiratory disease, acute bovine interdigital necrobacillosis (foot rot) and acute bovine metritis [164]. Furthermore, intramammary infusions of ceftiofur are approved to treat clinical mastitis in lactating cows and subclinical mastitis in dairy cattle at the time of dry-off [165]. Cephapirin is a first-generation cephalosporin only approved as an intramammary infusion for the treatment of mastitis in lactating cows [234]. Using ceftiofur for disease prevention is prohibited by the FDA's Center for Veterinary Medicine [234].

Despite the regulations, ceftiofur is one of the most used antibiotics in dairy farms [209,210]. However, prudent use of this and other antibiotic therapies should include testing to determine the most effective treatment. Enforcing the use of other antibiotic classes of lower importance to human health rather than choosing the last line of antibiotics could prevent the emergence of resistance threats in farm environments. Yet, more research is needed to ensure that other drug classes can lower the risk of selecting resistant threats such as ESBL Enterobacteriaceae since co-selection of ARGs has been observed priorly [218,228].

Given the increasing global population and the concomitant growth of intensive livestock production, it is expected that the demand for antibiotics will rise [3]. Consumer requests for products from "antibiotic-free" farms will also increase. Adopting regulations and incentives to reduce the use of antibiotics in food-producing animals is fundamental to fighting antibiotic resistance under a One Health approach. However, more scientific evidence is needed to guide effective policies while ensuring animal welfare. Alternative strategies to replace antibiotics include bacteriophages and antibiotic peptides [235,236]. Nonetheless, reducing the risk of infections in cattle farms through improving hygiene, vaccination, appropriate housing and husbandry practices are the best options to avoid the use of antibiotics.

Several countries already banned the use of certain antibiotics in food-producing animals. In 2005, FDA banned fluoroquinolone use in poultry, and in 2017 the use of antibiotics for growth promotion [162]. In 2006, the European Union prohibited the use of antibiotics for 'non-therapeutic' purposes [237]. While China withdrew the use of medicated feed additives through their National Action Plan to Combat Antibiotic Resistance from Animal Resources (2017–2020). The banned use of colistin in feed demonstrated a significant effect in reducing *mcr-1*-mediated resistance in humans and animals in China [230], proving the effectiveness of these policies in reducing the levels of antibiotic resistance in animal and human populations.

SUMMARY

Bovines have been one the most important animal species for the development of human societies [2]. Their complex GIT microbiome makes them the most efficient protein producers among food-producing animals [8]. As the demand for meat and dairy products is expected to increase with the human population, the adoption of intensive production systems will also escalate [2]. Despite the benefits of bovines, their expansion can result in increased negative footprints such as the transmission of foodborne pathogens and AR bacteria [2].

Cattle are the primary carriers of STEC and the main consumers of antibiotics worldwide. Preventing the transmission of STEC to humans is critical since no antibiotic therapies are recommended given that they increase toxicity with fatal consequences [65]. Understanding the role of the microbiome in STEC colonization can help identify alternative methods to decrease the prevalence of this pathogen in cattle farms.

Notably, medically important antibiotics for humans are also used in cattle farms in the U.S. [162,163]. Third-generation cephalosporins, for instance, are classified as critically important antibiotics of highest priority [161]. The overuse of third-generation cephalosporins is

associated with rising infections with ESBL Enterobacteriaceae which are the main cause of deaths attributed to MDR bacteria worldwide. Ceftiofur is a third-generation widely used in dairy farms to treat multiple infections including mastitis [164–166]. To prevent the misuse of this drug, the FDA prohibits the application of ceftiofur for extra labeling uses [234]. Nevertheless, policies are needed to prevent the overuse of ceftiofur in bovines, such as encouraging the use of other therapies or drugs with lower implications in the emergence of AR threats and the implementation of antibiotic resistance tests. Identifying the effects of ceftiofur application in the fecal resistome of cattle can help to enforce the judicious use of this last resort drug. Studies demonstrated the increase of ESBLs in the resistome of cattle after its parenteral application [207,227]. However, there is no evidence of the effect of intramammary ceftiofur application in the resistome of cattle feces. Reducing the prevalence of STEC and antibiotic resistance in cattle farms is critical to prevent infections in humans and ensuring the effectiveness of last-resort treatments. Studying the ecological interactions between the GIT microbiome, STEC, and AR through culture-depend and independent methods can provide valuable information to reduce the dispersion of bacterial threats in cattle farms.

STUDY AIMS

Despite the high prevalence of STEC and the use of third-generation cephalosporins in cattle farms, little is known about the ecological interactions of this pathogen and the effect of antibiotics on the cattle microbiome. Characterizing the GIT microbiome of cattle carrying STEC and the effects of antibiotic therapies in the abundance of resistant bacteria can help identify strategies to decrease the prevalence of resistant foodborne pathogens. To address this research gap culture-based methods, 16S *rRNA* sequencing, metagenomics, and metabolomics were applied to study the fecal microbiome, metabolome, and resistome of cattle.

Three main aims were developed in this study: 1) identify microbiome diversity and composition that may favor the colonization of STEC in the hindgut of cattle; 2) determine the impacts of IMM ceftiofur in dairy cattle at the time of dry-off in the abundance of resistant bacteria; and 3) characterize changes in the functional microbiome and metabolome associated with the IMM application of ceftiofur.

This study seeks to understand better the impacts of pathogens and antibiotic treatment on the cattle microbiome ecology. These findings could guide new prevention and control strategies to limit the spread of resistant bacteria and STEC in cattle farms.

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CHAPTER 2

Characterizing the cattle gut microbiome in farms with a high and low prevalence of Shiga toxin-producing *Escherichia coli.*

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ABSTRACT

Cattle are the main reservoirs of Shiga-toxin-producing *Escherichia coli* (STEC), a major foodborne pathogen associated with acute enteric disease and hemolytic-uremic syndrome in humans. A total of 397 beef and dairy cattle from 5 farms were included in this study, of which 660 samples were collected for 16S *rRNA* sequencing. The microbiota of farms with a high-STEC prevalence (HSP) had greater richness compared to farms with a low-STEC prevalence (LSP). Longitudinal analyses showed STEC-shedders from LSP farms had higher microbiome diversity; meanwhile, changes in the microbiome composition in HSP farms were independent of the STEC shedding status. Most of the bacterial genera associated with STEC shedding in dairy farms were also correlated with differences in the percentage of forage in diet and risk factors of STEC carriage such as days in milk, number of lactations, and warm temperatures. Identifying factors that alter the gut microbiota and enable STEC colonization in livestock could lead to novel strategies to prevent fecal shedding and the subsequent transmission to humans.

INTRODUCTION

Shiga-toxin producing *Escherichia coli* (STEC) is a foodborne pathogen causing 2.8 million cases of acute enteric disease and 230 deaths annually [1]. STEC infections are associated with the consumption of contaminated food and water or result from direct contact with cattle feces since cattle represent an important reservoir for this pathogen [2]. While livestock carriers of STEC are asymptomatic, humans can develop bloody diarrhea, hemolytic-uremic-syndrome, thrombotic thrombocytopenic purpura, or end-stage renal disease [1]. STEC virulence is caused by bacteriophage-encoded Shiga toxins (Stx1 and Stx2) that induce cellular apoptosis of endothelial cells in the gut, kidney and brain of humans [3–6]. Cattle are more tolerant to STEC due to the lack of Stx receptors (glycolipid globotriaosylceramide, Gb3) in the

intestinal tract as well as a lower receptivity of Gb3 receptors present in the kidney and brain [7]. Some STEC strains, classified as enterohemorrhagic *E. coli* (EHEC), possess the locus of enterocyte effacement (LEE) pathogenicity island that encodes for a type III secretion system and effectors, such as the intimin protein (*eae*), which are responsible for attaching and effacing (AE) lesion formation [8]. Adult cattle carrying EHEC are typically unaffected, though infected calves develop AE lesions on the apical epithelial surfaces of the recto-anal junction where the bacteria colonize [9].

Because cattle are important reservoirs of STEC, reducing carriage of this pathogen in livestock and preventing dissemination in food and the environment are priorities for preventing human infections [10]. Our previous study identified risk factors associated with high STEC prevalence in dairy farms including first lactation, less than 30 days in milk, and warm temperatures [11]. Meanwhile, protective factors identified in farms with low STEC prevalence included access to pasture, anthelmintic treatment, and antibiotic treatment for respiratory infections [11]. Nonetheless, it is not clear how factors associated with STEC prevalence influence the microbiota composition and potentially favor STEC colonization.

The gut microbiome is critical for the activation and regulation of the immune response and preventing pathogen colonization [12]. Some studies have analyzed the association between the gut microbiome and STEC in both humans and cattle. In humans, the gut microbiome of infected patients had a lower abundance of dominant taxa from Bifidobacteriales and Clostridiales [13]. We also previously showed that microbial communities from patients with acute enteric infections caused by STEC and other pathogens, had a lower bacterial richness with an increased abundance of Proteobacteria (genus *Escherichia*) and decreased abundance of Bacteroidetes compared to healthy communities [14,15]. With regard to cattle, varying results

have been observed in the richness and composition of the fecal microbiome between STEC shedders and non-shedders [16–24]. Within a specific farm, for example, some studies observed no difference in diversity among STEC shedders and non-shedders [19,21], whereas other studies have detected significantly higher [22] and lower [23] diversity in STEC shedders despite controlling for age, farm, and diet. This lack of consensus among previous reports compels further investigation.

Herein, we sought to compare the microbiota structure and function of cattle among farms with a high versus low STEC prevalence. Additionally, we aimed to determine whether STEC carriage is associated with changes in the microbiota composition over time. Characterizing a healthy cattle microbiome that does not support pathogen colonization and identifying key beneficial microorganisms (probiotics) can guide the development of new prevention protocols in an effort to eradicate STEC colonization in animal reservoirs.

MATERIALS AND METHODS

Sample collection

An initial study was carried out in the spring and summer of 2011 and 2012 in Michigan in which 1,096 samples were collected in eleven cattle farms including six dairy herds and five beef herds to determine STEC prevalence and risk factors [11]. Here, samples from five of those eleven cattle herds were selected for microbiome analysis, which was based on the varying prevalence of STEC in each herd. Specifically, a low STEC prevalence (LSP) was observed in one feedlot, 1B (8.2%), and two dairy farms, 2D (8.7%) and 4D (13.8%). Comparatively, the prevalence was considerably higher in feedlot 8B and dairy farm 9D (53.7 and 28.0%, respectively), which were considered high STEC prevalence (HSP) herds. The three dairy herds had Holstein cows (farms 2D, 4D, 9D), while the other two were beef feedlots with Crossbreed

(farm 1B) and Angus (farm 8B) breeds. Epidemiological information obtained from each herd included demographics, geographic location, husbandry practices, health management, and diet. Additional information including number of lactations, days in milk, and dry status was collected at the dairy farms.

Fecal grabs (FG) were collected by rectal palpation using obstetric sleeves (n = 308), while recto-anal junction (RAJ) samples were collected by swabbing the RAJ with a sterile cotton swab (n = 352) as described [11]. Roughly 256 pairs of fecal grabs (FG) and rectal-anal junction (RAJ) swabs were collected simultaneously from the same animal for microbiome comparison. A subset of cattle was also sampled over time at an interval of 2 to 3 weeks between each sampling point to examine microbiome changes and STEC shedding over time (**Table A.2**). In addition, blood samples were collected from the coccygeal or jugular vein of each animal for serology [25].

Pathogen identification

STEC was detected using CHROMOagar STEC and sorbitol MacConkey agar followed by PCR confirmation targeting key virulence genes [11]. Suspect isolates were classified as STEC if they were positive for any Shiga toxin gene (*stx*) subtypes with or without the intimin gene (*eae*). In addition, exposure to pathogens that can alter the gut microbiota was evaluated to account for confounding effects between STEC shedders and non-shedders. These pathogens included bovine leukemia virus (BLV), bovine viral diarrhea (BVD), and *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which were identified by serology using enzyme-linked immunosor-bent assays (ELISA) that detects antibodies specific for these pathogens as described [24].

Amplicon library processing

DNA was extracted from 660 samples recovered from cattle at each of the five farms using the QIAamp DNA Stool Mini Kit (QIAGEN; Valencia, CA). DNA was extracted from 250 mg of feces, or the RAJ swabs stored at -80ºC. A fragment of approximately 569 bp from the V3–V5 hypervariable region of the bacterial 16S *rRNA* gene was amplified using the linker primer 357F (5′-CCGTCAATTCMTTTRAGT-3′) and the reverse primer 926R (5′-CCTACGGGAGGCAGC AG -3′). A sample-specific barcode of 6-8 nucleotides was used to sequence samples in parallel on a single 454-sequencing plate. The amplification and pyrosequencing methods were described in our prior study [14].

The raw pyrosequencing reads were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) software v.1.9.1 workflow for 454 data [26]. First, the sequences were demultiplexed based on the nucleotide barcode and quality filtered using *split_libraries.py*. Then, *de novo* operational taxonomic units (OTUs) were identified with *pick_de_novo_otus.py* and the taxonomy was assigned with the SILVA database (v132) [27]; OTUs were clustered at 97% similarity. All samples were denoised with *denoise_wrapper.py* to reduce the amount of erroneous OTUs [28] and OTU chimera detection and filtering was done with VSEARCH [29]. Lastly, the sequences were aligned with *align_seqs.py* [30] and were converted into a phylogenetic tree using the QIIME 2 plugin '*qiime fragment-insertion sepp*' [31–33].

Microbiome analyses

The OTU table, taxonomy, metadata, and phylogenetic tree were imported into the R package Phyloseq v.1.24.2 [34]. Mitochondria and chloroplast OTUs were removed. Library rarefaction was applied to calculate alpha and beta diversities among samples. Alpha diversity was estimated to determine richness and evenness of OTUs with the Shannon index, and richness

based on the presence of rare OTUs (singletons and doubletons) was estimated with Chao1. The Wilcoxon and Kruskal-Wallis non-parametric tests were used to compare the alpha diversity estimates among LSP and HSP farms and STEC shedders and non-shedders. Beta diversity was also analyzed to compare the microbiome composition among groups using Bray-Curtis dissimilarity and Weighted UniFrac distances [35]. The ordination was calculated by principal coordinate analysis (PCoA), which was plotted with two axes. The difference between categorical variables and the microbial profiles were calculated with permutational multivariate analysis of variance (PERMANOVA) with 999 permutations using the Vegan package v.2.5-6 [36].

The differentially abundant taxa analysis was performed using differential expression analysis based on the negative binomial distribution DESeq2 (v.1.30.1) with default settings [37]. The R package metacoder v.0.3.3 [38] was used to visualize the taxa abundance as "heat trees" with the proportion of bacterial families. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST2) [39] was also used to predict metabolic pathways and enzymes based on 16S *rRNA* sequences. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) v.1.0 [40] was used to identify differentially abundant pathways.

RESULTS

Farm characteristics

Three dairy and two beef herds were included in this study based on their STEC prevalence [11]. One beef and two dairy farms (1B, 2D, and 4D) were classified as low STEC prevalence (LSP) farms, which were compared with farms with high STEC prevalence (HSP) represented by one beef and one dairy farm (8B and 9D). The five farms represented varying breeds and sizes with different healthcare and management practices (**Table 2.1**). Notably, the

LSP farms fed animals a lower percentage of forage in their diet (15-65%) and used anthelmintics, while HSP farms used a diet almost exclusively based on forage (80-100%) and did not provide anthelmintic treatments. Specific characteristics unique to the dairy farms, such as number of milkings per day, days in milk (DIM), and number of lactations are shown in **Table A.1**.

Table 2.1. Characteristics of each cattle farm examined.

Feature	Farm				
	2D	4D	9D	1B	8B
Breed	Holstein	Holstein	Holstein	Crossbreed	Angus
Herd	Dairy	Dairy	Dairy	Beef	Beef
Herd size	320	3000	243	136	54
STEC prevalence (%)	8.7	13.8	28.0	8.2	53.7
STEC prev. classification	LSP	LSP	HSP	LSP	HSP
No. samples[a]	213	81	77	206	83
Fecal grab	48	40	77	60	83
Recto anal junction	165	41	0	146	0
Mean age days (SD)	1382 (476)	NR	1362 (522)	372 (19)	442 (17)
Housing	Free stall; tie stall	Free stall	Access to pasture/dry lot; Free stall	Feedlot	Loose house
Diet % (SD)					
Forage	65.01 (18.76)	40.62 (9.47)	80 (0)	15 (0)	100 (0)
Concentrate	34.99 (18.76)	59.38 (9.47)	20 (0)	85 (0)	0
Corn silage	29.06 (8.82)	41.7 (3.74)	0	15 (0)	0
Cotton seed	1.60 (2.60)	0	0	0	0
Rumensin	No	Yes	No	Yes	No
Roughage, Protein	No	No	Yes	No	No
Season [b]	Summer	Summer	Summer	Spring	Summer
Temperature (°C)	25	25	36	4.4	36
Humidity (g/m^3)	66	68	31	75	42
Temp. max. 5 days [c]	23.44	29.89	37.11	20.33	29.33
Temp. avg 5 days [c]	19.22	16.89	30.11	13.89	22.78
Treatment					
Anthelmintic	Yes	Yes	No	Yes	No
Respiratory	Ceftiofur, Florfenicol	Ceftiofur	None	Ceftiofur, Tulathromycin	Florfenicol
Foot infection	Copper sulfate, Penicillin	Copper sulfate	Copper sulfate, Oxytetracycline, Ceftiofur	Oxytetracyclin e	Ceftiofur

NR=Not reported; SD=Standard deviation

[b] During sample collection; [c] Temperature five days prior to sample collection

Sequencing results

Twenty-eight 454-sequencing plates containing 660 samples yielded 1,937,794 reads of 569 bp paired-end fragments of the 16S *rRNA* gene. After trimming and quality filtering the sequences, the library size varied from 650 to 16,786 with a median library size of 2,332

sequences per sample. Following the *de novo* clustering, denoising, filtering chimera, and removal of chloroplast and mitochondria OTUs, 15,158 OTUs were detected.

Hindgut microbiota composition

The microbiota profiles for the 256 paired fecal grab (FG) and rectal-anal junction (RAJ) samples were similar ($p > 0.05$) and hence, these samples were combined into a single group representing the hindgut for downstream analyses (**Figures A.1 and A.2**). Overall, the hindgut microbiota was dominated by two phyla, Firmicutes (54.6%) and Bacteroidetes (38.9%), although varying proportions of other phyla were detected across farms with different forage percentages (**Figure 2.1A**). Indeed, the percentage of forage in the diet significantly influenced the microbial composition. Farms with low forage diets, for instance, had a lower abundance of Firmicutes and a higher abundance of Bacteroidetes ($p < 0.0001$). Classifying by family identified similar differences across farms with *Ruminococceaceae* predominating but increasing with the forage percentage ($p < 0.001$) (**Figure 2.1B**). Several additional bacterial families were significantly correlated with the percentage of forage in the diet (**Table A.3**).

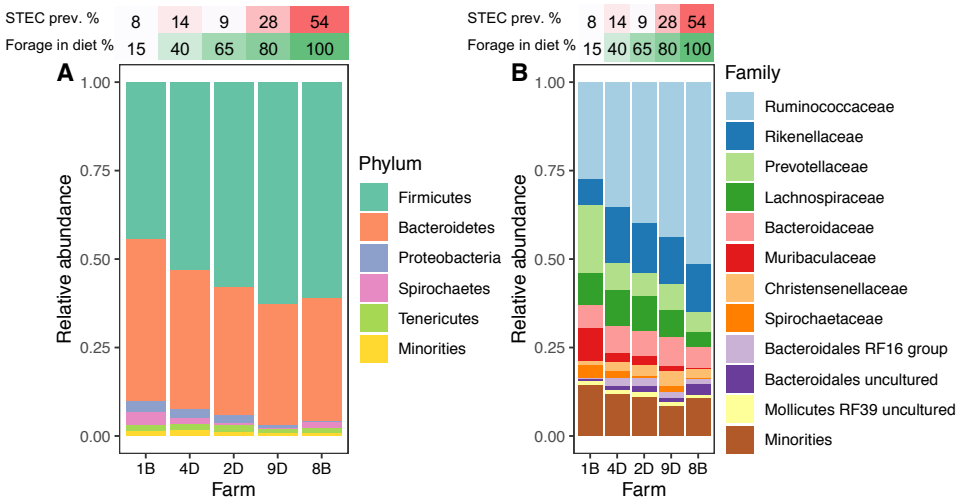

Figure 2.1. Hindgut microbiota composition of cattle from five farms with varying percentages of forage in the diet. Stacked bar charts show the relative abundance of bacterial A) phyla and B) families per farm. Less abundant taxa were grouped together and named "Minorities".

HSP farms characterized by forage-dominant diets exhibited higher alpha diversity and a distinct microbiota structure

To assess the association between STEC prevalence and the hindgut microbiota among farms, we analyzed the Shannon and Chao1 indices for alpha diversity and the Bray-Curtis dissimilarity and Weighted Unifrac for beta diversity. HSP farms exhibited greater richness than LSP farms, although no significant difference was observed using the Shannon index ($p = 0.67$) (**Figure 2.2A**). The Chao1 index, however, detected significantly greater diversity in HSP farms ($p = 1e\text{-}09$), indicating that a high number of OTUs were present in low proportions (singletons and doubletons) in the two HSP farms (**Figure 2.2B**). Notably, when comparing the alpha diversity indices between herds, the lowest and the highest OTU richness corresponded to farms 1B and 8B, respectively, which also had the lowest and the highest STEC prevalence (**Figure 2.3**). When the farms were plotted separately to evaluate beta diversity, the PCoA plot of Weighted Unifrac distances showed that the microbial communities from LSP farm 1B were the most divergent relative to the other four farms (**Figure 2.2C**). Comparatively, the farms were classified by STEC prevalence and a Bray-Curtis dissimilarity PCoA was generated (**Figure 2.2D**). This plot shows that cattle from the two HSP farms had a more similar microbiota structure that was significantly different than the microbiota profiles observed in the three LSP farms (PERMANOVA, $p < 0.0001$). HSP microbiota clustering was strongly associated with dominant forage diets (**Figure A.4**).

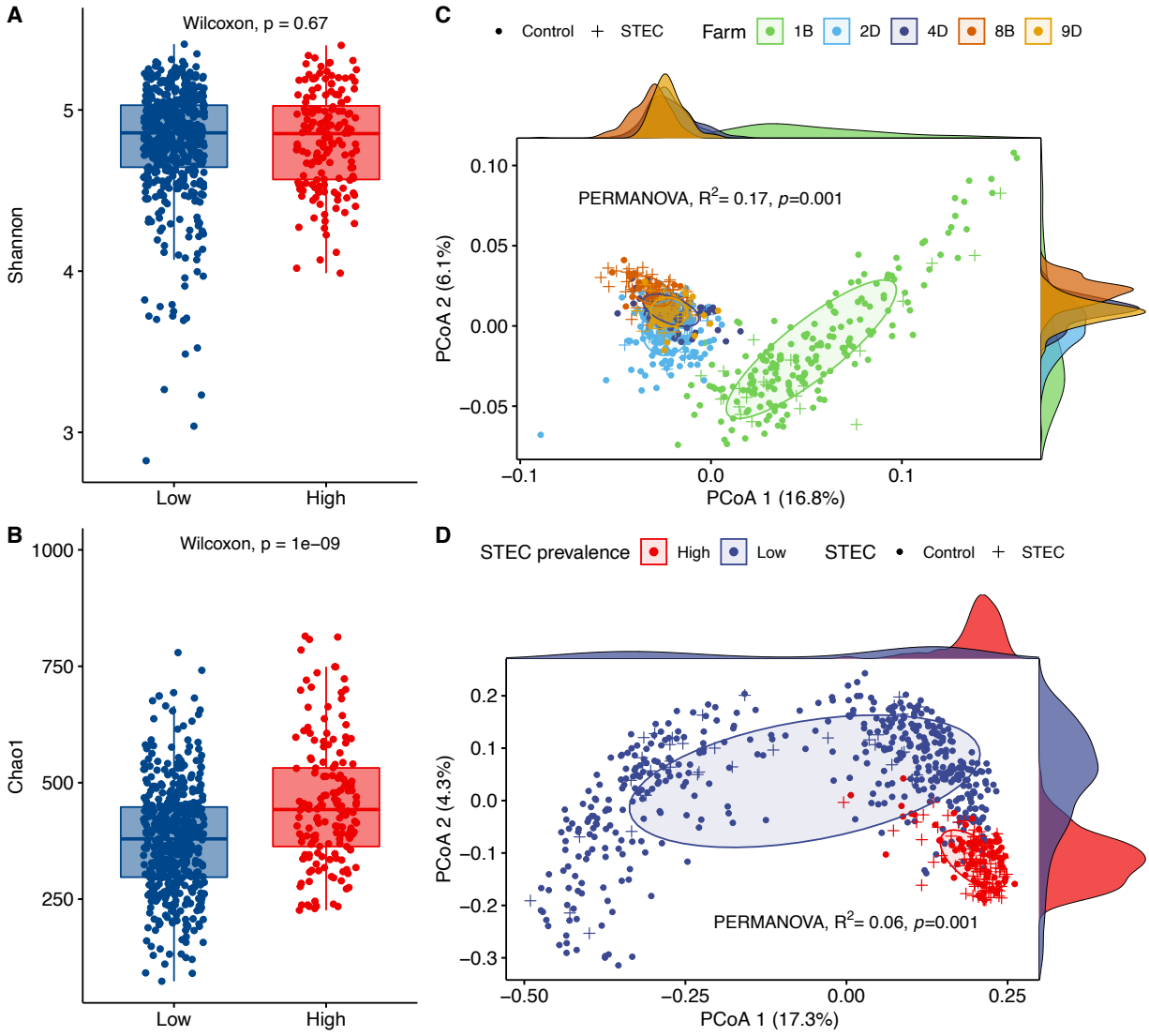

Figure 2.2. Cattle-hindgut microbiota alpha and beta diversities among farms with a low STEC prevalence (LSP) and high STEC prevalence (HSP). LSP Farms (1B, 2D, and 4D) were combined as were the two HSP farms (8B and 9D) to evaluate the alpha diversity using the A) Shannon index and B) Chao1 index. Beta diversity was evaluated by performing a C) Principal Coordinate Analysis (PCoA) of Weighted-Unifrac distances or and a D) PCoA of Bray-Curtis dissimilarity. The former plotted each farm separately along with the STEC shedders (+) and non-shedders (control, black circle), while the latter compared farms with LSP versus HSP.

Hindgut-microbiota diversity comparisons between STEC positive samples and controls

The microbiota profiles of STEC-shedders and non-shedders were examined in more detail regardless of farm. To do this, the animals were split into two comparison groups based on the recovery of isolates positive for *stx* and/or *eae* for comparison to cattle with isolates that were

negative for both virulence factors; 3-4 control animals were randomly selected from each of five farms for each case included in the analysis. The first comparison group examined 31 STEC positive (*stx* only) cattle from HSP farms and 85 STEC/EHEC negative (control) cattle including 20 from HSP and 65 from LSP farms. For the second comparison group, EHEC positive (*stx* and *eae* only) cattle (n = 52) were compared to a larger set of STEC/EHEC negative control animals (n = 205). EHEC comparison included cattle from HSP farms (cases = 34, controls = 20) and LSP herds (cases = 18, controls = 185). These groups excluded animals positive for BLV and MAP because they had significantly different microbiota profiles based on the alpha and beta diversity metrics ($p < 0.05$) (data not shown). Indeed, the exclusion of samples from MAP- and BLV-positive cattle was necessary given that prior studies showed that these pathogens were associated with important gut microbiome changes [41,42]. Notably, the STEC shedders possessing *stx* only had higher microbiota richness than the non-shedders (Shannon, $p = 0.19$; Chao1, $p = 0.008$), though only the Chao1 metric was significant (**Figures 2.3A & 2.3B**). By contrast, no difference in alpha diversity was observed when the EHEC shedders (*stx*-positive and *eae*-positive) were compared to the STEC/EHEC-negative controls (Shannon, $p = 0.27$; Chao1, $p = 0.41$) (**Figures 2.3C & 2.3D**). The microbiota structure of the STEC and EHEC shedders, however, was significantly different from the controls in both comparison groups (PERMANOVA, $p < 0.001$) (**Figures 2.3E & 2.3F**).

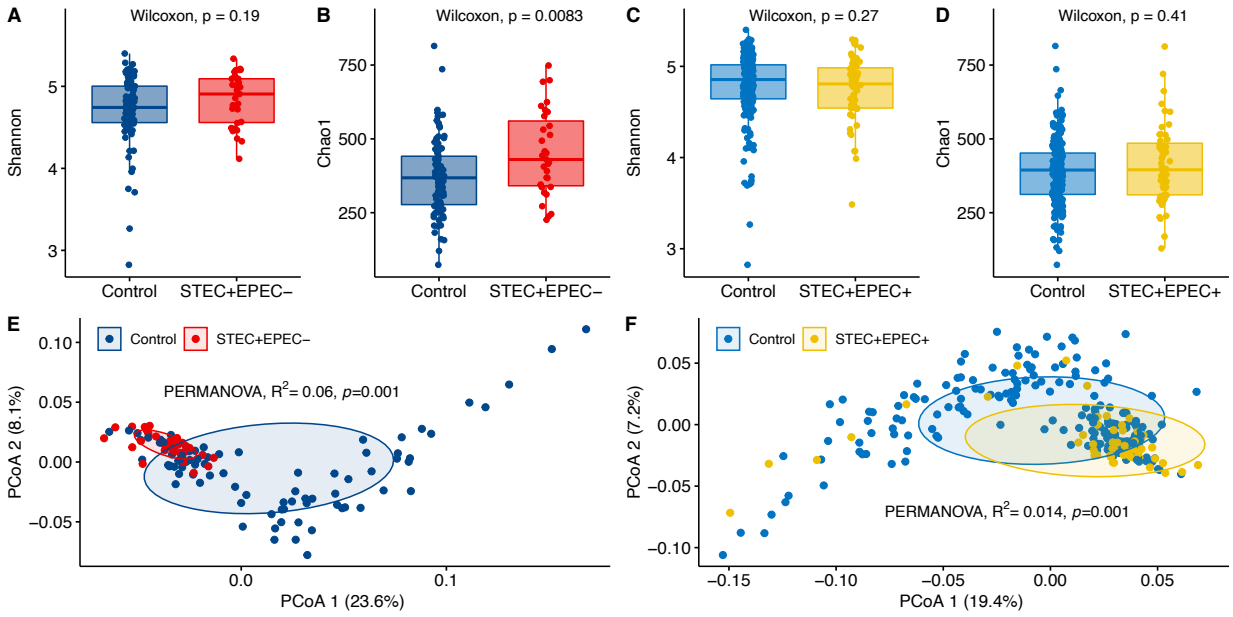

Figure 2.3. Cattle-hindgut microbiota diversity comparisons between animals shedding STEC or EHEC and non-shedders (controls). The alpha diversity was evaluated for 31 STEC-positive (*stx*-positive, *eae*-negative) cattle (red dots) for comparison to 85 STEC/EHEC-negative control cattle (blue dots) using the A) Shannon and B) Chao1 indices, while E) beta diversity was examined using a Principal Coordinate Analysis (PCoA) of Weighted Unifrac distances. The C) Shannon and D) Chao1 alpha diversity indices as well as a F) PCoA for beta diversity were also evaluated for the 52 EHEC shedders (*stx*-positive, *eae*-positive; light blue dots) for comparison to a larger sample of 205 randomly selected non-shedders (yellow dots) from the five herds.

STEC carriers from farms with LSP but not HSP showed changes in microbiota diversity over time

Among the 59 cattle evaluated longitudinally from five farms (**Table A.2**), we sought to determine how STEC shedding impacted the microbiota composition in the hindgut. Despite finding no significant modifications due to diet, management, and environmental conditions (**Table A.4**) were reported within the farms over time, all cattle had significant differences in the alpha and beta diversities across the four samplings or phases. In general, the STEC positive samples exhibited higher alpha diversity within each phase, particularly among the three LSP farms (**Figures 2.4A & 2.4B**), however, none of the animals from the LSP farms carried STEC

in more than one phase. By contrast, the cohorts from the HSP farms, 8B and 9D, had a high proportion of cattle shedding STEC in all four phases. Each animal from farm 8B (n = 13) shed STEC in two or more phases. Indeed, the biggest difference in alpha diversity over time was observed in the HSP 8B farm as both the Shannon and Chao1 indices were significantly different between STEC shedders and non-shedders over time. In farm 9D, 25% of animals shed the pathogen in two phases, while the remaining cattle shed in just one phase, and the alpha diversity was steady.

Differences in the microbiota composition or beta diversity, as determined by the PCoA, were also observed across samplings at each of the five farms evaluated (**Figure 2.4C**). Curiously, the Angus farm (8B) had two microbiota profiles that were not associated with STEC shedding status. In farm 8B, the microbiota profiles in phases 1 and 2 were similar, highly diverse, and dominated by Bacteroidetes (*log2 fold change* = 0.22; p = 0.0009). The microbiota profiles in phases 3 and 4, however, were distinct from those observed in phases 1 and 2, which coincided with a decreased alpha diversity and an increased abundance of Firmicutes (*log2 fold change* = 0.15*;* p = 0.05). Despite these differences, the proportion of STEC positive animals in farm 8B was steady across the four phases.

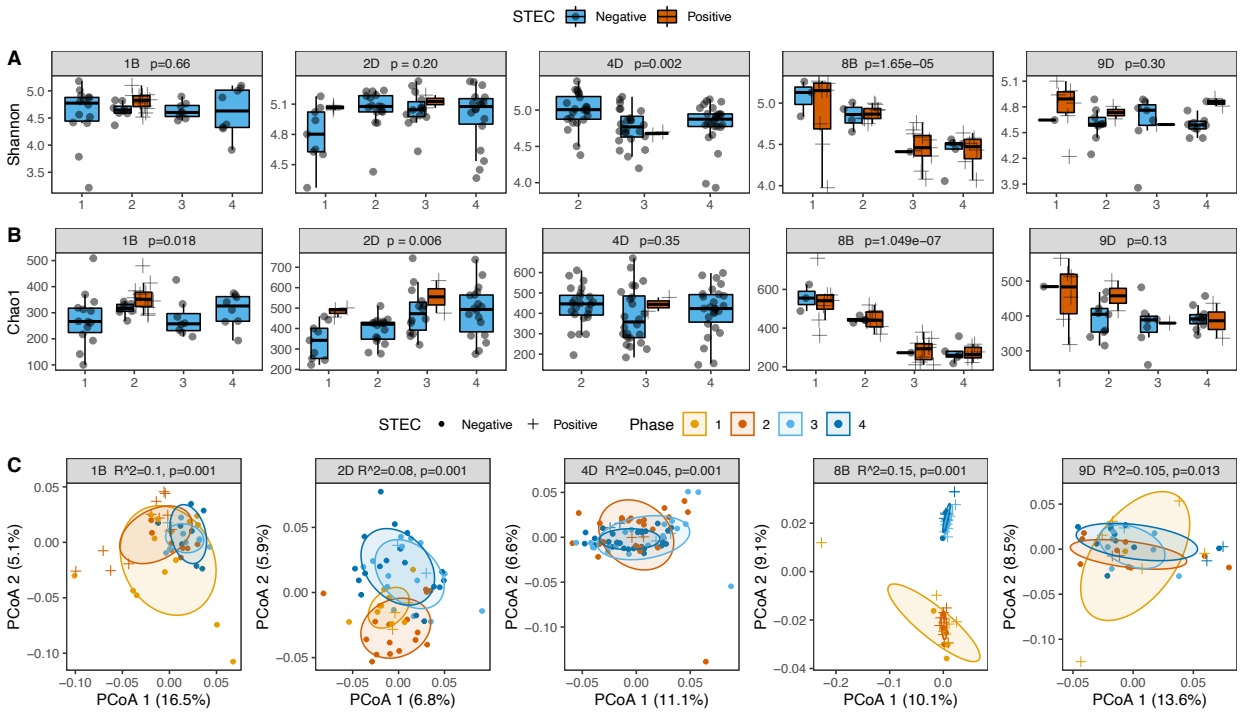

Figure 2.4. Temporal dynamics in microbiota diversity among 59 cattle from five farms. Alpha diversity was compared using the A) Shannon and B) Chao1 indices by farm and sampling period. Each box represents a different farm with the numbers on the x-axis representing the four sampling visits; significant differences were detected using the Kruskal-Wallis test. C) Beta diversity was also examined using a Principal Coordinate Analysis (PCoA) of the Weighted Unifrac distances; PERMANOVA results (R^2 and *p-value*) were calculated. STEC shedders (+) and non-shedders (circles) were plotted by phase, which is represented by four different colors.

Differentially abundant taxa among the STEC shedders from dairy farms

Among the three dairy farms, STEC shedders had a significantly greater abundance of Firmicutes and a lower abundance of Proteobacteria than the non-shedders ($p < 0.01$) (**Figure 2.5**). No taxa were identified when comparing between STEC-shedders and non-shedders among the dairy farms when controlling for farm. A total of 30 genera were found to be differentially abundant between STEC carriers and non-carriers (**Table A.5).**

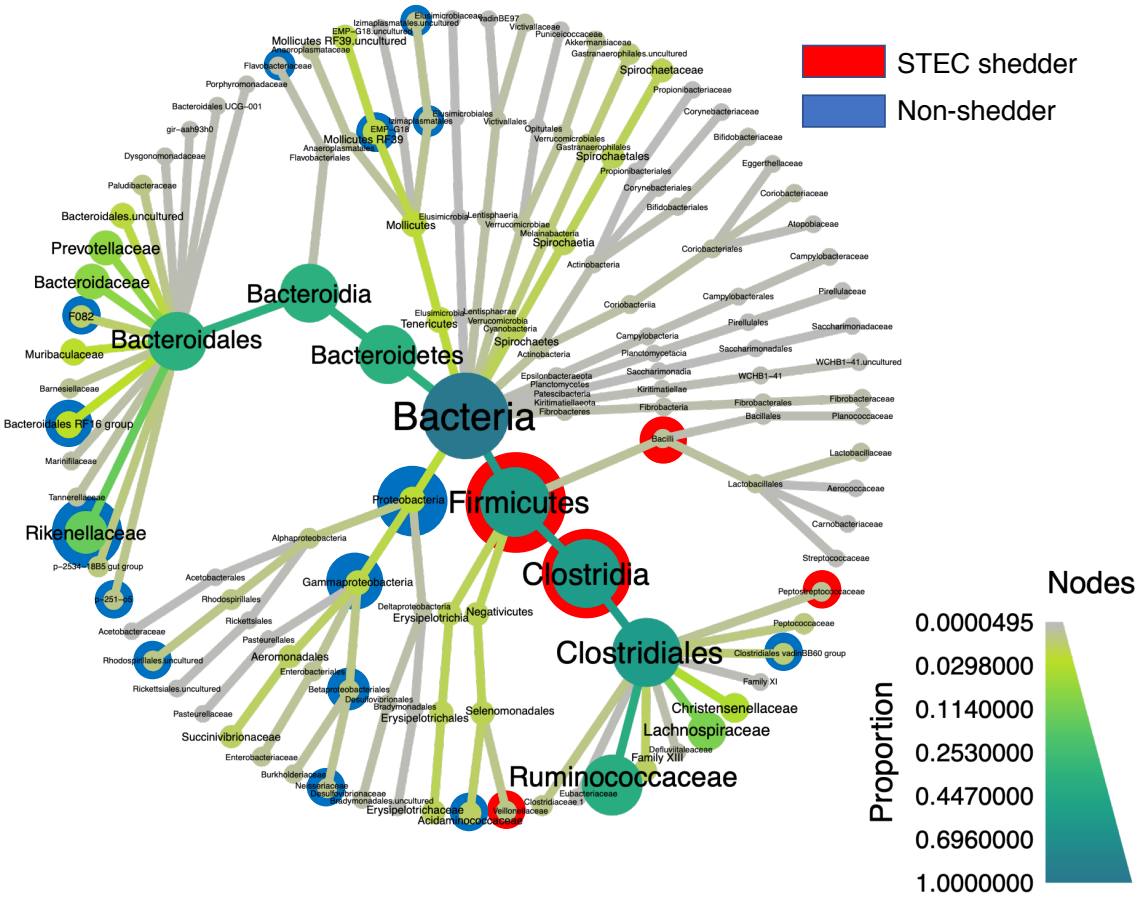

Figure 2.5. Heat tree showing the differentially abundant taxa found in the hindgut microbiota among STEC shedders (red) and non-shedders (blue) at three dairy farms. Only those taxa with a p-value lower than 0.01 were included. Node size and color correspond to the relative abundance at each taxonomic level.

Taxa correlated with factors associated with STEC carriage

Next, we analyzed how the microbiota composition can be disturbed by previously identified risk factors of STEC shedding in cattle [11] including maximum temperature 5 days prior to sampling, days in milk (DIM), and the number of lactations (**Figure 2.6, Table A.6**). Notably, temperature increases were associated with a differential abundance of 189 taxa including 42 observed among the STEC shedders (**Table A.5**). Similarly, the number of DIM was significantly correlated with 24 differentially abundant genera including those associated with STEC carriage such as *Actinobacteria*, *Anaeroporobacter*, *Kingella*, *Ruminococcaceae*

UCG-005, Tenericutes, Veillonellaceae, and *Eubacterium ruminantium group*. Finally, seven taxa were correlated with the number of lactations, including an increase of *Kingella* and Neisseriaceae and decrease of *Lentisphaerae* and *Ruminococcaceae UCG-011* as observed in non-STEC shedders. Forage percentage in diet across farms was associated with changes in 211 taxa of which 48 were associated with STEC shedding (**Figure 2.6**).

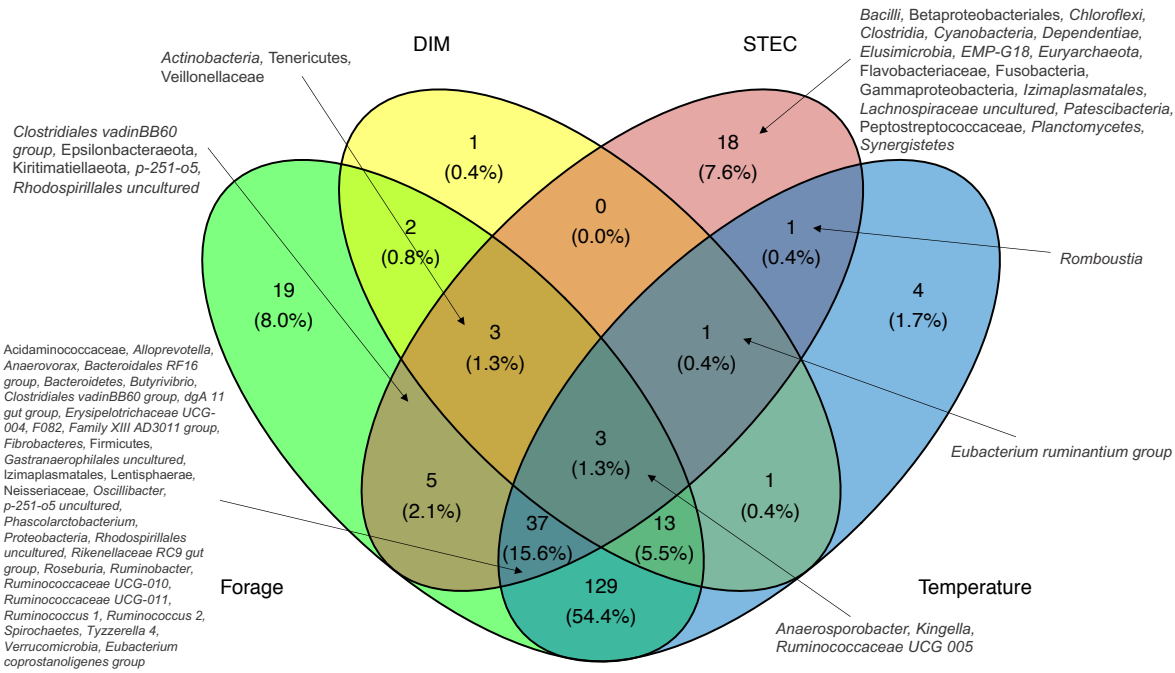

Figure 2.6. Venn diagram showing the number of differentially abundant genera associated with STEC shedding (STEC), percentage of forage in diet and risk factors of STEC in dairy farms (temperature and days in milk). Percentages represent the proportion of taxa intersected between different variables.

DISCUSSION

Preventing STEC shedding in livestock could significantly reduce the number of human infections. In this study, we sought to determine differences in the gut microbiota of bovines from farms with a low versus high STEC prevalence. In addition, we explored factors that could affect the microbial composition and contribute to STEC shedding. The diversity and composition of 16S *rRNA* sequences of 660 hindgut samples from five cattle farms (beef and

dairy) were analyzed in this study. Cattle from HSP farms, characterized by being fed a high percentage of forage in diet (80-100%), had a significantly higher richness of OTUs than LSP farms, which had a lower proportion of forage in diet (15-65%). Longitudinal analysis showed that most STEC-shedders from LSP farms had a greater microbial diversity than non-shedders; however, cattle from HSP farms showed changes in the microbial diversity that were not linked to the STEC carriage. Furthermore, bacterial taxa associated with STEC shedding was also correlated with diet and previously described risk factors of STEC. Meanwhile, significant differences in predicted metabolic pathways in animals from LSP and HSP farms reflect functional differences of the microbiota between herds that could affect STEC colonization.

The overall bacterial composition of the hindgut microbiota was similar to prior studies where Firmicutes and Bacteroidetes were the dominant taxa [43,44]. Though farm-specific composition was identified as previously observed in ten dairy farms with different housing, diet, and husbandry [43]. Notably, unlike LSP farms, HSP farms had in common a high-forage diet and did not administer anthelmintic treatment, which could affect the microbiome composition. The effect of diet in the gut microbiota was previously studied in cattle, where different ratios of forage:concentrate impacted changes in the microbiota [45–47]. As previously observed, a grain-based diet was associated with a higher abundance of Proteobacteria and lower abundance of Bacteroidetes [45,47]. Meanwhile, forage dominant diets were associated with a higher abundance of Firmicutes, *Ruminococcaceae* and *Paludibacter*, which have a critical function degrading forage [45,47]. Dietary interventions in beef cattle have been suggested to reduce the prevalence of STEC O157 as a preharvest intervention [48,49]. However, without knowledge of the microbiome and ecological interactions, those studies had conflicting results [48]. Furthermore, the effect of helminths in the cattle's microbiome has not yet been studied.

Anthelmintic treatment in dogs and horses, for instance, was not associated with shifts in the microbiome composition [50,51]. Humans treated with Albendazole, however, had a higher abundance of Clostridiales and a lower abundance of Enterobacteriales [52]. Meanwhile, helminthic infections were associated with a lower abundance of *Lachnospiraceae* in the human gut microbiome [53].

Higher alpha diversity identified among some STEC shedders has been observed in previous studies in both beef [54,55] and dairy cattle [22]. By contrast, some reports have found that STEC carriage in individual cattle was associated with lower alpha diversity [17,20,23,56]. Two of these reports were carried out in beef herds, where correlations between bacterial richness, STEC enumeration, and age (weaning to one year) were compared. While the authors found that older animals had higher microbial diversity and that younger animals (1-6 months) shed a higher number of STEC, the correlation between microbiome diversity and STEC shedding reflects factors related to age and dietary changes [17,20]. Other reports in dairy cattle that identified a lower alpha diversity in the gut microbiome were focused on shedding of STEC O157:H7 (O157), the serotype associated with a higher number of hospitalizations in humans. Stemkamp-Strahm et. al (2017) detected a weak association after removing outliers [23], while Mir et. al (2020) identified lower alpha diversity in STEC carriers only after vaccination and oral challenge with O157. Hence, these findings indicate that O157 carriage did not directly affect the microbiota, but that vaccination for O157 can alter the microbiota diversity [56]. Indeed, the longitudinal analysis of HSP farms examined herein and in our prior study [57], showed similar microbiota shifts as those observed in O157 vaccinated cattle, suggesting that STEC re-infection in cattle can be followed by a lower alpha diversity.

The microbiota composition of STEC-positive samples mostly overlapped with negative samples in the PCoA. Nevertheless, differentially abundant taxa have been documented among STEC shedders and non-shedders [17,19,23,24,55]. Zhao et. al (2013), for instance, found that butyrate-producing species were more abundant in low-STEC shedding cattle and were critical in avoiding RAJ lesions [17], suggesting the role of certain taxa as '*inhibitors'* or '*promoters'* [17]. Contrasting results have been observed among studies, but in general, there is a consensus that STEC shedders have a higher proportion of members from the order Clostridiales, the dominant order found in the bovine gut microbiome [18,22–24,55,56,58,59]. Consistent with other reports, a lower abundance of Proteobacteria were observed in STEC shedders [22,56]. Varying results in differentially abundant taxa among STEC shedders and non-shedders denote a high variability between species and strains within taxa, as well as differences between study approaches and farms.

In this study, the main microbial biomarkers of STEC shedders were *Romboustia* and *Alloprevotella,* implicated in the production of C_{12}-C_{19} fatty acids [60] and succinic acid [61], respectively. Other genera significantly higher in STEC shedders were associated with sugar fermentation and the production of acetic, formic, propionic, and succinic acids [62–64]. By contrast, the main biomarkers of non-shedders were *Kingella*, *Bacteroidales p-251-o5* and *Anaerosporobacter*. In humans, *Kingella* is implicated in invasive infections due to its cytotoxicity [65]. Butyrate-producing bacteria including *Butyrivibrio*, *Oscillibacter*, *Roseburia,* and *Ruminobacter*, were also found to be associated with non-shedders [66,67]. These families have previously been linked to a healthy human gut microbiota and were suggested to play a role in preventing chronic intestinal inflammation [68]. The functional role of these taxa in the bovine

microbiome or in immunomodulation as well as the correlation with pathogen colonization, however, requires further investigation.

Differences in predicted metabolic pathways observed between LSP and HSP farms suggest that distinct functional microbiomes could favor STEC carriage. For instance, metabolic-pathway prediction showed important differences between HSP and LSP farms associated with diet, where HSP farms had higher oxidation, production of short-chain fatty acids, degradation, and fermentation than LSP farms, which had higher biosynthesis of amino-acids and sugar degradation (**Figures 2.5 & 2.6**). Enhanced fermentation and fatty acid production in HSP farms could be influenced by forage dominant diets. Comparatively, the LSP farms had higher amino acid biosynthesis, suggesting differing amino acid availability in diets within the LSP and HSP farms. Higher inositol degradation in LSP farms shows enhanced cleav[39–42]age of phospholipid membranes that generate cell signaling molecules (i.e., inositol phosphate and diacylglycerol) important for microbial-host interactions [69]. Indeed, distinct metabolic profiles were suggested to be influenced by the diet, as a higher grain diet lowered the ruminal pH and altered the abundance of several metabolites including short-chain fatty acids, amino acids, ethanol, endotoxins, and biogenic amines [70]. Increasing amounts of grain in diets are also correlated with increasing concentrations of ethanolamine [70], the main product of enterocyte membranes, which is degraded to ethanol and acetate. Studies have shown that both *Salmonella* spp. and STEC O157 can use ethanolamine as a nitrogen source to outcompete commensal bacteria [71,72]. These studies, however, were carried out *in-vitro* under aerobic conditions, unlike the intestinal environment.

Microbiota diversity and STEC shedding are dynamic over time as different patterns were observed between farms with a low and high STEC prevalence. Longitudinal studies in

cattle found that the stability of the gut microbiota diversity and composition depends on the diet [73]. Once the animals are adapted to a specific diet, the microbial communities are relatively stable [73]. Unlike farms with low STEC prevalence, animals from farms with high STEC prevalence had access to pasture and a diet primarily based on forage. The grazing behavior and differences in forage composition in farms with high STEC prevalence could explain the high variability over time in their microbiota and a higher STEC detection. We identified that cattle from farms with low-STEC prevalence only shed the pathogen once in an 8-12-week period. Meanwhile, most of the cattle from farms with a HSP prevalence shed the bacteria more than once. We also identified STEC super shedders only in the HSP farms (data not shown). A longitudinal study carried in dairy cattle for a 12-month period identified a very low number of STEC super-shedders in farms with a low STEC prevalence (3.5-5%), and those animals only shed the bacteria once a year [74]. Other studies have reported that the within-farm proportion of super-shedders ranges from 3.8% to 25%, highlighting the importance of farm-specific differences on STEC prevalence and shedding levels [75–77].

The bioinformatics pipeline used in this study was designed to improve the quality of the pyrosequencing results. We used SATé-Enabled Phylogenetic Placement (SEPP) trees to more accurately identify the phylogenetic relationships between OTUs by including sequences of known species [33]. SEPP trees are strongly recommended to avoid incorrect results driven by erroneous phylogenetic placements as observed in *de-novo* trees [31]. Using SEPP trees was critical to account for differences in the beta diversity using Weighted UniFrac metrics and to predict metabolic pathways with PICRUSt2. In addition, we used non-linear approaches to identify differentially abundant taxa. Linear discriminant analyses which assume normality, showed similar results than DESeq though fewer taxa were identified as significantly different.

Prior studies looking for associations between the bacterial composition and STEC shedding used different techniques to identify taxa including denaturing gradient gel electrophoresis (DGGE) [17], pyrosequencing [16,20,24,55,78], and Illumina dye sequencing [19,22,23,56,58,59]. Despite differences in the pipeline, commonalities in the microbiota composition and differentially abundant taxa present in STEC shedders were observed across studies. As the sequencing techniques and bioinformatic tools evolve rapidly, high-resolution results will help to better understand more complex relationships within the microbiome. Along with defining the taxonomic composition, it is important to characterize molecular interactions between microorganisms and hosts by identifying KEGG pathways and metabolites that are common among STEC positive cattle.

Nonetheless, this study has several limitations and hence, the data should be interpreted carefully. For instance, we compared animals from cattle farms with different genetic backgrounds, diet, housing, locations, and husbandry practices, which could be confounding factors that also influence microbiota diversity and composition between farms. Furthermore, using pyrosequencing we were able to detect differences in numerically dominant taxa, limiting the identification of low abundant taxa that could also play a key role in defining the composition of the microbiota. For instance, the proportion of *Escherichia* was very low and absent in a large proportion of samples through pyrosequencing analysis. Metabolic pathways predicted from 16S *rRNA* showed significant differences between HSP and LSP farms (**Figure 2.5**) and between STEC shedders and non-shedders (**Figure 2.6**). These predictions, however, are not entirely accurate as they are based on metabolic reconstruction of a few representative species and do not account for genome differences between closely related strains. Despite this limitation, they provide clues that can be used to guide future studies aimed at defining the function of the cattle

gut microbiome in the presence and absence of STEC. Future studies should also use a longitudinal approach and consider the within-farm STEC prevalence to better identify changes in the microbiome among shedders. Understanding the role that anthelmintics play in STEC shedding should also be addressed, while metagenomic and metabolomic data should be evaluated to identify key metabolites, genes, and bacterial species that could inhibit STEC colonization and boost the gut immune response.

CONCLUSIONS

This study suggests that STEC carriage in cattle is favored by highly diverse microbiota profiles, which are associated with forage-dominant diets. In addition, multiple factors affect the abundance of taxa associated with STEC-shedding in dairy farms, including diet, number or lactations, DIM, and warm temperatures. Identifying healthy microbiomes could guide novel husbandry decisions that aim to decrease levels of pathogen shedding.

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APPENDIX

Table A.1. Characteristics of the three dairy farms examined in this study.

Feature	Dairy farm		
	2D	**4D**	**9D**
No. milkings/day	2 times	3 times	2-3 times
DIM (SD)	206.7 (134)	259 (118.5)	195.25 (136.43)
No. lactations			
0 (No. cows)	4	0	0
1 (No. cows)	80	36	34
≥ 2 (No. cows)	141	86	43
Dry (No. cows)	5	9	0
Treatment			
Clinic mastitis	Ceftiofur, Pirlimycin hydrochloride, Penicillin, Ampicillin, Oxytetracycline, Sulfadimethoxine	Penicillin G Procaine, Ceftiofur, Pirlimycin hydrochloride, Amoxicillin	Ceftiofur
Metritis	Oxytetracycline, Penicillin	Ceftiofur	Ceftiofur, Isoflupredone acetate
Dry	Penicillin-Novobiocin, Penicillin-dihydrostreptomycin	Penicillin-dihydrostreptomycin, Orbeseal	None

No.=Number; DIM=Days in milk; SD=Standard deviation

Table A.2. Number of animals sampled longitudinally per farm and number of days from each phase.

Farm	No. Animals	Days (No.)			
		Phase 1	Phase 2	Phase 3	Phase 4
1B	10	0	14	28	35
2D	10	0	10	38	67
4D	14	NA	0	21	42
8B	13	0	13	27	43
9D	12	0	29	43	49

Table A.3. Bacterial families differentially abundant in the bovine-gut microbiota of animals fed with different percentages of forage in diet.

Family	log2 Fold Change	Lfc SE	Stat (Wald)	p-value	p-adj
Ruminococcaceae	0.016471	0.0007213	22.8351089	2.05E-115	5.45E-114
Prevotellaceae	-0.0143815	0.00087637	-16.410246	1.62E-60	2.14E-59
Rikenellaceae	0.01722959	0.00105481	16.3343473	5.62E-60	6.62E-59
Lachnospiraceae	-0.0032235	0.00061375	-5.2521776	1.50E-07	4.83E-07
Bacteroidaceae	0.00520128	0.00117731	4.41791991	9.97E-06	2.78E-05
Muribaculaceae	-0.0407878	0.00118366	-34.459027	3.30E-260	3.50E-258
Christensenellaceae	0.02058984	0.0012825	16.0545191	5.31E-58	5.63E-57
Spirochaetaceae	-0.0285462	0.00210975	-13.53062	1.03E-41	7.81E-41
Acidaminococcaceae	-0.0117091	0.00101771	-11.505348	1.24E-30	6.92E-30
Succinivibrionaceae	-0.0390218	0.00304894	-12.798487	1.67E-37	1.18E-36
Planococcaceae	0.04576269	0.0056949	8.03572909	9.30E-16	3.52E-15
Clostridiaceae 1	-0.0332732	0.00220972	-15.057646	3.08E-51	2.72E-50
Veillonellaceae	-0.009967	0.00273246	-3.6476253	0.00026468	0.00066799
Bacteroidales RF16 group	0.02558717	0.00188224	13.5939779	4.35E-42	3.55E-41
Enterobacteriaceae	-0.0429341	0.00368522	-11.650361	2.28E-31	1.42E-30
Erysipelotrichaceae	-0.0057838	0.00099778	-5.7966039	6.77E-09	2.24E-08
p-2534-18B5 gut group	0.0655837	0.00367406	17.8504911	2.87E-71	5.06E-70
Gastranaerophilales uncultured	0.0086763	0.00191412	4.53278973	5.82E-06	1.67E-05
Fibrobacteraceae	-0.0196056	0.0045473	-4.3114863	1.62E-05	4.30E-05
Neisseriaceae	0.01931658	0.00612223	3.1551537	0.00160414	0.00377863
Tannerellaceae	-0.052279	0.0020561	-25.426363	1.29E-142	4.56E-141
Bacteroidales uncultured	0.03093304	0.00202808	15.2523773	1.59E-52	1.53E-51
Family XIII	0.01915138	0.00110293	17.3641336	1.54E-67	2.34E-66
p-251-o5	0.00772406	0.00306325	2.52152497	0.01168474	0.0269257
F082	0.07207967	0.00266119	27.0855519	1.46E-161	7.72E-160
Anaeroplasmataceae	-0.0182558	0.00241217	-7.568193	3.78E-14	1.38E-13
Barnesiellaceae	0.01620163	0.00214236	7.56250378	3.95E-14	1.40E-13
Burkholderiaceae	-0.0218915	0.0019579	-11.181093	5.05E-29	2.67E-28
Clostridiales vadinBB60 group	0.00752756	0.00174331	4.31797553	1.57E-05	4.28E-05
Peptococcaceae	0.01093276	0.00126473	8.64431919	5.41E-18	2.29E-17
Rhodospirillales uncultured	0.01311455	0.00191066	6.86389013	6.70E-12	2.29E-11
Paludibacteraceae	0.04345508	0.00236365	18.3847304	1.74E-75	3.69E-74
EMP G18 uncultured	-0.0361119	0.00345028	-10.466371	1.23E-25	5.68E-25
Akkermansiaceae	0.02514747	0.0029462	8.53556341	1.39E-17	5.69E-17
Bifidobacteriaceae	-0.0232566	0.00603712	-3.8522622	0.00011703	0.00030257
Izimaplasmatales uncultured	0.01951321	0.00220312	8.85709434	8.21E-19	3.63E-18
Bacteroidales UCG-001	0.04805996	0.00431028	11.150077	7.15E-29	3.61E-28
Victivallaceae	0.04549642	0.00394805	11.523781	1.00E-30	5.90E-30
WCHB1 41 uncultured	0.02284092	0.00464949	4.91256231	8.99E-07	2.80E-06
Eggerthellaceae	0.00785087	0.00387652	2.02523987	0.04284274	0.09461105
Desulfovibrionaceae	0.02477596	0.00300461	8.24599331	1.64E-16	6.43E-16
Campylobacteraceae	0.02246802	0.00664923	3.37904159	0.00072739	0.0017931
Marinifilaceae	0.03259325	0.00274105	11.8908113	1.32E-32	8.75E-32
Dysgonomonadaceae	0.0274436	0.00589212	4.65768126	3.20E-06	9.42E-06
Defluviitaleaceae	0.0236732	0.00218957	10.8118247	3.03E-27	1.46E-26
Eubacteriaceae	0.02711129	0.00552608	4.90606036	9.29E-07	2.81E-06
gir aah93h0	0.01833041	0.00576371	3.18031613	0.00147115	0.00354412
Pirellulaceae	0.01399368	0.00662901	2.1109765	0.03477433	0.07842722

* Coefficients and significance values were calculated with DESeq2.

Table A.4. Temperature (°C) and relative humidity (RH) the cattle farms during four-sampling phases.

	Farms									
	1B		**2D**		**4D**		**8B**		**9D**	
Phase	**°C**	**RH**	**°C**	**RH**	**°C**	**RH**	**°C**	**RH**	**°C**	**RH**
1	13.9	56.7	19.2	66.2	-	-	22.8	71.1	30.1	46.3
2	19.7	93.7	24.8	59.8	23.6	62.1	24.2	46.9	24.9	35.3
3	23.3	54.2	25.8	84.6	19.3	69.2	24.2	50.7	18.0	53.0
4	16.6	66.2	19.7	68.8	13.7	82.9	22.1	68.5	18.0	56.3
Mean	18.4	67.7	22.4	69.8	18.9	71.4	23.3	59.3	22.8	47.7
St. dev.	4.1	18.1	3.4	10.5	5.0	10.6	1.1	12.3	5.9	9.3

Table A.5. Differentially abundant taxa identified between STEC shedders and non-shedders among dairy farms.

Taxa	log2 Fold Change	Lfc SE	Stat (Wald)	p-value	p-adj
Acidaminococcaceae	-0.565	0.136	-4.144	3.42E-05	6.27E-04
Actinobacteria	-0.126	0.346	-0.365	7.15E-01	9.74E-01
Alloprevotella	0.704	0.192	3.670	2.43E-04	3.05E-03
Anaerosporobacter	-1.363	0.340	-4.011	6.04E-05	1.33E-03
Anaerovorax	-0.598	0.252	-2.369	1.78E-02	8.26E-02
Bacilli	2.008	0.469	4.282	1.86E-05	5.38E-04
Bacteroidales RF16 group	-0.382	0.161	-2.374	1.76E-02	9.64E-02
Bacteroidales RF16 group uncultured	-0.368	0.159	-2.312	2.08E-02	8.30E-02
Bacteroidetes	0.086	0.068	1.265	2.06E-01	8.74E-01
Betaproteobacteriales	-1.287	0.340	-3.788	1.52E-04	8.20E-03
Butyrivibrio	-0.706	0.290	-2.437	1.48E-02	7.24E-02
Chloroflexi	-0.138	4.294	-0.032	9.74E-01	9.74E-01
Clostridia	0.215	0.080	2.700	6.93E-03	6.70E-02
Clostridiales vadinBB60 group	-0.770	0.229	-3.366	7.64E-04	6.00E-03
Clostridiales vadinBB60 group uncultured	-0.779	0.225	-3.468	5.24E-04	5.50E-03
Cyanobacteria	-0.250	0.253	-0.987	3.24E-01	9.25E-01
Dependentiae	-0.148	4.294	-0.034	9.73E-01	9.74E-01
dgA 11 gut group	-0.666	0.230	-2.891	3.84E-03	2.39E-02
Elusimicrobia	-0.583	0.943	-0.618	5.37E-01	9.74E-01
EMP G18	-1.015	0.324	-3.137	1.71E-03	3.08E-02
Epsilonbacteraeota	0.387	0.650	0.596	5.51E-01	9.74E-01
Erysipelotrichaceae UCG 004	-0.768	0.236	-3.250	1.15E-03	9.23E-03
Euryarchaeota	-0.215	1.175	-0.183	8.55E-01	9.74E-01
F082	-0.777	0.264	-2.947	3.20E-03	2.20E-02
F082 uncultured	-0.741	0.258	-2.872	4.08E-03	2.39E-02
Family XIII AD3011 group	0.293	0.138	2.124	3.36E-02	9.87E-02
Fibrobacteres	-0.601	0.537	-1.118	2.63E-01	8.78E-01
Firmicutes	0.270	0.085	3.178	1.48E-03	1.48E-02
Flavobacteriaceae	-0.778	0.338	-2.300	2.15E-02	9.84E-02
Fusobacteria	-0.140	3.951	-0.036	9.72E-01	9.74E-01
Gammaproteobacteria	-0.970	0.298	-3.255	1.13E-03	1.64E-02
Gastranaerophilales uncultured	-0.561	0.262	-2.144	3.20E-02	9.87E-02
Izimaplasmatales	-0.872	0.265	-3.287	1.01E-03	2.73E-02
Izimaplasmatales uncultured	-1.034	0.279	-3.699	2.16E-04	2.97E-03
Kingella	-1.985	0.599	-3.315	9.15E-04	8.05E-03
Kiritimatiellaeota	-0.352	0.544	-0.648	5.17E-01	9.74E-01
Lachnospiraceae uncultured	-0.255	0.117	-2.171	3.00E-02	9.87E-02
Lentisphaerae	-0.560	0.455	-1.231	2.18E-01	8.74E-01
Neisseriaceae	-2.102	0.606	-3.468	5.24E-04	5.33E-03
Oscillibacter	-0.586	0.170	-3.449	5.63E-04	5.50E-03
p 251 o5	-1.479	0.332	-4.453	8.45E-06	4.65E-04
p 251 o5 uncultured	-1.473	0.327	-4.501	6.76E-06	5.95E-04
Patescibacteria	-0.333	0.847	-0.393	6.94E-01	9.74E-01
Peptostreptococcaceae	1.096	0.259	4.225	2.38E-05	6.27E-04
Phascolarctobacterium	-0.551	0.135	-4.072	4.66E-05	1.33E-03
Planctomycetes	0.072	0.694	0.103	9.18E-01	9.74E-01
Proteobacteria	-0.772	0.209	-3.696	2.19E-04	4.38E-03
Rhodospirillales uncultured	-0.599	0.254	-2.362	1.82E-02	9.64E-02
Rikenellaceae	-0.232	0.099	-2.340	1.93E-02	9.64E-02
Rikenellaceae RC9 gut group	-0.307	0.100	-3.063	2.19E-03	1.49E-02
Romboutsia	1.039	0.268	3.882	1.04E-04	1.83E-03
Roseburia	-0.971	0.226	-4.302	1.69E-05	7.45E-04
Ruminobacter	-1.070	0.434	-2.467	1.36E-02	7.05E-02
Ruminococcaceae UCG 005	0.220	0.087	2.520	1.17E-02	6.46E-02

Table A.5 (cont'd)

Taxa	log2 Fold Change	Lfc SE	Stat (Wald)	p-value	p-adj
Ruminococcaceae UCG 010	-0.366	0.172	-2.131	3.31E-02	9.87E-02
Ruminococcaceae UCG 011	0.761	0.244	3.116	1.83E-03	1.34E-02
Ruminococcus 1	0.308	0.142	2.172	2.98E-02	9.87E-02
Ruminococcus 2	-0.521	0.240	-2.174	2.97E-02	9.87E-02
Spirochaetes	0.202	0.235	0.858	3.91E-01	9.74E-01
Synergistetes	-0.162	4.294	-0.038	9.70E-01	9.74E-01
Tenericutes	0.011	0.108	0.106	9.16E-01	9.74E-01
Tyzzerella 4	0.404	0.174	2.319	2.04E-02	8.30E-02
Veillonellaceae	1.191	0.346	3.440	5.82E-04	5.33E-03
Verrucomicrobia	0.691	0.314	2.203	2.76E-02	1.84E-01
Eubacterium coprostanoligenes group	0.227	0.098	2.323	2.02E-02	8.30E-02
Eubacterium ruminantium group	0.578	0.262	2.208	2.72E-02	9.87E-02

Table A.6. Taxa correlated with factors associated with STEC carriage.

Risk factor	Taxa	log2 Fold Change	Lfc SE	Stat (Wald)	p-value	p-adj
DIM	*Acetitomaculum*	0.003	0.001	2.447	1.44E-02	9.94E-02
DIM	*Actinobacteria*	0.004	0.001	3.432	5.99E-04	5.99E-03
DIM	*Anaerosporobacter*	0.003	0.001	3.159	1.58E-03	4.15E-02
DIM	*Anaerovibrio*	-0.005	0.001	-4.567	4.94E-06	6.47E-04
DIM	*Cellulosilyticum*	-0.003	0.001	-2.631	8.52E-03	7.44E-02
DIM	*Coprococcus 3*	-0.001	0.001	-2.546	1.09E-02	8.34E-02
DIM	*Enterobacteriaceae*	-0.005	0.002	-3.417	6.34E-04	3.04E-02
DIM	*Escherichia Shigella*	-0.006	0.002	-3.382	7.19E-04	2.36E-02
DIM	*hoa5 07d05 gut group*	0.009	0.002	3.844	1.21E-04	6.85E-03
DIM	*Kingella*	0.004	0.002	2.528	1.15E-02	8.34E-02
DIM	*Mollicutes RF39 uncultured*	-0.001	0.000	-2.792	5.23E-03	6.86E-02
DIM	*Prevotellaceae UCG 003*	-0.001	0.000	-3.780	1.57E-04	6.85E-03
DIM	*Ruminiclostridium*	0.002	0.001	2.667	7.65E-03	7.44E-02
DIM	*Ruminiclostridium 1*	0.002	0.001	2.738	6.19E-03	7.37E-02
DIM	*Ruminiclostridium 6*	-0.002	0.001	-2.540	1.11E-02	8.34E-02
DIM	*Ruminococcaceae*	0.001	0.000	3.785	1.54E-04	1.48E-02
DIM	*Ruminococcaceae UCG 005*	0.001	0.000	2.850	4.37E-03	6.36E-02
DIM	*Ruminococcaceae UCG 009*	0.001	0.000	2.679	7.39E-03	7.44E-02
DIM	*Ruminococcaceae uncultured*	0.001	0.000	2.885	3.92E-03	6.36E-02
DIM	*Tenericutes*	-0.001	0.000	-3.494	4.75E-04	5.99E-03
DIM	*Turicibacter*	-0.003	0.001	-3.039	2.37E-03	5.18E-02
DIM	*Veillonellaceae*	-0.003	0.001	-3.125	1.78E-03	5.70E-02
DIM	*Veillonellaceae uncultured*	-0.003	0.001	-2.653	7.99E-03	7.44E-02
DIM	*Eubacterium ruminantium group*	-0.002	0.001	-2.864	4.18E-03	6.36E-02
Lactations	*Kingella*	0.610	0.167	3.644	2.69E-04	4.06E-02
Lactations	*Lentisphaerae*	-0.392	0.125	-3.135	1.72E-03	3.43E-02
Lactations	*Neisseriaceae*	0.631	0.169	3.738	1.86E-04	1.87E-02
Lactations	*Ruminococcaceae UCG 004*	-0.226	0.066	-3.409	6.52E-04	5.04E-02
Lactations	*Ruminococcaceae UCG 011*	-0.247	0.076	-3.267	1.09E-03	6.56E-02
Lactations	*Victivallaceae uncultured*	-0.530	0.143	-3.706	2.11E-04	4.06E-02
Lactations	*Eubacterium oxidoreducens group*	-0.177	0.052	-3.403	6.67E-04	5.04E-02
Temp. ave. 5 days	*Acetitomaculum*	0.060	0.026	2.342	1.92E-02	3.54E-02
Temp. ave. 5 days	*Acidaminococcaceae*	-0.073	0.007	-10.709	9.28E-27	7.66E-26
Temp. ave. 5 days	*Agathobacter*	-0.058	0.016	-3.659	2.53E-04	5.68E-04
Temp. ave. 5 days	*Akkermansia*	0.155	0.021	7.309	2.68E-13	1.11E-12
Temp. ave. 5 days	*Akkermansiaceae*	0.124	0.020	6.274	3.52E-10	1.01E-09
Temp. ave. 5 days	*Alistipes*	0.190	0.013	15.161	6.45E-52	2.87E-50
Temp. ave. 5 days	*Alloprevotella*	-0.082	0.011	-7.130	1.00E-12	4.03E-12
Temp. ave. 5 days	*Anaerofustis*	0.173	0.040	4.290	1.79E-05	4.27E-05
Temp. ave. 5 days	*Anaeroplasma*	-0.046	0.016	-2.781	5.42E-03	1.12E-02
Temp. ave. 5 days	*Anaeroplasmataceae*	-0.085	0.016	-5.210	1.89E-07	4.16E-07
Temp. ave. 5 days	*Anaerosporobacter*	-0.072	0.016	-4.544	5.51E-06	1.39E-05
Temp. ave. 5 days	*Anaerostipes*	-0.089	0.037	-2.370	1.78E-02	3.33E-02
Temp. ave. 5 days	*Anaerovorax*	0.084	0.015	5.414	6.17E-08	1.81E-07
Temp. ave. 5 days	*Angelakisella*	0.060	0.025	2.466	1.36E-02	2.63E-02
Temp. ave. 5 days	*Bacteroidaceae*	0.041	0.008	5.264	1.41E-07	3.21E-07
Temp. ave. 5 days	*Bacteroidales RF16 group*	0.108	0.013	8.197	2.47E-16	1.02E-15
Temp. ave. 5 days	*Bacteroidales RF16 group uncultured*	0.136	0.015	9.296	1.45E-20	9.29E-20
Temp. ave. 5 days	*Bacteroidales UCG 001*	0.263	0.033	7.860	3.85E-15	1.49E-14
Temp. ave. 5 days	*Bacteroidales UCG 001 uncultured*	0.327	0.036	9.182	4.23E-20	2.51E-19
Temp. ave. 5 days	*Bacteroidales uncultured*	0.137	0.015	9.228	2.76E-20	1.30E-19
Temp. ave. 5 days	*Bacteroidales uncultured uncultured*	0.177	0.017	10.592	3.26E-26	2.70E-25
Temp. ave. 5 days	*Bacteroides*	0.081	0.009	8.750	2.13E-18	1.10E-17
Temp. ave. 5 days	*Bacteroidetes*	-0.027	0.003	-10.258	1.09E-24	1.09E-23
Temp. ave. 5 days	*Barnesiellaceae*	0.088	0.014	6.140	8.26E-10	2.18E-09
Temp. ave. 5 days	*Barnesiellaceae uncultured*	0.126	0.016	7.981	1.45E-15	7.07E-15
Temp. ave. 5 days	*Blautia*	-0.054	0.012	-4.320	1.56E-05	3.77E-05
Temp. ave. 5 days	*Breznakia*	0.057	0.022	2.573	1.01E-02	2.01E-02
Temp. ave. 5 days	*Burkholderiaceae*	-0.124	0.013	-9.305	1.34E-20	6.82E-20
Temp. ave. 5 days	*Butyrivibrio*	-0.097	0.015	-6.493	8.42E-11	3.08E-10
Temp. ave. 5 days	*Campylobacter*	0.125	0.045	2.765	5.68E-03	1.16E-02
Temp. ave. 5 days	*Campylobacteraceae*	0.083	0.044	1.878	6.04E-02	9.73E-02

Table A.6 (cont'd)

Risk factor	Taxa	log2 Fold Change	Lfc SE	Stat (Wald)	p-value	p-adj
Temp. ave. 5 days	*Candidatus Soleaferrea*	0.106	0.012	8.614	7.08E-18	3.60E-17
Temp. ave. 5 days	*Candidatus Stoquefichus*	0.153	0.029	5.343	9.14E-08	2.65E-07
Temp. ave. 5 days	*Caproiciproducens*	0.153	0.021	7.251	4.14E-13	1.69E-12
Temp. ave. 5 days	*Christensenellaceae*	0.092	0.009	10.064	8.00E-24	4.40E-23
Temp. ave. 5 days	*Christensenellaceae R 7 group*	0.134	0.011	12.767	2.51E-37	4.80E-36
Temp. ave. 5 days	*Christensenellaceae uncultured*	0.161	0.021	7.670	1.72E-14	7.40E-14
Temp. ave. 5 days	*Clostridiaceae 1*	-0.098	0.016	-6.157	7.40E-10	2.03E-09
Temp. ave. 5 days	*Clostridiales vadinBB60 group uncultured*	0.052	0.014	3.774	1.61E-04	3.67E-04
Temp. ave. 5 days	*Clostridium sensu stricto 1*	-0.086	0.015	-5.645	1.65E-08	5.08E-08
Temp. ave. 5 days	*Clostridium sensu stricto 6*	0.192	0.068	2.849	4.39E-03	9.11E-03
Temp. ave. 5 days	*Coprobacillus*	0.147	0.025	5.934	2.96E-09	9.46E-09
Temp. ave. 5 days	*Coprococcus 3*	0.077	0.011	6.808	9.88E-12	3.67E-11
Temp. ave. 5 days	*Defluviitaleaceae*	0.108	0.015	7.402	1.34E-13	4.91E-13
Temp. ave. 5 days	*Defluviitaleaceae UCG 011*	0.150	0.016	9.208	3.33E-20	2.02E-19
Temp. ave. 5 days	*Desulfovibrionaceae*	0.079	0.020	3.846	1.20E-04	2.47E-04
Temp. ave. 5 days	*dgA 11 gut group*	0.251	0.020	12.338	5.67E-35	8.83E-34
Temp. ave. 5 days	*Dielma*	0.207	0.018	11.579	5.26E-31	5.95E-30
Temp. ave. 5 days	*Dorea*	0.140	0.015	9.211	3.22E-20	2.01E-19
Temp. ave. 5 days	*Dysgonomonadaceae*	0.085	0.039	2.145	3.20E-02	5.41E-02
Temp. ave. 5 days	*Dysgonomonadaceae uncultured*	0.129	0.041	3.151	1.62E-03	3.40E-03
Temp. ave. 5 days	*Eisenbergiella*	0.113	0.022	5.089	3.61E-07	9.76E-07
Temp. ave. 5 days	*EMP G18 uncultured*	-0.219	0.025	-8.912	5.01E-19	2.21E-18
Temp. ave. 5 days	*EMP G18 uncultured uncultured*	-0.171	0.024	-7.026	2.13E-12	8.42E-12
Temp. ave. 5 days	*Enterobacteriaceae*	-0.278	0.026	-10.904	1.10E-27	1.04E-26
Temp. ave. 5 days	*Enterorhabdus*	0.078	0.032	2.403	1.62E-02	3.06E-02
Temp. ave. 5 days	*Erysipelatoclostridium*	0.134	0.019	6.993	2.68E-12	1.04E-11
Temp. ave. 5 days	*Erysipelotrichaceae*	-0.039	0.007	-5.951	2.66E-09	6.76E-09
Temp. ave. 5 days	*Erysipelotrichaceae UCG 004*	-0.118	0.012	-9.503	2.03E-21	1.41E-20
Temp. ave. 5 days	*Erysipelotrichaceae uncultured*	0.061	0.012	5.000	5.74E-07	1.54E-06
Temp. ave. 5 days	*Escherichia Shigella*	-0.251	0.026	-9.621	6.52E-22	4.64E-21
Temp. ave. 5 days	*Eubacteriaceae*	0.125	0.039	3.205	1.35E-03	2.62E-03
Temp. ave. 5 days	*F082*	0.279	0.021	13.111	2.83E-39	6.23E-38
Temp. ave. 5 days	*F082 uncultured*	0.307	0.023	13.170	1.30E-39	4.04E-38
Temp. ave. 5 days	*Faecalibacterium*	-0.294	0.024	-12.485	9.01E-36	1.50E-34
Temp. ave. 5 days	*Faecalitalea*	0.089	0.024	3.764	1.67E-04	3.79E-04
Temp. ave. 5 days	*Family XIII*	0.084	0.008	10.242	1.29E-24	7.72E-24
Temp. ave. 5 days	*Family XIII AD3011 group*	0.120	0.010	11.735	8.45E-32	1.00E-30
Temp. ave. 5 days	*Family XIII UCG 001*	0.147	0.019	7.822	5.18E-15	2.39E-14
Temp. ave. 5 days	*Fibrobacter*	-0.065	0.032	-2.025	4.29E-02	7.68E-02
Temp. ave. 5 days	*Fibrobacteraceae*	-0.092	0.031	-3.006	2.65E-03	4.86E-03
Temp. ave. 5 days	*Fibrobacteres*	-0.113	0.030	-3.738	1.85E-04	5.30E-04
Temp. ave. 5 days	*Firmicutes*	0.024	0.003	7.693	1.43E-14	9.56E-14
Temp. ave. 5 days	*Fournierella*	-0.183	0.042	-4.386	1.15E-05	2.82E-05
Temp. ave. 5 days	*Gastranaerophilales uncultured*	0.025	0.013	1.936	5.28E-02	8.72E-02
Temp. ave. 5 days	*Gastranaerophilales uncultured uncultured*	0.065	0.015	4.439	9.03E-06	2.23E-05
Temp. ave. 5 days	*GCA 900066225*	0.057	0.022	2.597	9.40E-03	1.89E-02
Temp. ave. 5 days	*gir aah93h0 uncultured*	0.099	0.039	2.526	1.15E-02	2.26E-02
Temp. ave. 5 days	*GWE2 31 10*	0.152	0.036	4.258	2.06E-05	4.89E-05
Temp. ave. 5 days	*hoa5 07d05 gut group*	-0.319	0.032	-10.065	7.85E-24	5.92E-23
Temp. ave. 5 days	*Hydrogenoanaerobacterium*	0.136	0.029	4.736	2.18E-06	5.53E-06
Temp. ave. 5 days	*Intestinimonas*	0.117	0.050	2.351	1.87E-02	3.48E-02
Temp. ave. 5 days	*Izimaplasmatales uncultured*	0.057	0.015	3.772	1.62E-04	3.24E-04
Temp. ave. 5 days	*Izimaplasmatales uncultured uncultured*	0.091	0.017	5.286	1.25E-07	3.50E-07
Temp. ave. 5 days	*Kingella*	-0.156	0.041	-3.815	1.36E-04	3.14E-04
Temp. ave. 5 days	*Lachnoclostridium*	-0.069	0.014	-4.900	9.58E-07	2.48E-06
Temp. ave. 5 days	*Lachnoclostridium 10*	0.122	0.018	6.886	5.72E-12	2.19E-11
Temp. ave. 5 days	*Lachnospira*	0.120	0.027	4.483	7.35E-06	1.83E-05
Temp. ave. 5 days	*Lachnospiraceae FCS020 group*	0.087	0.011	7.911	2.55E-15	1.22E-14
Temp. ave. 5 days	*Lachnospiraceae NC2004 group*	0.100	0.039	2.546	1.09E-02	2.15E-02
Temp. ave. 5 days	*Lachnospiraceae NK3A20 group*	0.105	0.021	4.966	6.85E-07	1.79E-06
Temp. ave. 5 days	*Lachnospiraceae NK4A136 group*	0.084	0.008	11.208	3.72E-29	3.20E-28

Table A.6 (cont'd)

Risk factor	Taxa	log2 Fold Change	Lfc SE	Stat (Wald)	p-value	p-adj
Temp. ave. 5 days	*Lachnospiraceae UCG 001*	0.041	0.012	3.494	4.75E-04	1.03E-03
Temp. ave. 5 days	*Lachnospiraceae UCG 008*	0.122	0.018	6.866	6.58E-12	2.48E-11
Temp. ave. 5 days	*Lachnospiraceae UCG 010*	0.090	0.025	3.604	3.13E-04	6.84E-04
Temp. ave. 5 days	*Lentisphaerae*	0.118	0.024	4.853	1.22E-06	4.06E-06
Temp. ave. 5 days	*Lysinibacillus*	0.333	0.044	7.569	3.76E-14	1.59E-13
Temp. ave. 5 days	*Mailhella*	0.119	0.022	5.303	1.14E-07	3.22E-07
Temp. ave. 5 days	*Marinifilaceae*	0.133	0.019	7.179	7.03E-13	2.44E-12
Temp. ave. 5 days	*Marvinbryantia*	0.035	0.018	1.913	5.57E-02	9.84E-02
Temp. ave. 5 days	*Mogibacterium*	0.153	0.025	6.026	1.68E-09	5.67E-09
Temp. ave. 5 days	*Mollicutes RF39 uncultured*	0.024	0.009	2.730	6.33E-03	1.13E-02
Temp. ave. 5 days	*Mollicutes RF39 uncultured uncultured*	0.056	0.010	5.630	1.80E-08	5.48E-08
Temp. ave. 5 days	*Muribaculaceae*	-0.186	0.010	-18.328	4.94E-75	3.26E-73
Temp. ave. 5 days	*Muribaculaceae uncultured*	-0.156	0.010	-15.156	6.90E-52	2.87E-50
Temp. ave. 5 days	*Muribaculum*	-0.102	0.042	-2.423	1.54E-02	2.92E-02
Temp. ave. 5 days	*Negativibacillus*	0.143	0.012	11.494	1.41E-30	1.53E-29
Temp. ave. 5 days	*Neisseriaceae*	-0.196	0.041	-4.768	1.86E-06	3.96E-06
Temp. ave. 5 days	*Odoribacter*	0.198	0.021	9.368	7.39E-21	4.84E-20
Temp. ave. 5 days	*Oscillibacter*	-0.073	0.009	-7.775	7.56E-15	3.42E-14
Temp. ave. 5 days	*Oscillospira*	-0.147	0.029	-5.138	2.77E-07	7.58E-07
Temp. ave. 5 days	*p 1088 a5 gut group*	0.102	0.045	2.299	2.15E-02	3.91E-02
Temp. ave. 5 days	*p 251 o5 uncultured*	0.060	0.022	2.735	6.24E-03	1.26E-02
Temp. ave. 5 days	*p 2534 18B5 gut group*	0.324	0.027	12.144	6.15E-34	6.76E-33
Temp. ave. 5 days	*p 2534 18B5 gut group uncultured*	0.348	0.028	12.279	1.17E-34	1.71E-33
Temp. ave. 5 days	*Paeniclostridium*	0.071	0.029	2.498	1.25E-02	2.43E-02
Temp. ave. 5 days	*Paludibacter*	0.133	0.041	3.251	1.15E-03	2.43E-03
Temp. ave. 5 days	*Paludibacteraceae*	0.183	0.017	10.595	3.13E-26	2.29E-25
Temp. ave. 5 days	*Paludibacteraceae uncultured*	0.240	0.019	12.580	2.71E-36	4.81E-35
Temp. ave. 5 days	*Papillibacter*	0.205	0.022	9.454	3.27E-21	2.20E-20
Temp. ave. 5 days	*Parabacteroides*	-0.215	0.016	-13.027	8.61E-39	2.38E-37
Temp. ave. 5 days	*Parasutterella*	0.119	0.019	6.212	5.24E-10	1.79E-09
Temp. ave. 5 days	*Peptococcaceae*	0.046	0.009	5.331	9.77E-08	2.30E-07
Temp. ave. 5 days	*Peptococcaceae uncultured*	0.089	0.010	8.887	6.30E-19	3.48E-18
Temp. ave. 5 days	*Phascolarctobacterium*	-0.039	0.008	-4.863	1.16E-06	2.97E-06
Temp. ave. 5 days	*Planococcaceae*	0.294	0.041	7.109	1.17E-12	3.86E-12
Temp. ave. 5 days	*Prevotella 2*	-0.635	0.037	-17.189	3.23E-66	4.02E-64
Temp. ave. 5 days	*Prevotella 9*	-0.442	0.034	-12.852	8.38E-38	1.90E-36
Temp. ave. 5 days	*Prevotellaceae*	-0.080	0.006	-12.926	3.22E-38	5.32E-37
Temp. ave. 5 days	*Prevotellaceae Ga6A1 group*	0.107	0.028	3.869	1.09E-04	2.54E-04
Temp. ave. 5 days	*Prevotellaceae NK3B31 group*	-0.243	0.041	-5.942	2.81E-09	9.09E-09
Temp. ave. 5 days	*Prevotellaceae UCG 001*	-0.077	0.012	-6.343	2.26E-10	7.93E-10
Temp. ave. 5 days	*Prevotellaceae UCG 003*	0.089	0.010	8.760	1.95E-18	1.03E-17
Temp. ave. 5 days	*Prevotellaceae UCG 004*	0.247	0.017	14.828	9.69E-50	3.45E-48
Temp. ave. 5 days	*Prevotellaceae uncultured*	-0.435	0.034	-12.972	1.77E-38	4.40E-37
Temp. ave. 5 days	*Proteobacteria*	-0.134	0.012	-11.193	4.40E-29	8.79E-28
Temp. ave. 5 days	*Rhodospirillales uncultured*	0.040	0.013	3.071	2.13E-03	4.02E-03
Temp. ave. 5 days	*Rikenellaceae*	0.079	0.008	10.351	4.16E-25	2.74E-24
Temp. ave. 5 days	*Rikenellaceae RC9 gut group*	0.094	0.009	9.998	1.55E-23	1.14E-22
Temp. ave. 5 days	*Romboutsia*	0.033	0.014	2.331	1.98E-02	3.62E-02
Temp. ave. 5 days	*Roseburia*	0.020	0.010	1.971	4.88E-02	8.68E-02
Temp. ave. 5 days	*Ruminiclostridium*	0.220	0.018	12.235	2.02E-34	2.80E-33
Temp. ave. 5 days	*Ruminiclostridium 1*	0.209	0.020	10.580	3.71E-26	2.98E-25
Temp. ave. 5 days	*Ruminiclostridium 5*	0.115	0.015	7.858	3.89E-15	1.83E-14
Temp. ave. 5 days	*Ruminiclostridium 6*	0.069	0.019	3.619	2.95E-04	6.51E-04
Temp. ave. 5 days	*Ruminiclostridium 9*	0.045	0.014	3.276	1.05E-03	2.24E-03
Temp. ave. 5 days	*Ruminobacter*	-0.135	0.023	-5.850	4.91E-09	1.55E-08
Temp. ave. 5 days	*Ruminococcaceae*	0.075	0.006	12.879	5.90E-38	7.79E-37
Temp. ave. 5 days	*Ruminococcaceae NK4A214 group*	0.213	0.012	17.462	2.80E-68	6.96E-66
Temp. ave. 5 days	*Ruminococcaceae UCG 002*	0.125	0.014	9.070	1.19E-19	6.89E-19
Temp. ave. 5 days	*Ruminococcaceae UCG 004*	0.158	0.014	11.283	1.59E-29	1.42E-28
Temp. ave. 5 days	*Ruminococcaceae UCG 005*	0.148	0.009	16.907	3.98E-64	3.30E-62
Temp. ave. 5 days	*Ruminococcaceae UCG 008*	0.104	0.019	5.479	4.27E-08	1.27E-07

Table A.6 (cont'd)

Risk factor	Taxa	log2 Fold Change	Lfc SE	Stat (Wald)	p-value	p-adj
Temp. ave. 5 days	Ruminococcaceae UCG 009	0.151	0.013	11.823	2.97E-32	3.70E-31
Temp. ave. 5 days	Ruminococcaceae UCG 010	0.150	0.013	11.455	2.21E-30	2.20E-29
Temp. ave. 5 days	Ruminococcaceae UCG 011	0.110	0.017	6.343	2.25E-10	7.93E-10
Temp. ave. 5 days	Ruminococcaceae UCG 013	0.061	0.010	5.952	2.64E-09	8.77E-09
Temp. ave. 5 days	Ruminococcaceae UCG 014	0.048	0.010	4.969	6.73E-07	1.78E-06
Temp. ave. 5 days	Ruminococcaceae uncultured	0.164	0.010	16.411	1.60E-60	9.96E-59
Temp. ave. 5 days	Ruminococcus 1	0.093	0.008	11.305	1.24E-29	1.14E-28
Temp. ave. 5 days	Ruminococcus 2	0.112	0.015	7.675	1.65E-14	7.23E-14
Temp. ave. 5 days	Saccharofermentans	0.171	0.032	5.304	1.14E-07	3.22E-07
Temp. ave. 5 days	Solibacillus	0.470	0.074	6.321	2.60E-10	9.01E-10
Temp. ave. 5 days	Sphaerochaeta	0.143	0.026	5.583	2.36E-08	7.08E-08
Temp. ave. 5 days	Spirochaetaceae	-0.087	0.015	-5.738	9.57E-09	2.34E-08
Temp. ave. 5 days	Spirochaetes	-0.106	0.015	-6.891	5.55E-12	2.78E-11
Temp. ave. 5 days	Subdoligranulum	-0.256	0.022	-11.442	2.58E-30	2.47E-29
Temp. ave. 5 days	Succinivibrio	-0.132	0.025	-5.214	1.84E-07	5.10E-07
Temp. ave. 5 days	*Succinivibrionaceae*	-0.145	0.021	-6.811	9.67E-12	2.90E-11
Temp. ave. 5 days	*Sutterella*	-0.290	0.025	-11.484	1.59E-30	1.65E-29
Temp. ave. 5 days	*Tannerellaceae*	-0.245	0.016	-15.034	4.39E-51	1.45E-49
Temp. ave. 5 days	*Treponema 2*	-0.067	0.016	-4.240	2.23E-05	5.24E-05
Temp. ave. 5 days	*Turicibacter*	-0.151	0.020	-7.720	1.17E-14	5.18E-14
Temp. ave. 5 days	*Tyzzerella 4*	0.057	0.010	5.743	9.31E-09	2.90E-08
Temp. ave. 5 days	*Verrucomicrobia*	0.116	0.019	6.061	1.35E-09	5.42E-09
Temp. ave. 5 days	*Victivallaceae*	0.200	0.028	7.059	1.67E-12	5.26E-12
Temp. ave. 5 days	*Victivallaceae uncultured*	0.297	0.033	8.977	2.79E-19	1.58E-18
Temp. ave. 5 days	*WCHB1 41 uncultured*	0.076	0.032	2.396	1.66E-02	2.88E-02
Temp. ave. 5 days	*WCHB1 41 uncultured uncultured*	0.123	0.034	3.628	2.86E-04	6.35E-04
Temp. ave. 5 days	*Anaerorhabdus furcosa group*	0.162	0.013	12.808	1.49E-37	3.09E-36
Temp. ave. 5 days	*Eubacterium brachy group*	0.138	0.017	8.144	3.83E-16	1.91E-15
Temp. ave. 5 days	*Eubacterium coprostanoligenes group*	0.088	0.010	8.801	1.36E-18	7.35E-18
Temp. ave. 5 days	*Eubacterium eligens group*	0.170	0.029	5.945	2.77E-09	9.07E-09
Temp. ave. 5 days	*Eubacterium nodatum group*	0.194	0.016	11.889	1.34E-32	1.76E-31
Temp. ave. 5 days	*Eubacterium oxidoreducens group*	0.106	0.010	10.217	1.67E-24	1.30E-23
Temp. ave. 5 days	*Eubacterium ruminantium group*	0.060	0.018	3.370	7.53E-04	1.62E-03
Temp. ave. 5 days	*Eubacterium ventriosum group*	-0.074	0.034	-2.160	3.08E-02	5.55E-02
Temp. ave. 5 days	*Ruminococcus torques group*	-0.111	0.017	-6.423	1.34E-10	4.82E-10
Temp. ave. 5 days	*X28 4*	0.076	0.031	2.464	1.37E-02	2.63E-02

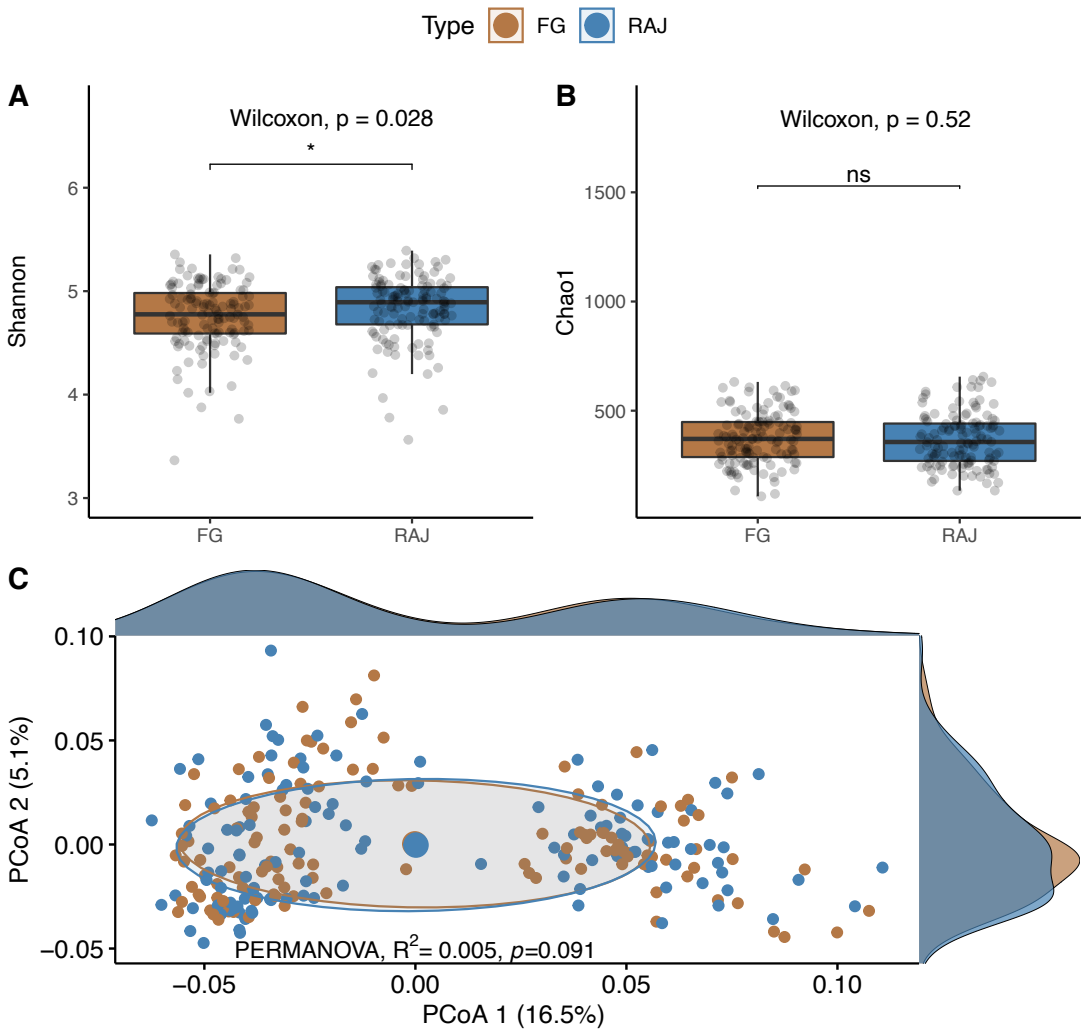

Figure A.1. Paired comparison of fecal grab (FG) and recto-anal junction (RAJ) microbiome alpha and beta diversities. A) Shannon index boxplots. B) Chao1 index boxplots. C) Principal coordinate analysis of Weighted Unifrac ordination. The centroids of FG and RAJ distributions are shown with a bigger point size.

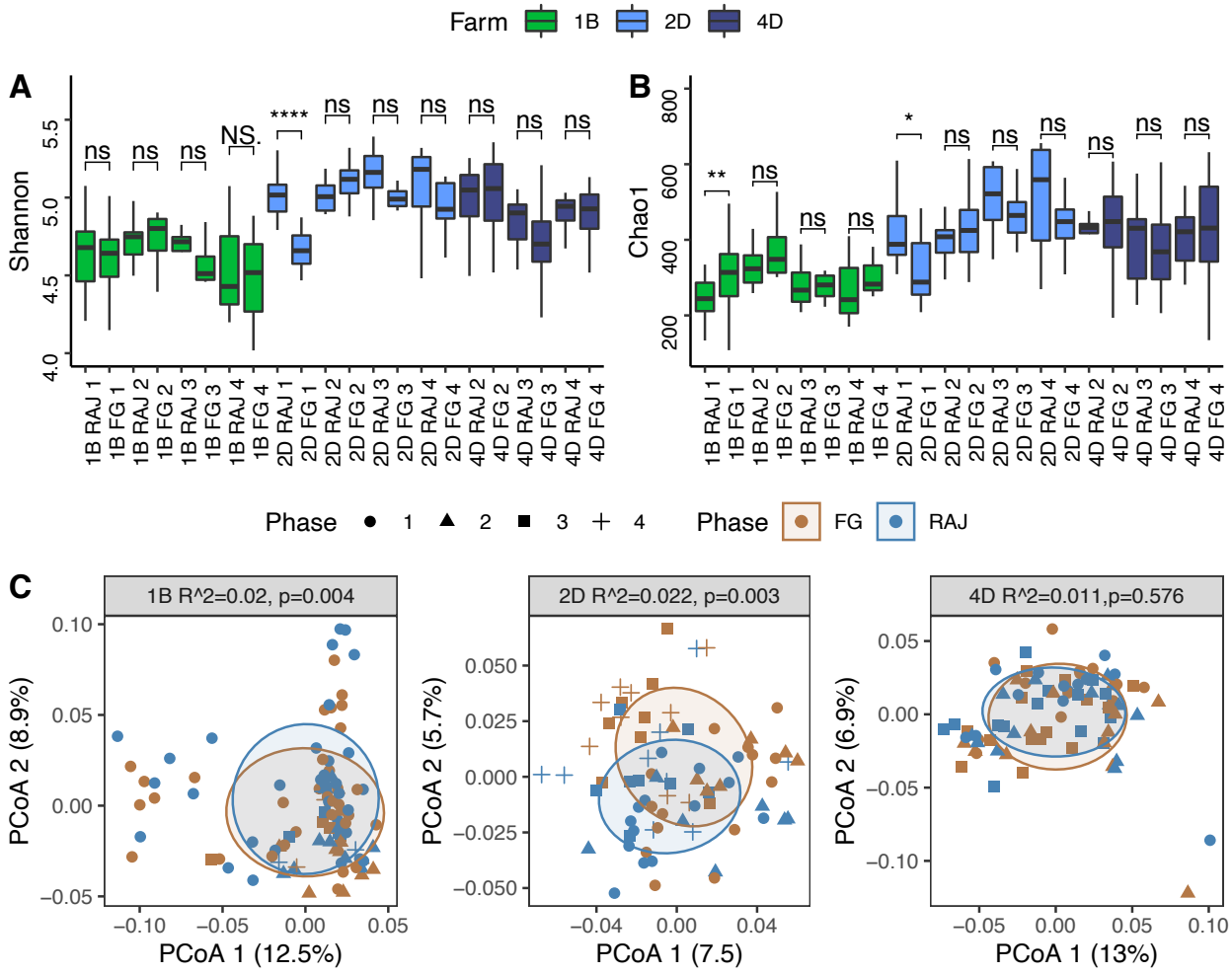

Figure A.2. Paired comparison of fecal grab (FG) and recto-anal junction (RAJ) microbiome alpha and beta diversities per farm and sampling phase. The *p-values* in A and B were calculated with the Wilcoxon test and are represented with symbols "****" ≤ 0.0001, "*" ≤ 0.05, and "*ns*" not significant. A) Shannon index boxplots, B) Chao1 index boxplots. C) PCoA of Weighted Unifrac ordination; each box corresponds to a farm; the R^2 and *p-value* are from the PERMANOVA test between RAJ and FG.

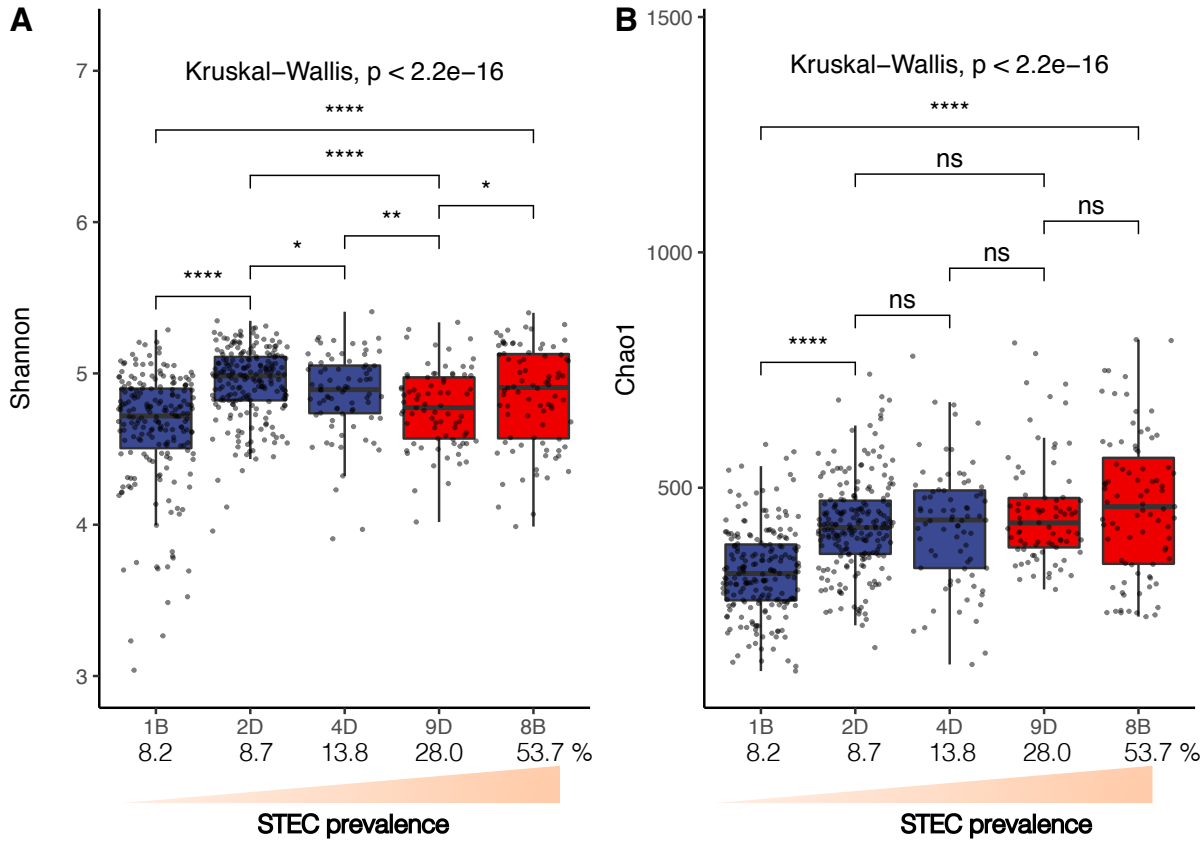

Figure A.3. Cattle-hindgut microbiota alpha diversity among five farms. Farms classified as low STEC prevalence (LSP) are shown in blue, and farms with a high STEC prevalence (HSP) are in red. *p-value*: **** ≤ 0.0001, *** ≤ 0.001, ** ≤ 0.01, ns not significant.

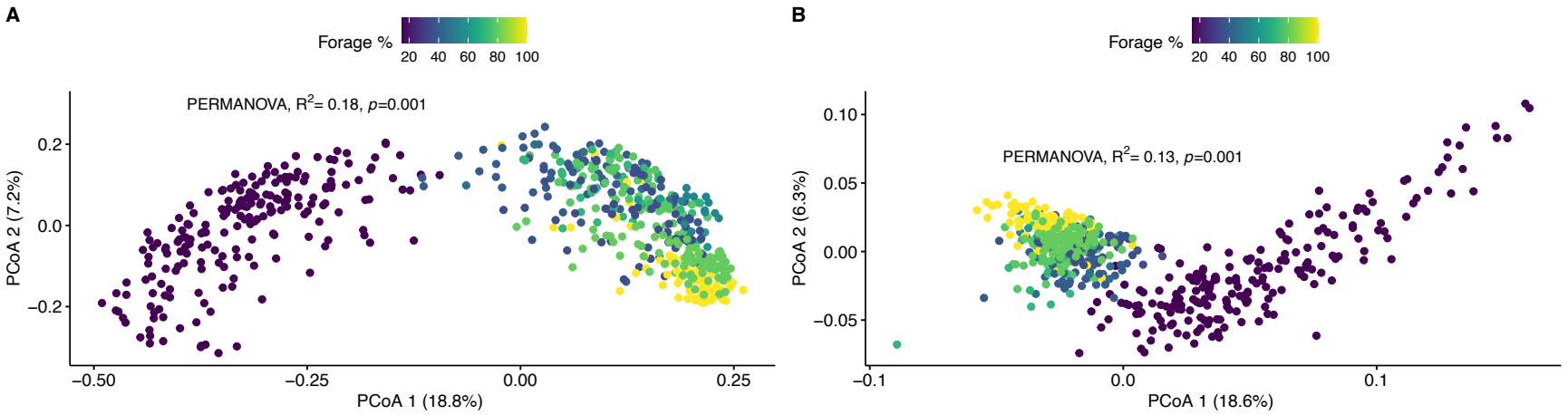

Figure A.4. Principal coordinate analysis showing differences in the microbiome composition associated with the percentage of forage in diet. A) Bray-Curtis dissimilarity distances. B) Weighted Unifrac ordination.

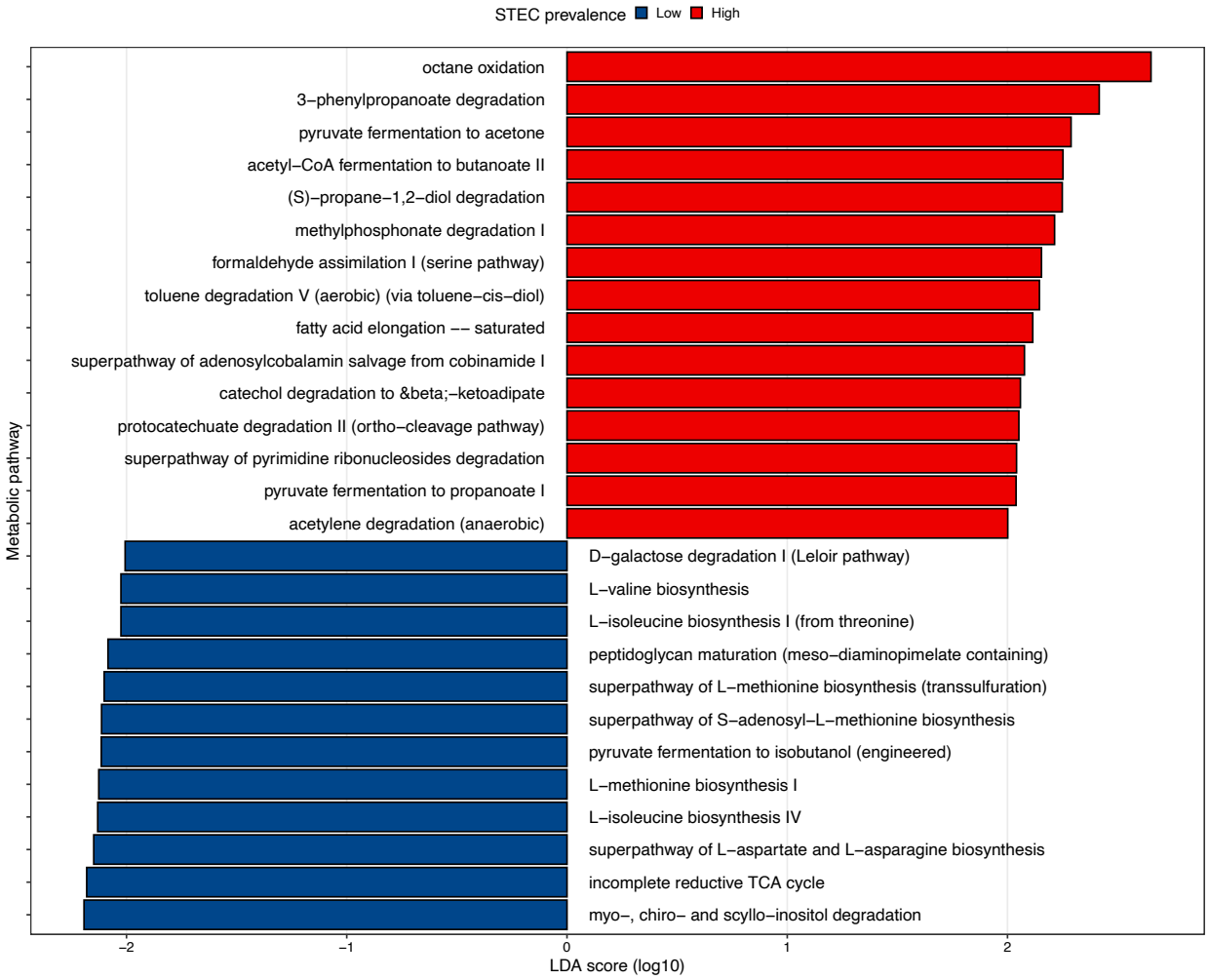

Figure A.5. Linear discriminant effect sizes of metabolic pathways inferred from 16S *rRNA* sequences with PICRUSt2, between farms with low and high STEC prevalence. A total of 487 MetaCyc pathways were predicted from 16S *rRNA* sequences. LDA effect sizes of 5.5% of the pathways were significantly different between farms with a high and low STEC prevalence.

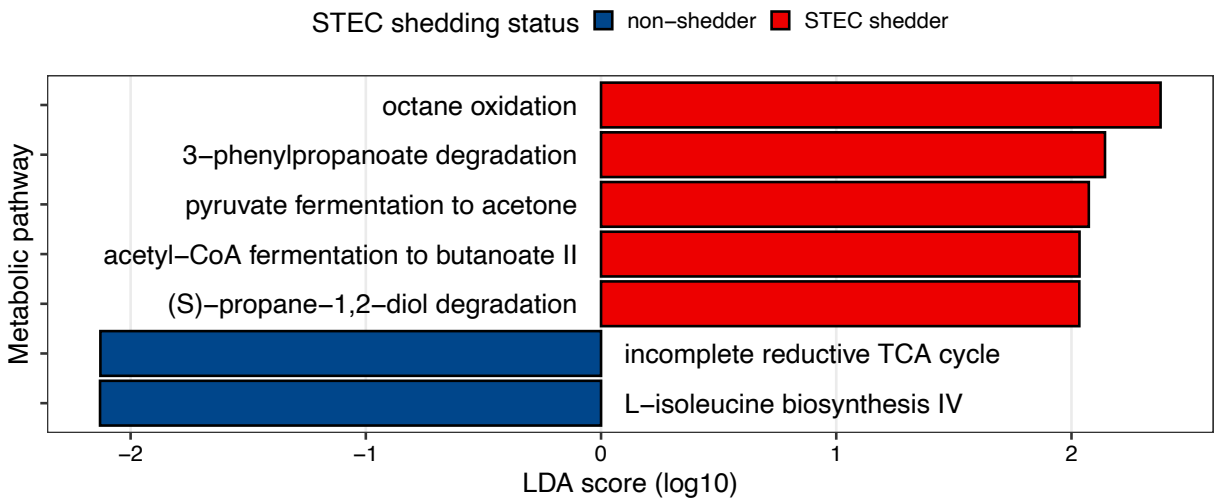

Figure A.6. Linear discriminant effect sizes of metabolic pathways inferred from 16S *rRNA* sequences between STEC shedders and non-shedders.

CHAPTER 3

Perturbations in intestinal antibiotic-resistant bacterial populations in dairy cattle following intramammary treatment with a third-generation cephalosporin.

This chapter is from a manuscript submitted for publication:

Karla Vasco, Samantha Carbonell, Rebekah E. Sloup, Bailey Bowcutt, Rita R. Colwell, Karlis Graubics, Ronald Erskine, Bo Norby, Pamela L. Ruegg, Lixin Zhang, Shannon D. Manning. "Perturbations in intestinal antibiotic-resistant bacterial populations in dairy cattle following intramammary treatment with a third-generation cephalosporin". (2022). Manuscript submitted for publication.

ABSTRACT

Background. Intramammary (IMM) ceftiofur treatment is commonly used in dairy farms to prevent mastitis, though its impact on the cattle gut microbiome and selection of antibiotic-resistant bacteria has not been elucidated. Herein, we enrolled 40 healthy dairy cows after lactation: 20 were treated with IMM ceftiofur (Spectramast®DC) and a non-antibiotic internal teat sealant (bismuth subnitrate) and 20 (controls) received only bismuth subnitrate. Fecal samples were collected before (day -1) and after treatment (weeks 1, 2, 3, 5, 7, and 9) for quantifying resistant bacteria and metagenomic next-generation sequencing.

Results. Overall, no difference was observed in the quantity of resistant bacteria or prevalence of β-lactam resistance between treatment groups, yet 90% and 24% of the 278 samples had Gram-negative bacteria with resistance to ampicillin and ceftiofur, respectively. Although the number of Gram-negative ceftiofur-resistant bacteria increased in treated cows up to 2 weeks after treatment, considerable variation was observed across animals. Indeed, only a subset (25%) of cows shed higher levels of ceftiofur-resistant bacteria post-treatment. Treated cows had lower microbiome richness during weeks 5 and 9 with a decreased abundance of Proteobacteria, Tenericutes, Verrucomicrobia, and Firmicutes and higher abundance of Bacteroidetes and Actinobacteria. A greater abundance of extended-spectrum β-lactamase (ESBL) genes (*bla-CFXA*, *bla-ACI-1*, and *bla-CMY*) and *blc* genes was also observed at weeks 1, 5 and 9 ($LDA > 3.3$; $P < 0.03$), while a network analysis detected correlations between β-lactam resistance genes and phages, mobile genetic elements, and genera 1-week post-treatment. Specific commensal bacteria possessed ESBL genes as did members of Enterobacteriaceae, though in lower proportions.

Conclusion. This study highlights variable, long-term effects of IMM ceftiofur treatment on the gut microbiome and resistome in dairy cattle. Indeed, treated cattle had an increased abundance of specific taxa and genes encoding ESBL production that persisted for up to 9 weeks. Fecal shedding of ESBL-producing Enterobacteriaceae, which are a serious public health threat, varied across animals and hence, additional research should focus on identifying factors associated with shedding levels as well as the dissemination and persistence of antibiotic resistance determinants on dairy farms.

INTRODUCTION

Approximately three quarters of the quantity of antimicrobials produced globally are employed in production of food-producing animals [1]. Thus, effects of these drugs on the emergence of antimicrobial resistant bacteria require analysis. In particular, extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae are the most concerning and economically impactful antimicrobial-resistant threats [2]. Use of third generation cephalosporins to treat humans and for livestock production may contribute to the emergence of ESBL-producing Enterobacteriaceae. Indeed, bacteria carrying ESBL genes have widespread resistance to clinically important antibiotics such as the penicillins and cephalosporins; resistance to these drugs has been linked to an increased risk of hospitalization and death in humans [3].

In the U.S., 81% of the total kilograms of cephalosporins used in livestock are consumed on cattle farms [4]. In addition, ~90% of dairy farms use intramammary (IMM) β-lactam antibiotics during the dry-off period to treat and prevent mastitis, an infection of the mammary gland [5–7]. Ceftiofur, a third-generation cephalosporin, is one of the most common β-lactam antibiotics used intramammarily as a dry-cow therapy. These broad-spectrum drugs have bactericidal activity against both Gram-negative and Gram-positive bacterial populations, low

toxicity potential, and excellent penetration of most body fluids. Consequently, β-lactams are used to treat a variety of pathologies in humans such as septicemia, urinary tract infections, respiratory infections, meningitis, and peritonitis. In dairy cattle, ceftiofur is recommended for the IMM treatment of mastitis as well as respiratory disease, metritis, and footrot. Although cephalosporins like ceftiofur are mainly excreted in the urine (61-77%), they have been also found in the biliary system (~30%) [8], ileum and colon (20% of plasmatic concentration) [9]. However, the effects of IMM ceftiofur treatment on the fecal microbiome and resistome have not yet been determined.

A prior study using mathematical modeling predicted that parenteral ceftiofur therapy would reduce the total concentration of *E. coli* in cattle, but would lead to an increase in the fraction of ESBL-resistant *E. coli* [10]. However, there is no significant evidence that ceftiofur treatment is associated with an increase in the emergence of ESBL-producing bacterial populations [9,11,12]. One study of cows in early lactation demonstrated that systemic ceftiofur temporarily increased the abundance of resistant Enterobacteriaceae for 7-8 days but not over the long-term (29-35 days) [13]. Similarly, in feedlot cattle, the combined treatment of chlortetracycline and ceftiofur was shown to contribute to an increase in the number of resistant *E. coli* and ESBL genes [14], suggesting co-selection of these antibiotic resistance genes (ARGs). Because of these prior associations, we sought to determine how IMM ceftiofur treatment at dry off impacts the cattle gut microbiome and abundance of antibiotic resistant bacterial populations over a 9-week period through the dry period and early part of the subsequent lactation.

MATERIALS AND METHODS

Animals and treatments

The aim of this study was to assess the long-term effects of IMM ceftiofur hydrochloride (CHCL) treatment on the gut microbiome of dairy cows at dry-off, the last milking before the dry period. This study was conducted in 2019 (June-November) at the Dairy Cattle Teaching and Research Center at Michigan State University, which contained ~230 lactating dairy cows. Forty healthy Holstein cows were enrolled at dry-off if they met the following inclusion criteria: no antibiotic treatment during the last 90 days of lactation, and a somatic cell count (SCC) of <150,000 cells/mL using the most recent Dairy Herd Improvement Association (DHIA) test (**Figure 3.1**). Cows were matched based on parity and monthly milk production, while data about health status, ambient temperature, and diet were recorded. The treated group (n = 20) received 4 IMM infusions (1 per mammary gland) that each contained 500 mg ceftiofur (SpectramastDC®; Zoetis Animal Health) after the last milking and an internal IMM teat sealant containing bismuth subnitrate (Orbeseal®; Zoetis Animal Health). The second (control) group received only the internal IMM teat sealant. Cows were randomly assigned to treatment and researchers were blinded to treatment during sampling collection and laboratory analyses. Fecal grab samples were collected using clean obstetric sleeves one day before treatment (day -1) and in weeks 1, 2, 3, 5, 7, and 9 post-treatment. Each sample was homogenized by hand massage in a whirl-pak bag and immediately aliquoted for bacterial culture and DNA extraction for metagenomic next-generation sequencing (mNGS).

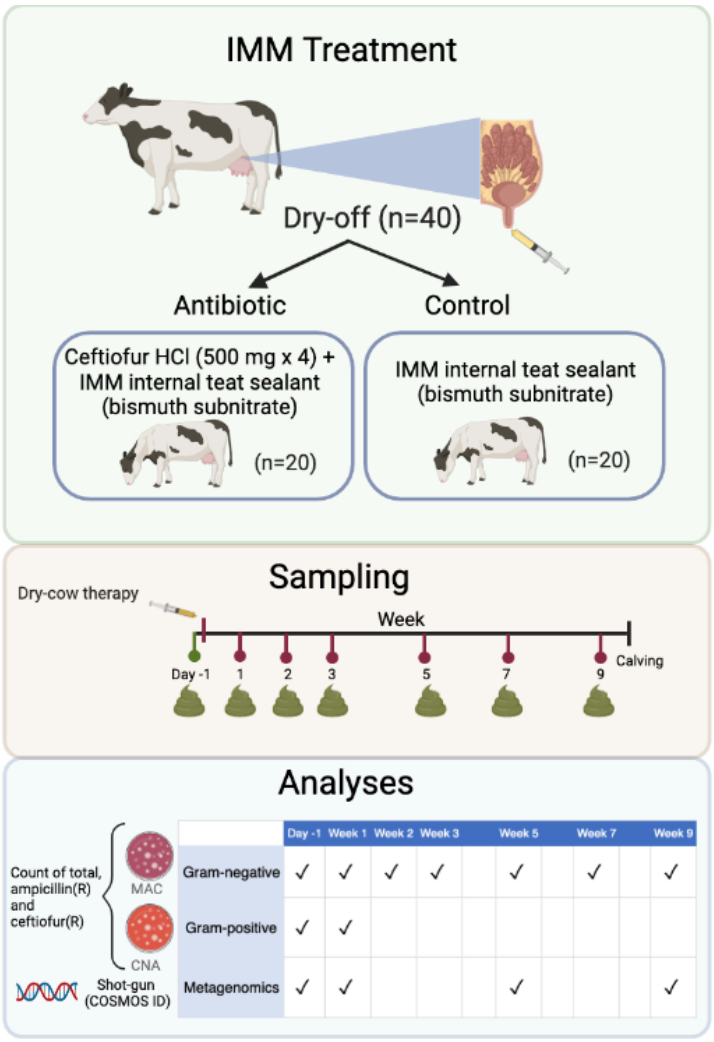

Figure 3.1. Methods used to evaluate long-term changes in the gut microbiome and resistome following dry-cow therapy with ceftiofur.

Quantification of antibiotic-resistant bacteria

The total bacterial counts were quantified and presented as colony-forming units (CFUs) per gram (g) of feces. The percentage of ceftiofur- and ampicillin-resistance were also quantified for Gram-positive (samples collected on day -1 and week 1) and Gram-negative (day-1 through week 9) bacterial populations. Fecal samples were diluted at a concentration of 10^{-1} using 1X PBS and plated in duplicate on media with and without antibiotics (ampicillin or ceftiofur) using a spiral autoplater (Neutec Group Inc.). Gram-negative bacteria were quantified on MacConkey

lactose agar (MAC; Criterion®), while Columbia Nalidixic Acid agar (CNA; BD Difco ®) with 5% sheep blood was used for Gram-positive bacteria. Amphotericin B (4 µg/ml) was added to the media to inhibit fungal growth. The ceftiofur concentration was 8 µg/ml for both Gram-negative and -positive bacteria [15], whereas 32 µg/ml and 25 µg/ml of ampicillin were used for Gram-negative and Gram-positive bacteria, respectively, per the Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. The plates were incubated at 37°C for 24 hours under aerobic conditions (MAC) or in the presence of 5% carbon dioxide (CNA). The volumes of 10^{-1} dilution of fresh feces plated to quantify Gram-negative bacteria were 40 µl (C mode in spiral plater) for total count, 100 µl (C mode) for ampicillin resistant, and 400 µl (linear mode) for ceftiofur resistant CFUs. Whereas, total Gram-positive were quantified from frozen stocks preserved in a 1:1 dilution with glycerol. The 10^{-1} dilution of frozen feces consisted of 2 g of the preserved stock with 8 ml of 1X PBS, which was plated in the following volumes: 40 µl (C mode) for total count, 200 µl (linear mode) for ampicillin resistant, and 100 µl (C mode) for ceftiofur resistant CFUs. The volumes used for quantification of Gram-negative and Gram-positive bacteria were determined after comparing different modes with samples from this farm that enable identifying isolated CFUs and enhancing the limits of detection for resistant bacteria.

Additionally, media controls were plated to test each batch of MAC for the ability to inhibit Gram-positives with *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212. The antibiotic concentration on MAC necessary to inhibit susceptible bacteria and to enable the growth of resistant strains was tested with the following control strains: *E. coli* ATCC 25922 (Amp^S, Cef^S), *E. coli* ATCC 35218 (Amp^S, Cef^S), *E. coli* strain TW18741 (Amp^R, Cef^S), three ESBL *E. coli* strains (Amp^R, Cef^R) obtained from clinical samples in a prior study [17]. CNA media controls included ATCC 29212 (Amp^S, Cef^R), ATCC 29213 (Amp^S, Cef^S),

Listeria monocytogenes ATCC 3382 (Amp^S, Cef^R), *Listeria monocytogenes* ATCC 19115 (Amp^S, Cef^R), *Streptococcus pneumoniae* ATCC 49619 (Amp^S, Cef^S), *Streptococcus equi* subsp. *Zooepidemicus* ATCC 700400 (Amp^S, Cef^S), and *Streptococcus agalactiae* strain COH1 (Amp^S, Cef^S). Inhibition of the Gram-negative bacteria was tested with *E. coli* ATCC 25922 and ESBL *E. coli* strains. Paired non-parametric tests, Wilcoxon and Friedman, were used to compare the number of CFU/g and proportion of resistant bacteria between treatment groups and time points.

Metagenomic sequencing

Fecal DNA of samples collected on day -1 and weeks 1, 5, and 9, were extracted with the DNeasy PowerSoil Pro Kit (Qiagen, Germantown, MD, USA). Genomic DNA (102.18 ± 24.84 ng/µl) was sent to CosmosID (Rockville, MD, USA) for mNGS. Libraries were prepared with the Nextera™ XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and sequenced on the Illumina HiSeq X platform 2x150 bp. Shotgun metagenomic datasets were analyzed using the CosmosID bioinformatics software package to characterize the microbiome, resistome, and virulome. The CosmosID pipeline includes a reference GeneBook® database that comprises genomes and gene sequences from multiple publicly available databases as described [18]. Reads were assigned to a species or gene with the highest aggregation statistics. Each GeneBook® database was screened and cleaned for bovine (*Bos taurus*) genome sequences.

Metagenome characterization

The CosmosID bioinformatics platform was used to compare bacteria, eukaryotes, fungi, phages, antibiotic resistance genes (ARGs), and virulence genes between the two treatment groups. Relative abundance estimates, abundance scores, total matches percentage, unique matches percentage, and read frequencies were determined. Sample richness was calculated with the Shannon and Chao1 alpha diversity indices, while beta diversity was estimated with the

Bray-Curtis dissimilarity ordination and depicted with principal coordinate analysis (PCoA). Normalized abundance scores were used for statistical analyses and visualizations using the following R packages as described [19]: Phyloseq v.1.34.0 [20], vegan v.2.5-7 [21], and Metacoder v.0.3.5 [22].

The Wilcoxon and Friedman paired non-parametric tests were used to compare alpha diversity estimates between treatment groups and sampling points. Meanwhile, permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was used to compare the median composition between treatment groups and sampling points in the PCoA of the Bray-Curtis dissimilarity ordination. The analysis of differentially abundant taxa was carried out with four different approaches: 1) DESeq2, which uses a negative binomial distribution [23] with three settings (Wald test with a local fit, Wald test with Gamma-Poisson Generalized Linear Model, and likelihood ratio test (LRT) with Gamma-Poisson Generalized Linear Model); 2) Linear Discriminant Analysis (LDA) Effect Size (LEfSe), which identifies the effect relevance of a differential feature based on an algorithm that includes non-parametric tests and LDA [24]; 3) Analysis of compositions of microbiomes with bias correction (ANCOM-BC), which uses linear regression models and corrects for bias induced by sample differences [25]; and 4) Microbiome Multivariable Associations with Linear Models (MaAsLin2) [26] that uses generalized linear and mixed models. A consensus approach was used to ensure robust identification of differentially abundant taxa; only differentially abundant features ($P < 0.05$) identified with two or more pipelines were reported as was suggested in a prior study [27].

ARG host association analyses

Biochemical identification of Gram-negative ceftiofur resistant strains was done with oxidase tests (OxiStrips^{TM}, Hardy Diagnostics) and Chromocult® Coliform agar (Merck KGaA,

Darmstadt, Germany) to test β-glucuronidase and β-galactosidase activity. Additionally, metagenomic contigs over 500 bp containing β-lactam resistance genes were assembled with metaSPADES [28] and identified with DeepARG [29] for those ARGs with at least 70% query coverage and 80% identity. These ARG-carrying contigs (ACCs) were extracted with seqtk and translated to amino acid sequences with Prodigal (PROkaryotic DYnamic programming Gene-finding ALgorithm) [30]. The amino acid sequences were taxonomically classified with BLASTP [31] and with the contig annotation tool (CAT) v.5.2.3 [32].

Network analysis

Correlations between ARGs, mobile genetic elements (MGEs), and bacterial genera were identified by calculating Spearman's correlation coefficients; only coefficients (ρ) greater than 0.75 and p-values <0.01 were included in the networks. Significant correlations were analyzed in R v.4.1.2. with the package Hmisc [33] and visualized with Gephi v.0.9.2 [34]. Network statistics were calculated in Gephi including the degree of centrality, weighted degree of centrality, graph density, modularity, and clustering coefficient. The comparisons of centrality measures among β-lactam ARGs were analyzed between treatment groups and time points using non-parametric statistics.

RESULTS

Phenotypic identification of resistant bacteria

Incidence of β-lactam resistant bacteria. Gram-positive bacteria with resistance to both ampicillin and ceftiofur were recovered from all samples (100%) from both antibiotic treated and control animals. Comparatively, the percentage of samples with Gram-negative bacteria resistant to ampicillin and ceftiofur was 90% and 24%, respectively, and was not associated with the IMM treatment ($P > 0.69$).

Gram-positive bacteria quantifications. Overall, the number of total Gram-positive CFU/g was 7.96E+05 (±9.68E+05) which was similar between treatment groups ($P = 0.127$) (**Figure 3.2B**). Though, one week after IMM treatment, control animals had higher counts of Gram-positive ($P = 0.029$) bacteria relative to the antibiotic group. A greater proportion of Gram-positive bacteria were resistant to ceftiofur (28.16% ±21.82%) than ampicillin (4.81% ± 6.06%) ($P < 2.2e-16$). The proportion of ampicillin resistant Gram-positives was significantly higher in cows treated with ceftiofur ($P = 0.0413$) at week 1 as compared to controls (**Figure B.1**). However, no difference was observed in the number of Gram-positive CFUs/g resistant to ceftiofur and ampicillin between day -1 and week 1 after the treatment with ceftiofur, or between treatment groups (**Figures 3.2D and 3.2F**).

Gram-negative bacteria quantifications. The average total Gram-negative CFU/g was 9.11E+05 (±1.16E+06), which was significantly lower in cows treated with ceftiofur ($P = 0.003$). The lower abundance of Gram-negative CFUs, however, was observed one day before treatment, when fecal samples from control cows had twice the number of Gram-negative bacteria than cows that were going to be treated with ceftiofur (*control* = $8.25x10^5$ vs. *antibiotic* = $4.41x10^5$; $P = 0.027$) (**Figure 3.2A**). Lower total Gram-negative bacteria were also observed one week after the IMM treatment ($P = 0.0148$), but not in further sampling points ($P > 0.05$). For this reason, the FC was calculated by dividing the logarithm with base 10 ($\log_{10}$) of the CFU/g at each time point by the $\log_{10}$ of the CFU/g on the day before the treatment for each animal. Using this approach, no significant differences in the fold-change (FC) were observed between treatment groups for total Gram-negatives ($P = 0.215$).

Regardless of treatment, fecal samples had a greater proportion of Gram-negative CFUs resistant to ampicillin (2.81%±10.60%) than to ceftiofur (0.02%±0.09%) ($P < 2.2e-16$).

Considerable variation was observed in the percentage of Gram-negative bacteria resistant to ampicillin ($P = 0.001$) and ceftiofur ($P = 0.0015$) across animals at the different time points (**Figure B.1**). The average number of ceftiofur resistant bacteria was ~14 times higher in the antibiotic treated group during weeks 1 (197 CFU/g) and 2 (335 CFU/g) relative to the control group (*week 1:* 24 CFU/g; *week 2:* 14 CFU/g) (**Figure 3.2C**). This difference was not significant ($P > 0.2$), which is likely because only a subset (25%) of the treated cows shed more ceftiofur-resistant bacteria. By contrast, the number of ampicillin resistant Gram-negatives was significantly higher in the control group in weeks 1 ($P = 0.041$) and 2 ($P = 0.021$) compared to day -1, but no difference was observed in the ceftiofur treated group ($P > 0.3$) (**Figure 3.2E**). Intriguingly, the total number of Gram-negative bacteria and the number of ampicillin resistant Gram-negative colonies increased at week 9 during pre-calving ($P = 0.033$) in both the treatment groups (**Figures 3.2C and 3.2E**). The number of Gram-negatives resistant to ceftiofur was also significantly higher in both groups at week 9 compared to weeks 5 and 7 ($P <0.006$), which was also true for Gram-negatives resistant to ampicillin ($P <0.01$).

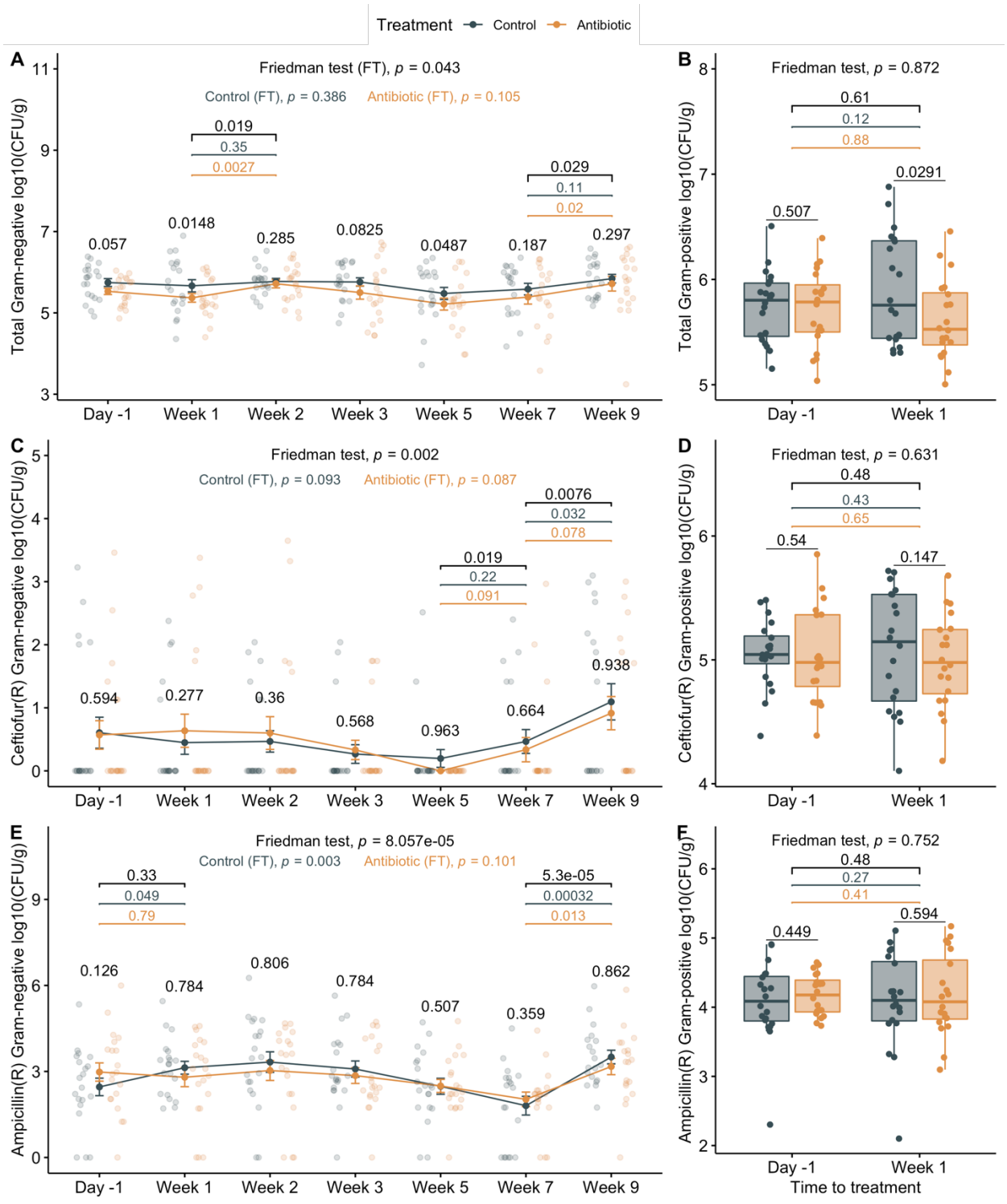

Figure 3.2. Number of bacterial colony-forming units (CFUs) per gram of feces. Total number of A) Gram-negatives and B) Gram-positives; ceftiofur resistant C) Gram-negatives and D) Gram-positives; and ampicillin-resistant E) Gram-negatives and F) Gram-positives with (orange) and without (gray) intramammary ceftiofur treatment. Numbers are plotted before (Day -1) and after treatment for Gram negatives through 9 weeks and Gram-positives after 1 week.

Figure 3.2 (cont'd)
Line plots show means and standard error bars with sample counts represented as dots. Boxplots indicate the median, lower, and upper quartiles, and the whiskers represent extreme values in the distribution. The per animal variability over time was calculated with the Friedman test (FT), which is shown per treatment group for Gram-negatives. Significant p-values between sampling points are represented for all animals (black) as well as for control (grey) and antibiotic (orange) groups.

Metagenome analysis

The metagenomic composition of cattle feces was analyzed for a total of 159 samples collected one day prior to treatment (day -1) and at weeks 1, 5, and 9 post-treatment. There was no difference in the number of reads per sample ($P = 0.067$) between groups, resulting in an average of 5.74 ($\pm$1.1) million reads. The microbiome was dominated by bacteria (92.51%) followed by archaea (4.27%), eukaryotes (1.65%), viruses (1.54%), and fungi (0.03%). The alpha diversity of bacteria was significantly lower during dry-off and increased during pre-calving ($P < 0.001$) (**Figures 3.3A and 3.3B**). Treatment with IMM ceftiofur at dry off did not affect bacterial richness in the short-term; though a significant reduction in the Chao1 index was observed in week 5 ($P = 0.014$) (**Figure 3.3B**) and in the Shannon diversity in week 9 ($P = 0.053$) (**Figure 3.3A**) compared to the control group. Likewise, the resistome alpha diversity was similar between groups ($P > 0.24$), though the richness of ARGs increased during pre-calving regardless of treatment ($P < 0.0001$) (**Figures 3.3C and 3.3D**).

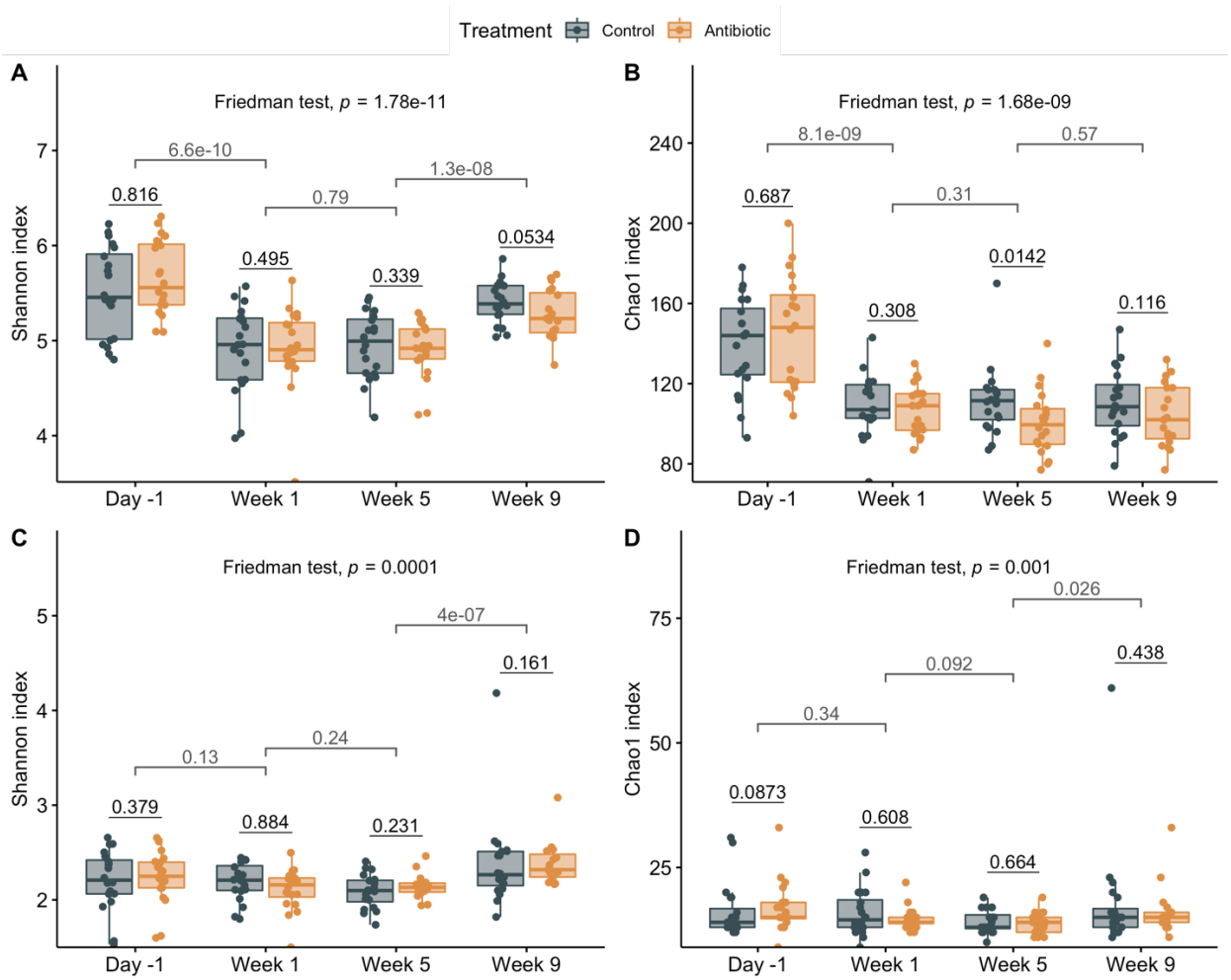

Figure 3.3. Alpha diversity in the microbiome (A and B) and resistome (C and D) of dairy cattle. Alpha diversity was determined using the Shannon and Chao1 indices over a 9-week period after dry-off in cows with (orange) and without (grey) intramammary ceftiofur treatment. P-values were calculated with the Wilcoxon test to compare treatment groups within a sampling point (black) and changes in diversity between sampling points (grey) regardless of the treatment group. Friedman test shows per animal variability in the alpha diversity over time. Each boxplot shows the median, lower, and upper quartiles with the whiskers representing extreme values in the distribution.

Similarly, the Bray-Curtis dissimilarity ordination showed significant changes in the bacterial composition over the sampling period (*PERMANOVA*, $F = 3.93$, $P = 0.001$) (**Figure 3.4A**). These changes were also detectable in the phyla distribution visualized in a relative abundance plot (**Figure 3.4B**). Specifically, the day before the dry-off (Day -1) was characterized by a higher abundance of Actinobacteria, Firmicutes and Proteobacteria compared

to weeks 1, 5, and 9 regardless of treatment status. An analysis of the resistome composition also showed significant differences in the Bray-Curtis dissimilarity ordination over time (*PERMANOVA*, $F = 4.28$, $P = 0.001$), which is likely due to distribution of points at week 9 (**Figure 3.4C**). Despite this difference, the relative abundance of ARGs was highly similar over the sampling period (**Figure 3.4D**). Genes encoding tetracycline resistance were the most abundant followed by those for macrolides and the β-lactams.

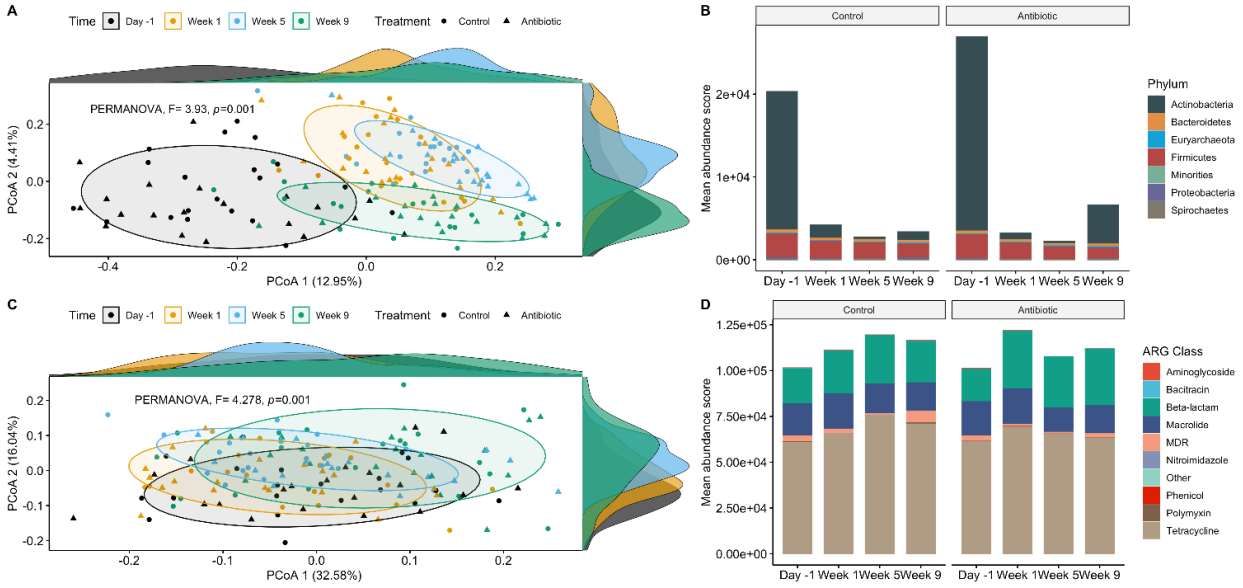

Figure 3.4. Fecal microbiome and resistome composition of dairy cows during the dry-off period by treatment group. PCoAs of the Bray-Curtis dissimilarity are shown for the A) microbiome and C) resistome clustered by sampling point (ellipses contain at least 90% of the samples). The densities on the X and Y axis show the distribution of samples per sampling point in each principal coordinate. The mean abundance score of the B) phyla and D) antibiotic resistance gene (ARG) drug classes are shown for each treatment group and sampling point. This normalized metric takes the genome size and number of reads into consideration and can be used to calculate the relative abundance (%).

Additionally, three viruses affecting cattle were identified at different time points including *Bos taurus* polyomavirus which was identified in one control animal on week 9, Pestivirus G in one control in week 5, and Bovine papillomavirus in three animals including 2 control and 1 antibiotic-treated cows, which were identified in more than once per individual

including day -1, week 1, and week 5 after dry-cow therapy. Other viruses identified included Baculoviruses and Granuloviruses that affect insects, two viruses from plants, 1 virus from *Penicillium*, and viruses from other mammal species including Torque teno midi virus 2 (animals, $n = 3$), murine mastadenovirus B ($n = 1$), and mouse mammary tumor virus ($n = 1$).

Differentially abundant taxa were also identified over time after stratifying by treatment group. Cows treated with IMM ceftiofur, for instance, had lower proportions of phyla Proteobacteria ($P < 0.02$) and Tenericutes ($P < 0.007$) compared to controls 1-week following treatment (**Figure 3.5**). By contrast, a lower abundance of Chloroflexi and Firmicutes ($P < 0.0001$) and a higher abundance of Actinobacteria ($P < 0.0001$) and Bacteroidetes ($P < 0.0001$) were observed at week 9 in the ceftiofur-treated cows as compared to controls. Bacteroidetes was also more abundant at week 5 in the treated animals. Further classification at the genus level identified a greater abundance of *Garciella* and *Pseudobutyrivibrio* in the treated cows as well as a decreased abundance of *Clostridium, Desulfosporosinus, Neorickettsia, Paeniclostridium, Phascolarctobacterium, Romboutsia, Ruminiclostridium, Terrisporobacter* and *Turicibacter* (**Table B.1**). IMM treatment with ceftiofur was also associated with a differential proportion of other genera in the long term. The abundance of *Clostridium* and *Turicibacter*, for example, was lower in weeks 1 and 9, whereas the abundance of genus *Peptostreptococcaceae bacterium VA2* was higher. During weeks 5 and 9, however, a higher proportion of genus *Bacillales bacterium* UBA1231 and a lower proportion of *Hungatella hathewayi, Methanobacteriaceae archaeon* UBA237 and *Ruminococcaceae bacterium* UBA2851 were identified.

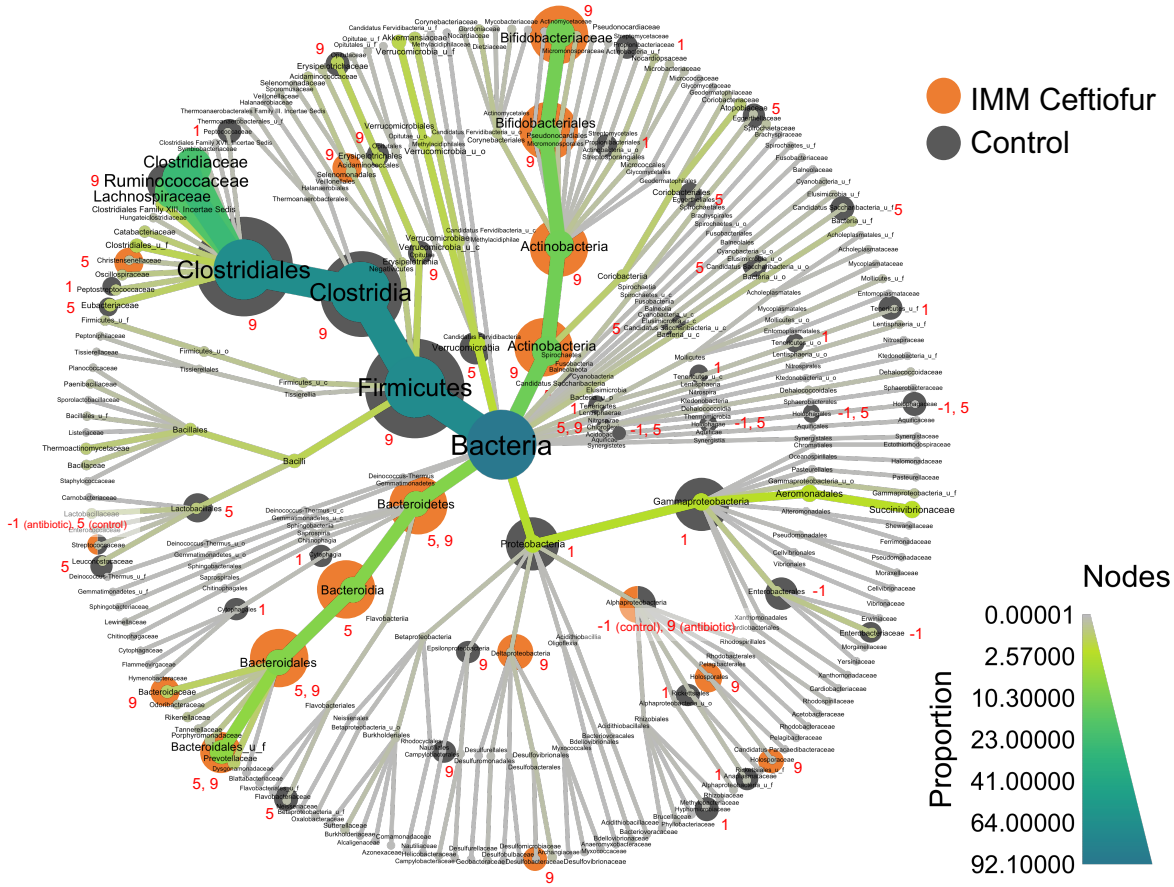

Figure 3.5. Heat tree showing the differential abundance of taxa from phylum to family over the 9-week sampling period. For each taxonomic level, the size of the node represents the proportion of a taxa within the microbiome. Significantly different taxa are noted for controls (grey) and antibiotic-treated cows (orange). The red numbers at the significant nodes indicate the sampling time (e.g., day (-1) or week (1, 5, or 9)) when taxa were found to be differentially abundant.

A similar analysis of the resistome composition also detected differentially abundant resistance genes in the treated and untreated animals over the sampling period (**Table B.2**). For instance, the untreated (control) cows had a greater abundance of genes encoding resistance to macrolides in weeks 1 and 5, and to tetracycline and multidrug resistance at weeks 1 and 9 (**Figure 3.6**). The ceftiofur-treated cows, however, had a greater proportion of β-lactam resistance genes at all three post-treatment samplings (weeks 1, 5, and 9). Upon further analysis,

the abundance of β-lactam resistance genes was significantly higher 1 week after treatment in the ceftiofur-treated cows, with similar levels persisting through week 9 (**Figure 3.7A**). Indeed, the treated cows had a greater abundance of *bla-CFXA, bla-ACI-1,* and *bla-CMY*, which contribute to ESBL production, as well as *blc* (outer membrane lipoprotein) over the 9-week period (*LDA* > 4.3; $P < 0.03$). A significant increase in the abundance of *bla-CFXA* and *bla-ACI-1* was observed in the treated cows at 1 week following treatment (**Figures 3.7B and 3.7C**). The increased abundance of these genes (*bla-CFXA* and *bla-ACI-1*) was maintained through the 9-week period, though some fluctuations were observed. Increased abundance of *blc* was only observed at week 1 with no difference by treatment group (**Figure 3.7D**), whereas *bla-CMY* was only significantly more abundant in the treated animals at week 9 (**Figure 3.7E**). Unlike the Wilcoxon test results presented in Figure 3.7, use of other statistical tests including DESeq2, LEfSe, ANCOM-BC and MaAsLin2, identified significant differences for both *blc* and *bla-CMY* at weeks 1, 5, and 9 (**Table B.2**).

Taxonomic identification of bacteria resistant to β-lactams

From 882 Gram-negative bacterial isolates resistant to ceftiofur, a total of 146 Gram-negative isolates were preserved for further analyses (72 from controls and 74 from ceftiofur-treated animals). These strains were recovered at day -1 ($n = 26$), week 1 ($n = 25$), week 2 (n=17), week 3 ($n = 17$), week 5 ($n = 5$), week 7 ($n = 10$), week 9 ($n = 44$), and week 11 ($n = 2$). Through biochemical identification, 94 isolates were identified as *E. coli*, while 25 were classified as other members of Enterobacteriaceae and 27 were classified as non-Enterobacteriaceae.

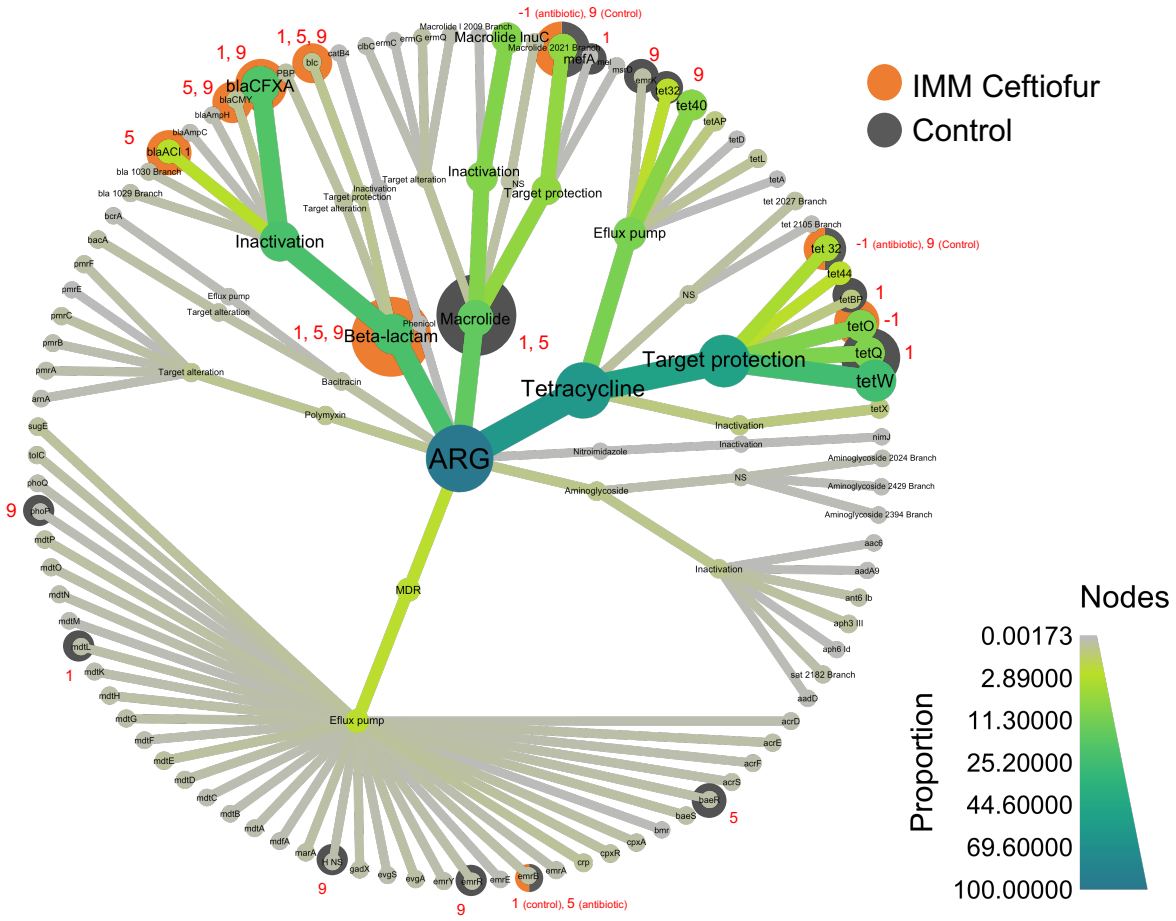

Figure 3.6. Heat tree showing the differential abundance of antibiotic resistance genes (ARGs) at the class, mechanism, and gene level. ARGs that were significantly different between controls (grey) and antibiotic treated cows (orange) are highlighted. For each level, the size of the node represents the proportion of each within the microbiome, while the red numbers indicate the day (-1) or week (1, 5, or 9) when the genes were differentially abundant.

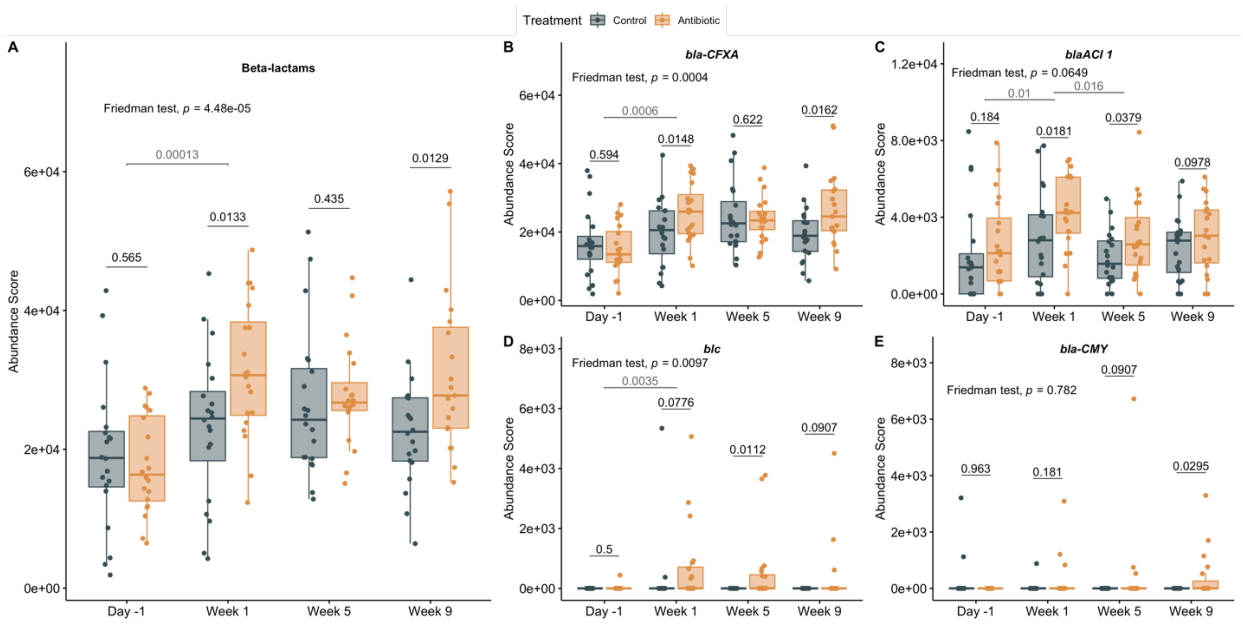

Figure 3.7. Abundance of β-lactam resistance genes before and after intramammary ceftiofur treatment over the 9-week period. Boxplots show the abundance score for A) the β-lactam antibiotic resistance gene class; B) *bla-CFXA*; C) *bla-ACI-1*; D) *blc*; and E) *bla-CMY*. The median, lower, and upper quartiles are shown in each boxplot with the whiskers representing extreme values in the distribution. P-values were calculated with the Wilcoxon test to compare treatment groups within a sampling point (black) and changes in abundance between sampling points (grey) regardless of the treatment group. The Friedman test assesses variability in class and gene abundance per animal over the sampling period.

In all, 121 contigs contained β-lactam resistance genes that could be classified with DeepARG [29] and analyzed with BLASTP [31] and CAT [32]. Of these, 77.98% of the BLASTP hits were identified as members of the Cytophaga, Fusobacterium, and Bacteroides (CFB) group, followed by Firmicutes (9.67%) and Gamma-Proteobacteria (4.65%); 7.7% were classified as "other". At the genus level, 44.34% of the hits belonged to *Bacteroides*, while a lower proportion were classified as *Prevotella* (6.76%), *Parabacteroides* (4.41%), *Escherichia* (1.57%), *Salmonella* (0.31%), *Campylobacter* (0.04%), and other (62.05%) (**Table B.3**). Among these ARG-carrying contigs (ACCs), most were associated with Bacteroidetes (n = 30), Firmicutes (n = 23), and Proteobacteria (n = 6) by CAT, though 62 could not be identified and were considered unknown. At the order level, a subset of these ACCs was associated with

Bacteroidales (n = 30), Clostridiales (n = 8), and Aeromonadales (n = 6) using CAT, whereas only 9 contigs could be classified at the species level. These species include *Bacteriodales bacterium* (n = 3), *Bacteroides* sp. (n = 1), *Ruminococcaceae bacterium* (n = 4), and "uncultured prokaryote" (n = 1).

Network analyses

Co-occurrence networks compared the interconnections among β-lactam ARGs and other resistance genes, MGEs, and bacterial genera between controls and ceftiofur-treated cows over time (**Figure B.2**). At week 1, for instance, the network for ceftiofur-treated animals showed β-lactam resistance genes with a higher degree of centrality [*number of connections*] (P = 0.033; **Figure 3.8A**) and betweenness centrality [*number of times a node stands between two random nodes*] (P = 0.004; **Figure 3.8B**) than before IMM treatment. This network included a wide range of bacterial species, phages, and plasmid genes. Meanwhile, the control animals had a similar degree of centrality in the β-lactam resistance genes before and 1 week after dry-off (P = 0.79), though this metric was significantly higher than in the antibiotic-treated cows at day -1 (P = 0.043). At week 5, both groups had a similar number of connections for the β-lactam resistance genes (P = 0.86) and a similar betweenness centrality value (P =0.95), which was significantly lower than the value observed at week 1 ($P < 0.02$).

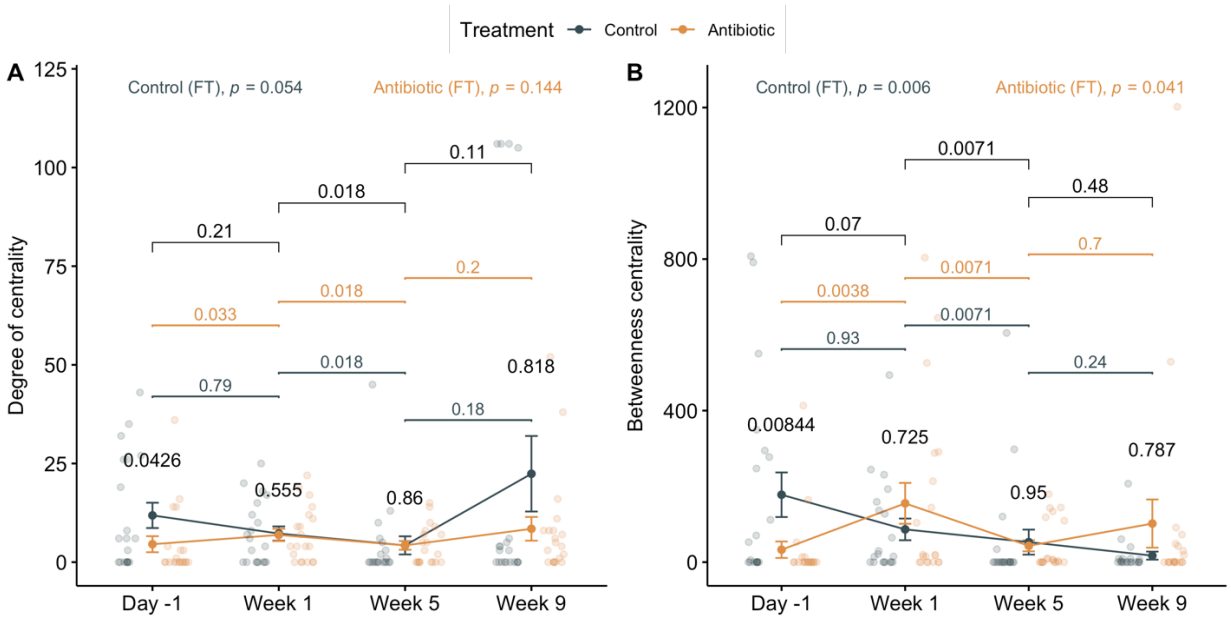

Figure 3.8. Average of centrality measures between β-lactam resistant genes and MGEs, phages and bacterial genera. A) Degree of centrality B) Betweenness centrality. Values are plotted over a 9-week period following IMM treatment. Line plots show means and standard error bars, while gene values are represented as dots. P-values indicate differences between treatment groups by time point and between sampling points despite the treatment (black bracket) or within treatment group (*control* = gray; *antibiotic* = orange brackets). Friedman test shows per gene variability in the centrality measures over time.

Interestingly, during pre-calving (week 9), the networks showed a greater number of interconnections between β-lactam resistance genes, other ARG, MGEs, and taxa in both groups but there was no difference relative to week 5 ($P > 0.2$). This result is likely due to the heterogeneity of connections among the different genes important for β-lactam resistance. For instance, on week 9, the control group had a network with a high number of edges (*density* = 0.139; *connected components* = 95; *average weighted degree* = 36.32) but with few clusters (*modularity* = 0.074) (**Figure B.2**). The main cluster in the control group at week 9 contained the β-lactam resistance genes *blaAmpC*, *blaAmpH*, *pbp2*, and *pbp4*, which shared connections with the same nodes (*average degree of centrality* = 106) as phages (e.g., *Escherichia* virus phiV10, Stx2-converting phage 86, *Salmonella* phage vBSemP, and others), transposases, plasmids,

virulence genes, and bacterial taxa (*Pseudoxanthomonas*, Enterobacteriaceae, and *Shewanella*). The ESBL genes, *bla-CFXA* and *bla-ACI*, in the control group, however, were not linked with the main cluster at week 9 and had very few edges (*average degree of centrality* = 1.56) with commensal bacteria such as *Halocynthiibacter*, *Holophagacae*, *Lachnobacterium*, *Lachnospira*, *Methylobacterium*, *Ureaplasma*, and phages from *Shigella* and *Salmonella*.

In contrast, the network for ceftiofur-treated cows at week 9 had a smaller number of edges (*density* = 0.032, *average weighted degree* = 7.336) but a greater number of connected components (*n* = 128) and clusters (*modularity* = 0.586). The ESBL genes, for instance, had a greater degree of centrality (*average degree* = 8.95) than in the control networks (*P* = 0.008) when excluding those β-lactam genes (*blaAmpC*, *blaAmpH*, *pbp2,* and *pbp4*) found in the main cluster of controls. In the ceftiofur-treated cows, the ESBL genes were linked with several taxa including *Cetobacterium, Clostridiales, Coprococcus, Corynebacterium, Dorea, Gallibacterium, Gordonia, Halorubrum, Kocuria, Microbacterium, Microvirga, Paracaedibacteriacea, Rickettsiales, Saccharibacillus, Saccharomonospora, Thermoactinomyces, Thermobifida, Treponema, Turicibacter, Tyzzerella,* and *Yersinia*. Linkages with phages from *Shigella*, *Escherichia*, and *Salmonella* were also identified. On average, the *bla* genes with the highest betweenness centrality were *bla-CFXA*, *bla-CMY*-13, *bla-ACI-1*, *pbp2*, *bla*-1030 Branch, *pbp4*, and *blaAmpH*. Notably, *bla-CMY-13* had the highest value of betweenness centrality at week 9 in the antibiotic-treated group (*n* = 1202.04) when compared to the other *bla* genes identified.

DISCUSSION

It was estimated that ~90% of dairy farms use IMM β-lactam antibiotics during the dry-off period to treat and prevent mastitis [5–7] despite the possibility of selecting for resistant bacterial populations. Of great concern is the emergence and selection of ESBL-producing

Enterobacteriaceae, which are classified as a serious public health threat [2]. Although the effect of IMM ceftiofur treatment has been studied in the milk microbiome, including five days with IMM 125 mg/day [35,36] and a single application of 2 g of CHCL [37], the impact of this treatment on the gut microbiome has not been elucidated. Through this study, however, we have demonstrated long-term effects on the fecal microbiome due to a single 2g dose of IMM ceftiofur via culture-based analyses and metagenomics. Indeed, compared to controls, antibiotic-treated cows had reduced microbial richness over time, differentially abundant taxa, and an increased abundance and persistence of β-lactam resistance genes that were associated with Enterobacteriaceae hosts and commensal bacteria. A subset of the treated cows also had greater concentrations of ceftiofur-resistant Gram-negative bacterial populations.

Following subcutaneous treatment, a prior study showed that Holstein steers had higher concentrations of CHCL in the gastrointestinal tract compared to ceftiofur crystalline-free acid (CCFA) [9], though only CCFA resulted in decreased fecal *E. coli* concentrations for up to two weeks. Similarly, parenteral ceftiofur treatment resulted in lower fecal *E. coli* concentrations for 3 days [12] and up to a month post-treatment [13] in two other studies. In the latter study of 96 dairy cows, systemic ceftiofur administration resulted in a significant increase in the level of ceftiofur-resistant Enterobacteriaceae, though the concentrations returned to baseline levels after one week [13]. Consistent with these findings, we observed a reduction in the total number of Gram-negative bacteria one week after IMM ceftiofur treatment, and an increased number of ceftiofur-resistant Gram-negative bacteria in 25% of the treated cows. Enhanced recovery of Gram-negative bacteria with resistance to ceftiofur was observed for two weeks after the treatment. Re-emergence of ceftiofur resistance was also observed in the Gram-negative bacterial populations at 9 weeks (pre-calving) in both the treated and untreated animals, which is

consistent with data generated in another study [13]. This increase could be linked to environmental acquisition of resistant strains, horizontal gene transfer, peri-parturient immune suppression, higher contact with personnel, or modifications in the diet formulation during pre-calving, all of which could contribute to the expansion of resistant Enterobacteriaceae populations. While these data are intriguing, it is important to note that *in vitro* bacterial quantifications do not distinguish between acquired and intrinsic antimicrobial resistance. Hence, future studies should involve isolating the resistant strains for further identification using biochemical tests and whole-genome sequencing, which can also be used to define the mechanisms of resistance.

Similar to a prior report of six healthy 1st lactation cows given 2 subcutaneous doses of CCFA [38], we observed no difference in fecal microbiome diversity after IMM ceftiofur treatment in the short term. The abundance of some taxa belonging to *Turicibacter*, Peptostreptococcaceae, and Tenericutes, however, decreased a week after treatment. A decrease was also observed in Gammaproteobacteria, which are Gram-negatives of the order Enterobacterales (e.g., *E. coli*) that are typically susceptible to β-lactam antibiotics, and Epsilonproteobacteria comprising *Campylobacter*. Members of the latter group are mostly resistant to third-generation cephalosporins with the exception of *Campylobacter fetus* [39]. A greater abundance of *Campylobacter* was observed during pre-calving (9 weeks) in the control group, which was correlated with the multidrug-resistant genes *cpxA*, *crp*, *gadX*, and *mdtK*. By contrast, Erysipelotrichia, including *Turicibacter* and Peptostreptococcaceae, are Gram-positives susceptible to penicillin and cephalosporins [40]. Because Tenericutes typically have intrinsic resistance to β-lactams due to the lack of a cell wall, the observed decrease in abundance at week 1 was unexpected. Despite a prior report that steers treated with subcutaneous ceftiofur had a

lower abundance of Firmicutes and increased Bacteroidetes for two weeks [9], we only observed changes in these phyla at weeks 5 and 9 post-treatment. The effects of these changes on cattle health and production, however, could not be elucidated.

A key finding of this study was the exclusive and persistent increase in the abundance of ESBL genes (*bla-CFXA, bla-ACI-1*, and *bla-CMY*) in the fecal resistome of cows given IMM ceftiofur treatment. Although increases in the abundance of ESBL genes following parenteral ceftiofur treatment have been reported [12,14,38,41,42], no prior studies have examined the effect of IMM treatment. Steers receiving subcutaneous CCFA, for example, had a higher abundance of bacterial isolates harboring *bla-CMY-2* up to 4 days post-treatment, which resulted in co-selection of isolates containing *tet(A)* and *bla-CMY-2* after a subsequent chlortetracycline treatment for up to 26 days [14]. Similarly, Holstein cows treated with systemic CCFA showed a higher abundance of genes encoding *bla-CFXA* β-lactamases three days after treatment [38], while other studies reported an increase of *bla-CMY-2* in cattle feces for up to 10 days post-treatment when pure cultures were analyzed [12,43]. In addition, our observation of a persistent increase in the abundance of the gene encoding Blc (bacterial lipocalin), an outer membrane lipoprotein, suggests a stress response related to the use of β-lactams. A prior study demonstrated that Blc production is linked to a starvation response and β-lactam resistance in *E. coli*, which is activated by the sigma factor, RpoS, between exponential and stationary growth phases [44]. Consequently, the higher abundance of *blc* observed during weeks 1, 5 and 9 after IMM ceftiofur treatment indicates lasting effects of a single IMM application of ceftiofur on the gut microbiota.

Despite observing an increased abundance of ESBL genes, no increment in resistant third-generation cephalosporin CFUs was identified in cows treated with IMM ceftiofur in this study. This inconsistency between culture-dependent and independent methods could be

attributed to the oxygenic environment used to identify bacteria on plates. The hindgut microbiome is composed predominantly of anaerobic bacteria; thus, aerobic and microaerophilic conditions used for the quantification of Gram-negative and Gram-positive bacteria represented a small fraction of the microbiota. For instance, Bacteroidetes (e.g., *Prevotella* and *Bacteroides*), are predominant anaerobic Gram-negative bacteria in the hindgut, priorly identified as the main carriers of the ESBL gene *bla-CFXA* [45], which was the most abundant β-lactamase gene identified by metagenomics. Likewise, *bla-ACI1* was the second most abundant ESBL gene, priorly reported in the Gram-negative Firmicutes *Acidaminococcus* [46] and the Gram-positive genus *Bifidobacterium* [47]. Consistently, a significantly higher abundance of Bacteroidetes, Acidaminoccocales, and *Bifidobacterium* was identified in cows treated with IMM ceftiofur when compared with controls. These results suggest that the increased abundance of ESBLs identified after IMM ceftiofur treatment was associated with changes in the abundance of anaerobic bacteria.

The identification of bacterial hosts harboring β-lactam resistance genes is also critical for developing new interventions as well as enhancing understanding of the ecology of resistance within the cattle microbiome and risks associated with carriage of specific genes. Indeed, our use of different approaches including *in-vitro* and *in-silico* analyses, allowed us to identify those bacterial hosts more accurately. While culture identification of the resistant bacteria indicated 64% of the isolates were *E. coli*, metagenomic analyses showed most (44%) of the β-lactamases were associated with Bacteroidetes and only 1.57% were associated with *E. coli*. Similarly, co-occurrence networks indicated that bacteriophages of Enterobacteriaceae and other genera were associated with ESBL genes. Network analysis also revealed changes in the interconnections of ESBL genes over the dry-off period, as a significant increase in the degree of centrality of *bla*

genes was observed in the antibiotic-treated cows one week after treatment and during pre-calving. This finding suggests that disturbances in the microbiome composition likely contributed to the increased distribution of ESBL genes across bacterial strains, potentially aided by connections with MGEs. During pre-calving, for instance, the control group had high levels of centrality among ARGs, MGEs, virulence genes, and potential pathogens compared to the ceftiofur-treated cows. However, ESBL genes that significantly increased after IMM ceftiofur treatment were not part of that cluster. Differences in networks between controls and antibiotic-treated cows before treatment (day -1) could therefore be related to the higher number of Gram-negative bacteria recovered in the control group, compromising comparisons between these two groups. To our knowledge, this is the first study to report network dynamics of ARGs during dry-off in dairy cows.

An increased proportion of ESBL genes in cattle manure following IMM ceftiofur administration could contribute to enhanced dissemination and persistence of resistance in the farm environment. For example, one study demonstrated that use of manure from dairy cows treated with IMM antibiotics (pirlimycin and cephapirin) in unamended soils resulted in a significant 2.2X increase in ARG abundance [48]. Composting and a 120-day waiting period, however, reduced the total ARG relative abundance as well as the co-occurrence of ARGs, MGEs and pathogens [48]. Enterobacteriaceae one week after IMM ceftiofur treatment and during pre-calving in both the antibiotic-treated and control groups. These findings highlight the need for additional measures such as separating manure from antibiotic-treated and control animals during peripartum, to prevent the spread and persistence of resistant bacteria and resistance determinants in the farm environment.

Intriguingly, the abundance of Actinobacteria was significantly higher on day -1 compared to further time points. The most abundant family of phylum Actinobacteria was Bifidobacteriaceae represented mainly by the genus *Bifidobacterium*. Bifidobacteriaceae are implicated in the utilization of oligosaccharides in the colon resulting in the production of VFAs [49]. Differences in the composition of the fecal microbiome, primarily caused by the abundance of Actinobacteria, observed on day -1 could be associated with differences in the diet given in late lactation which has higher levels of dry matter intake and metabolizable energy and protein than during dry off (weeks 1 – 7) and fresh cows (week 9). However, further analyses of microbial metabolic pathways and metabolite composition are necessary to better explain the impacts of differentially abundant taxa on cattle's performance.

Although this study is the first to describe the impact of IMM ceftiofur treatment on the gut microbiome, it is important to highlight a few limitations. For instance, current resistome databases do not include all known ARGs from cattle samples and hence, novel resistance determinants may remain unclassified. The identification of species, ARGs, and ACCs can also be limited by a low number of metagenomic reads, as sequencing depth of ≥50 million reads is needed for complex microbial communities such as those residing in the bovine gut [50]. Since the proportion of microbial phyla and ARG classes was constant across various sequencing depths [50], we were able to detect the predominant and differential metagenomic features. Sequencing depth, assembly method, and size of DNA segments (150 bp) are also important for the identification of bacterial hosts through ACCs. Future work involving use of third-generation sequencing platforms that sequence ultralong DNA segments such as the PacBio (40-70 kbp) or Oxford Nanopore Technologies (>100 kbp), however, would be highly beneficial for confirmation and characterization of these regions [51]. Since the identification of differentially

abundant features, including bacterial taxa and genes, tends to vary across bioinformatic pipelines, we applied six different approaches but only reported those features with significant p-values using at least two pipelines, as suggested previously [27]. Altogether, our analyses highlight those metagenomic features that are most impacted by IMM ceftiofur treatment.

CONCLUSIONS

One application of IMM ceftiofur (2 g) at dry off contributed to an increase in the abundance of genes encoding ESBLs in the fecal samples of antibiotic-treated cattle that persisted for nine weeks. These genes were associated with Enterobacteriaceae hosts, which illustrates how ESBL-producing pathogens emerge and are selected for in this niche. While most of the cows given the prophylactic IMM ceftiofur treatment did not have an increase in the number of resistant bacteria, 25% had an increased level of ceftiofur-resistant Gram-negative bacteria. Indeed, the recovery of resistant isolates was 14X greater in the antibiotic-treated versus control cows for up to two weeks after treatment, highlighting significant variation in fecal shedding levels across animals. Future studies should therefore focus on understanding the association between shedding and the dissemination and persistence of antibiotic resistance determinants in dairy farm environments.

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APPENDIX

Table B.1. Differentially abundant bacterial taxa identified with four approaches: LEfSe, DESeq2 (three settings), MaAsLin2, and ANCOM-BC. Taxa with a significant p-value to at least two of the six algorithms were reported in Figure 3.5.

Time point	Level	Taxa	Group	# Tests TRUE
Week 1	Species	*[Clostridium] dakarense*	Control	6
Week 1	Species	*[Clostridium] viride*	Control	3
Day -1	Phylum	Acidobacteria	Control	4
Week 5	Phylum	Acidobacteria	Control	2
Week 9	Class	Actinobacteria	Antibiotic	4
Week 9	Phylum	Actinobacteria	Antibiotic	4
Day -1	Class	Alphaproteobacteria	Control	6
Week 9	Class	Alphaproteobacteria	Antibiotic	3
Week 1	Family	Anaplasmataceae	Control	2
Day -1	Species	*Aspergillus_u_s*	Antibiotic	3
Week 9	Species	*Bacillales bacterium UBA1231*	Antibiotic	3
Day -1	Species	*Bacillales bacterium UBA2753*	Control	3
Week 1	Species	*Bacillales bacterium UBA5578*	Control	5
Week 9	Family	Bacteroidaceae	Antibiotic	2
Week 5	Order	Bacteroidales	Antibiotic	5
Week 9	Order	Bacteroidales	Antibiotic	2
Week 5	Family	Bacteroidales_u_f	Antibiotic	3
Week 9	Family	Bacteroidales_u_f	Antibiotic	2
Week 5	Genus	*Bacteroidales_u_g*	Antibiotic	3
Week 9	Genus	*Bacteroidales_u_g*	Antibiotic	3
Week 9	Genus	*Bacteroides*	Antibiotic	3
Day -1	Species	*Bacteroides barnesiae*	Control	2
Week 5	Species	*Bacteroides thetaiotaomicron*	Control	3
Week 5	Species	*Bacteroides vulgatus*	Control	2
Week 5	Phylum	Bacteroidetes	Antibiotic	5
Week 9	Phylum	Bacteroidetes	Antibiotic	3
Week 5	Class	Bacteroidia	Antibiotic	5
Week 9	Family	Bifidobacteriaceae	Antibiotic	2
Week 9	Order	Bifidobacteriales	Antibiotic	2
Week 9	Genus	*Bifidobacterium*	Antibiotic	3
Day -1	Species	*Bifidobacterium animalis*	Antibiotic	4
Day -1	Species	*Bifidobacterium breve*	Antibiotic	3
Day -1	Species	*Bifidobacterium choerinum*	Antibiotic	5
Week 1	Species	*Bifidobacterium pseudolongum*	Control	2
Week 9	Species	*Bifidobacterium pseudolongum*	Antibiotic	3
Day -1	Species	*Bifidobacterium thermacidophilum*	Antibiotic	2
Week 1	Species	*Blautia sp UBA2945*	Antibiotic	2
Week 5	Species	*Butyrivibrio sp.*	Control	2
Week 9	Order	Campylobacterales	Control	2
Week 5	Phylum	Candidatus Saccharibacteria	Control	5
Week 5	Class	Candidatus Saccharibacteria_u_c	Control	5
Week 5	Family	Candidatus Saccharibacteria_u_f	Control	5
Week 5	Genus	*Candidatus Saccharibacteria_u_g*	Control	6
Week 5	Order	Candidatus Saccharibacteria_u_o	Control	5
Week 5	Species	*Catabacter_u_s*	Antibiotic	3
Week 9	Genus	*Cellulosilyticum*	Control	3
Week 9	Species	*Cellulosilyticum lentocellum*	Control	3
Week 5	Phylum	Chloroflexi	Control	3
Week 9	Phylum	Chloroflexi	Control	2

Table B.1 (cont'd)

Time point	Level	Taxa	Group	# Tests TRUE
Week 5	Species	*Christensenella timonensis*	Antibiotic	2
Week 9	Class	Clostridia	Control	5
Week 9	Order	Clostridiales	Control	2
Week 1	Species	*Clostridiales bacterium*	Antibiotic	5
Day -1	Species	*Clostridiales bacterium UBA2907*	Control	2
Week 1	Species	*Clostridiales bacterium UBA3705*	Control	3
Week 1	Species	*Clostridiales bacterium UBA4638*	Control	2
Week 5	Species	*Clostridiales bacterium UBA643*	Antibiotic	3
Week 9	Species	*Clostridiales bacterium UBA7081*	Antibiotic	3
Week 9	Species	*Clostridioides mangenotii*	Control	4
Week 1	Genus	*Clostridium*	Control	6
Week 9	Genus	*Clostridium*	Control	3
Week 9	Species	*Clostridium bornimense*	Control	4
Week 9	Species	*Clostridium butyricum*	Control	5
Week 9	Species	*Clostridium sp. CL-2*	Control	2
Week 5	Genus	*Corynebacterium*	Control	3
Week 1	Species	*Corynebacterium sputi*	Control	2
Week 5	Genus	*Cytophaga*	Antibiotic	2
Week 5	Species	*Cytophaga_u_s*	Antibiotic	2
Week 1	Order	Cytophagales	Control	3
Week 1	Class	Cytophagia	Control	2
Week 9	Class	Deltaproteobacteria	Antibiotic	3
Week 9	Family	Desulfomicrobiaceae	Antibiotic	4
Week 9	Genus	*Desulfomicrobium*	Antibiotic	3
Week 9	Species	*Desulfomicrobium_u_s*	Antibiotic	4
Week 1	Genus	*Desulfosporosinus*	Control	2
Week 1	Species	*Desulfosporosinus_u_s*	Control	2
Week 5	Genus	*Desulfovibrionaceae_u_g*	Control	2
Day -1	Genus	*Diutina*	Control	3
Day -1	Species	*Diutina catenulata*	Control	3
Week 5	Family	Eggerthellaceae	Control	3
Week 5	Species	*Eggerthellaceae bacterium UBA3686*	Control	3
Week 5	Genus	*Eggerthellaceae_u_g*	Control	3
Week 5	Order	Eggerthellales	Control	3
Week 5	Genus	*Elizabethkingia*	Control	3
Week 5	Species	*Elizabethkingia_u_s*	Control	3
Day -1	Order	Enterobacterales	Control	3
Week 1	Species	*Enterobacteria phage mEp460*	Control	4
Week 9	Species	*Enterobacteria phage SfI*	Control	2
Day -1	Family	Enterobacteriaceae	Control	3
Week 1	Species	*Enterococcus faecium*	Control	2
Week 9	Class	Epsilonproteobacteria	Control	2
Week 9	Family	Erysipelotrichaceae	Control	5
Day -1	Species	*Erysipelotrichaceae bacterium*	Control	2
Week 5	Species	*Erysipelotrichaceae bacterium NK3D112*	Control	3
Week 9	Genus	*Erysipelotrichaceae_u_g*	Control	4
Week 9	Order	Erysipelotrichales	Control	5
Week 9	Class	Erysipelotrichia	Control	6
Day -1	Genus	*Escherichia*	Control	3
Day -1	Species	*Escherichia coli*	Control	3
Week 9	Species	*Escherichia virus pro483*	Control	2
Week 5	Family	Eubacteriaceae	Control	3
Week 5	Genus	*Eubacterium*	Control	3
Week 9	Phylum	Firmicutes	Control	2
Week 5	Species	*Firmicutes bacterium UBA3738*	Control	6

Table B.1 (cont'd)

Time point	Level	Taxa	Group	# Tests TRUE
Week 5	Family	Flavobacteriaceae	Control	2
Week 1	Class	Gammaproteobacteria	Control	5
Day -1	Species	*Gammaproteobacteria bacterium UBA2804*	Control	2
Week 1	Genus	*Garciella*	Antibiotic	3
Day -1	Family	Holophagaceae	Control	4
Week 5	Family	Holophagaceae	Control	2
Day -1	Genus	*Holophagaceae_u_g*	Control	4
Week 5	Genus	*Holophagaceae_u_g*	Control	2
Day -1	Species	*Holophagaceae_u_s*	Control	4
Week 5	Species	*Holophagaceae_u_s*	Control	2
Day -1	Class	Holophagae	Control	3
Week 5	Class	Holophagae	Control	2
Day -1	Order	Holophagales	Control	4
Week 5	Order	Holophagales	Control	2
Week 9	Genus	*Holospora*	Antibiotic	3
Week 9	Species	*Holospora_u_s*	Antibiotic	4
Week 9	Family	Holosporaceae	Antibiotic	4
Week 9	Order	Holosporales	Antibiotic	4
Week 5	Genus	*Hungatella*	Control	6
Week 5	Species	*Hungatella hathewayi*	Control	6
Week 9	Species	*Hungatella hathewayi*	Control	4
Day -1	Species	*Kandleria sp. UBA2934*	Antibiotic	2
Week 9	Genus	*Kocuria*	Control	2
Week 9	Genus	*Lachnobacterium*	Control	3
Week 5	Genus	*Lachnoclostridium*	Antibiotic	3
Day -1	Species	*Lachnospiraceae bacterium 10-1*	Antibiotic	2
Week 5	Species	*Lachnospiraceae bacterium UBA2825*	Control	4
Day -1	Species	*Lachnospiraceae bacterium UBA2860*	Control	5
Week 5	Species	*Lachnospiraceae bacterium UBA2860*	Control	6
Week 5	Species	*Lachnospiraceae bacterium UBA2906*	Antibiotic	2
Week 5	Species	*Lachnospiraceae bacterium UBA3632*	Control	3
Week 5	Species	*Lachnospiraceae bacterium UBA4348*	Control	5
Week 5	Species	*Lachnospiraceae bacterium UBA7023*	Control	3
Week 5	Order	Lactobacillales	Control	5
Day -1	Genus	*Lactobacillus*	Antibiotic	3
Week 5	Species	*Lactobacillus curvatus*	Control	2
Day -1	Species	*Lactobacillus mucosae*	Antibiotic	5
Day -1	Species	*Lactobacillus phage phiAT3*	Control	2
Day -1	Species	*Lactobacillus sp.*	Antibiotic	5
Week 5	Family	Leuconostocaceae	Control	5
Week 5	Species	*Methanobacteriaceae archaeon UBA237*	Control	6
Week 9	Species	*Methanobacteriaceae archaeon UBA237*	Control	2
Week 5	Species	*Methanobacteriaceae archaeon UBA541*	Control	4
Week 5	Genus	*Methanobacteriaceae_u_g*	Control	5
Week 5	Genus	*Methanosphaera*	Control	3
Week 5	Species	*Methanosphaera stadtmanae*	Control	3
Week 1	Family	Methylobacteriaceae	Control	5
Week 5	Species	*Microbacterium_u_s*	Antibiotic	3
Day -1	Genus	*Monascus*	Control	3
Day -1	Species	*Monascus ruber*	Control	4
Week 1	Genus	*Neorickettsia*	Control	2
Week 1	Species	*Neorickettsia_u_s*	Control	3
Week 1	Genus	*Paeniclostridium*	Control	5
Week 1	Species	*Paeniclostridium sordellii*	Control	5
Day -1	Species	*Pediococcus pentosaceus*	Control	2

Table B.1 (cont'd)

Time point	Level	Taxa	Group	# Tests TRUE
Week 1	Family	Peptococcaceae	Control	6
Week 1	Family	Peptostreptococcaceae	Control	6
Week 1	Species	*Peptostreptococcaceae bacterium VA2*	Control	6
Week 9	Species	*Peptostreptococcaceae bacterium VA2*	Control	3
Week 9	Genus	*Peptostreptococcaceae_u_g*	Control	5
Week 1	Species	*Peptostreptococcaceae_u_s*	Antibiotic	3
Week 1	Genus	*Phascolarctobacterium*	Control	3
Day -1	Genus	*Porphyromonas*	Control	2
Week 1	Species	*Prevotella stercorea*	Control	3
Week 1	Family	Propionibacteriaceae	Control	3
Week 1	Order	Propionibacteriales	Control	3
Week 1	Phylum	Proteobacteria	Control	5
Week 1	Genus	Pseudobutyrivibrio	Antibiotic	3
Day -1	Genus	Punavirus	Antibiotic	3
Week 1	Order	Rickettsiales	Control	3
Day -1	Genus	Robinsoniella	Antibiotic	3
Week 1	Genus	*Romboutsia*	Control	6
Week 1	Genus	*Ruminiclostridium*	Control	3
Week 9	Genus	*Ruminobacter*	Control	3
Week 9	Species	*Ruminobacter sp. RM87*	Control	4
Week 9	Family	Ruminococcaceae	Control	4
Week 5	Species	*Ruminococcaceae bacterium UBA2851*	Control	3
Week 9	Species	*Ruminococcaceae bacterium UBA2851*	Control	4
Week 5	Species	*Ruminococcaceae bacterium UBA2854*	Control	3
Day -1	Species	*Ruminococcaceae bacterium UBA642*	Control	2
Week 1	Species	*Ruminococcaceae bacterium UBA642*	Antibiotic	5
Week 9	Genus	*Ruminococcaceae_u_g*	Control	6
Week 5	Species	*Ruminococcaceae_u_s*	Control	4
Day -1	Genus	*Ruminococcus*	Control	2
Week 1	Species	*Ruminococcus flavefaciens*	Control	5
Week 1	Species	*Ruminococcus sp.*	Antibiotic	5
Day -1	Family	Saccharomycetales_u_f	Control	3
Week 9	Order	Selenomonadales	Antibiotic	2
Week 5	Genus	*Selenomonas*	Antibiotic	2
Day -1	Species	*Shigella virus Sf6*	Control	3
Week 1	Family	Siphoviridae	Control	5
Week 1	Genus	*Siphoviridae_u_g*	Control	6
Day -1	Genus	*Solobacterium*	Control	2
Week 5	Genus	*Stomatobaculum*	Antibiotic	2
Week 5	Species	*Stomatobaculum longum*	Antibiotic	2
Day -1	Family	Streptococcaceae	Antibiotic	3
Week 5	Family	Streptococcaceae	Control	2
Day -1	Genus	*Streptococcus*	Antibiotic	2
Week 9	Species	*Succinivibrionaceae_u_s*	Antibiotic	6
Week 1	Phylum	Tenericutes	Control	5
Week 1	Class	Tenericutes_u_c	Control	6
Week 1	Family	Tenericutes_u_f	Control	6
Week 1	Genus	*Tenericutes_u_g*	Control	6
Week 1	Order	Tenericutes_u_o	Control	6
Week 1	Genus	*Terrisporobacter*	Control	6
Week 9	Species	*Terrisporobacter othiniensis*	Control	5
Week 1	Species	*Terrisporobacter_u_s*	Control	6
Week 9	Species	*Terrisporobacter_u_s*	Antibiotic	2
Week 9	Genus	*Turicibacter*	Control	6
Week 1	Genus	*Turicibacter*	Control	4

Table B.1 (cont'd)

Time point	Level	Taxa	Group	# Tests TRUE
Week 9	Species	*Turicibacter sanguinis*	Control	3
Week 9	Species	*uncultured Bacteroides sp*	Antibiotic	2
Week 1	Species	*uncultured Clostridium sp*	Control	3
Week 9	Species	*uncultured Desulfovibrio sp.*	Antibiotic	3
Day -1	Species	*uncultured Dialister sp*	Antibiotic	2
Week 1	Species	*uncultured Garciella sp*	Antibiotic	2
Week 1	Species	*uncultured Prevotella sp*	Control	2
Week 1	Species	*uncultured Prevotellaceae bacterium*	Control	3
Day -1	Species	*uncultured Robinsoniella sp*	Antibiotic	2
Week 5	Species	*uncultured Ruminococcaceae bacterium*	Antibiotic	2
Week 1	Species	*uncultured Tenericutes bacterium*	Control	6
Week 5	Phylum	Verrucomicrobia	Control	3
Week 1	Species	*Verrucomicrobia bacterium UBA2808*	Antibiotic	5
Week 5	Species	*Verrucomicrobia bacterium UBA3761*	Control	2
Week 1	Species	*Verrucomicrobia bacterium UBA3841*	Control	3
Week 5	Genus	*Weissella*	Control	5
Week 5	Species	*Weissella cibaria*	Control	3

Table B.2. Differentially abundant ARGs identified with LEfSe, DESeq2 (three settings), MaAsLin2, and ANCOM-BC. Genes with a significant p-value to at least two of the six methods were included in the Heat-Tree of Figure 3.6.

Time point	Level	Gene	Group	# Tests TRUE
Week 5	Gene	*baeR*	Control	2
Week 1	Class	Beta-lactam	Antibiotic	5
Week 5	Class	Beta-lactam	Antibiotic	2
Week 9	Class	Beta-lactam	Antibiotic	5
Week 5	Gene	*blaACI 1*	Antibiotic	2
Week 1	Gene	*blaCFXA*	Antibiotic	4
Week 9	Gene	*blaCFXA*	Antibiotic	5
Week 5	Gene	*blaCMY*	Antibiotic	2
Week 9	Gene	*blaCMY*	Antibiotic	5
Week 1	Gene	*blc*	Antibiotic	2
Week 5	Gene	*blc*	Antibiotic	5
Week 9	Gene	*blc*	Antibiotic	2
Week 1	Gene	*emrB*	Control	2
Week 5	Gene	*emrB*	Antibiotic	2
Week 9	Gene	*emrK*	Control	2
Week 9	Gene	*emrR*	Control	5
Week 9	Gene	*H NS*	Control	2
Week 1	Class	Macrolide	Control	3
Week 5	Class	Macrolide	Control	3
Day -1	Gene	*Macrolide 2021 Branch*	Antibiotic	3
Week 9	Gene	*Macrolide 2021 Branch*	Control	2
Week 1	Gene	*mdtL*	Control	5
Week 1	Gene	*mefA*	Control	2
Week 9	Gene	*phoP*	Control	2
Day -1	Gene	*tet32*	Antibiotic	2
Week 9	Gene	*tet32*	Control	2
Week 1	Gene	*tetBP*	Control	2
Day -1	Gene	*tetO*	Antibiotic	2
Week 1	Gene	*tetQ*	Control	2

Table B.3. Taxonomic classification of contigs carrying β-lactam resistant genes analyzed with the Contig Annotation Tool (CAT).

Taxa	Count of ACCs
Firmicutes	20
Clostridia	8
Clostridiales	8
Ruminococcaceae	4
Ruminococcaceae bacterium	4
Bacteroidetes	27
Bacteroidia	27
Bacteroidales	27
Bacteroidales bacterium	3
Bacteroidaceae	1
Bacteroides	1
Bacteroides sp.	1
Muribaculaceae	16
Proteobacteria	3
Gammaproteobacteria	3
Aeromonadales	3
Succinivibrionaceae	1
uncultured prokaryote	1

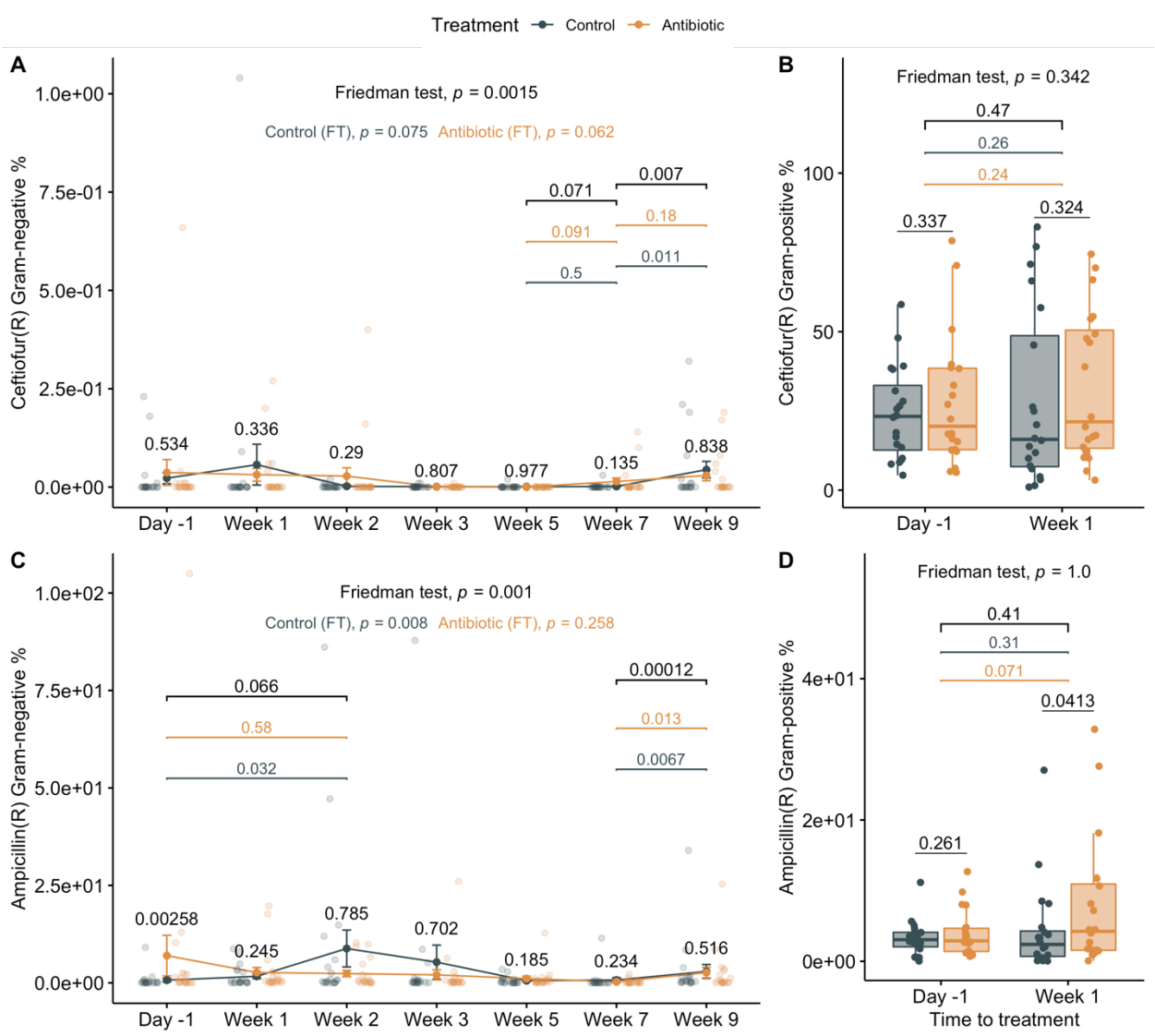


Figure B.1. Percentage of resistant bacteria to ceftiofur (A) Gram-negative and (B) Gram-positive; and resistant to ampicillin (C) Gram-negative and (D) Gram-positive. The percentages were calculated based on the total number of CFU/g of feces.

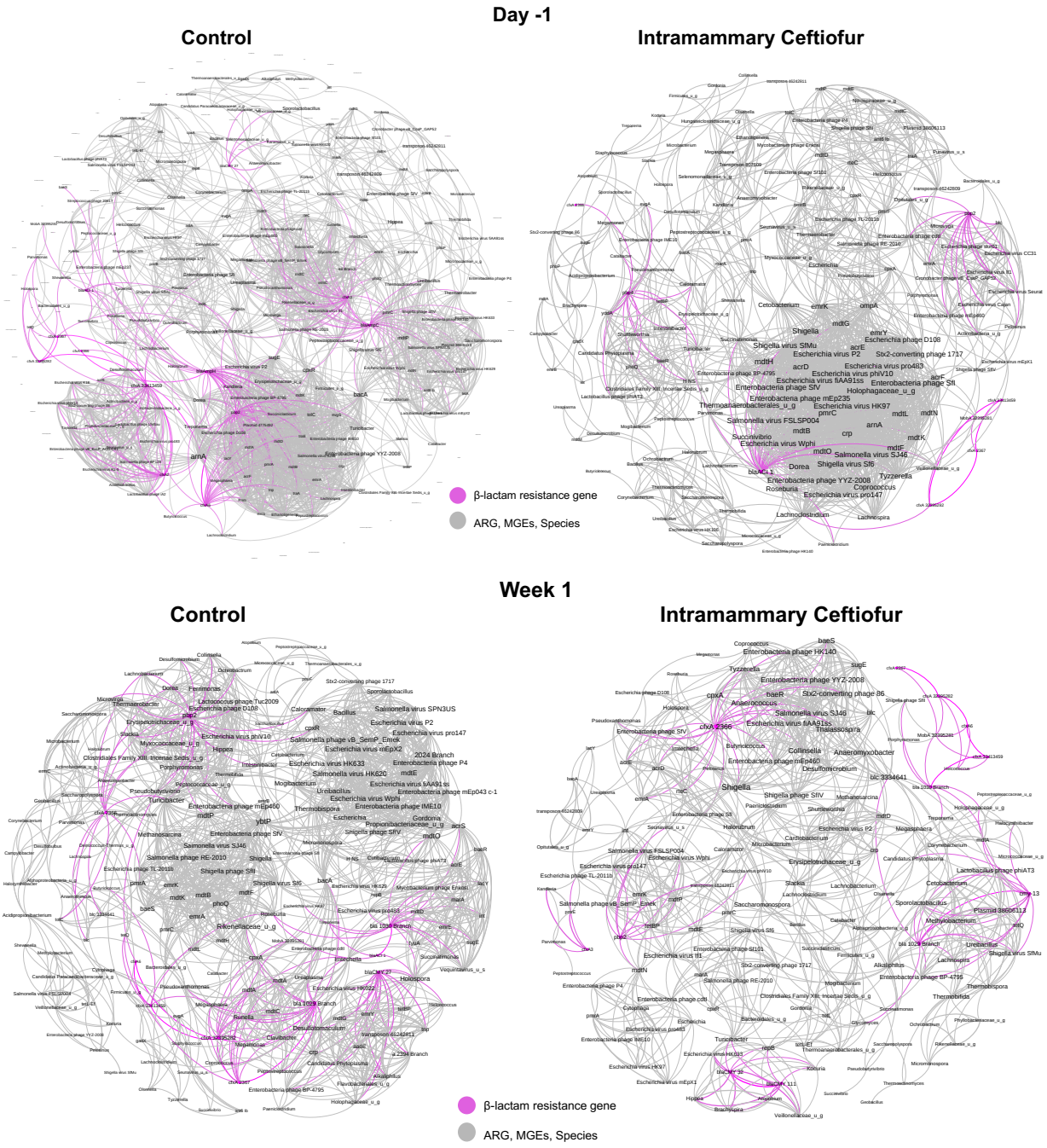
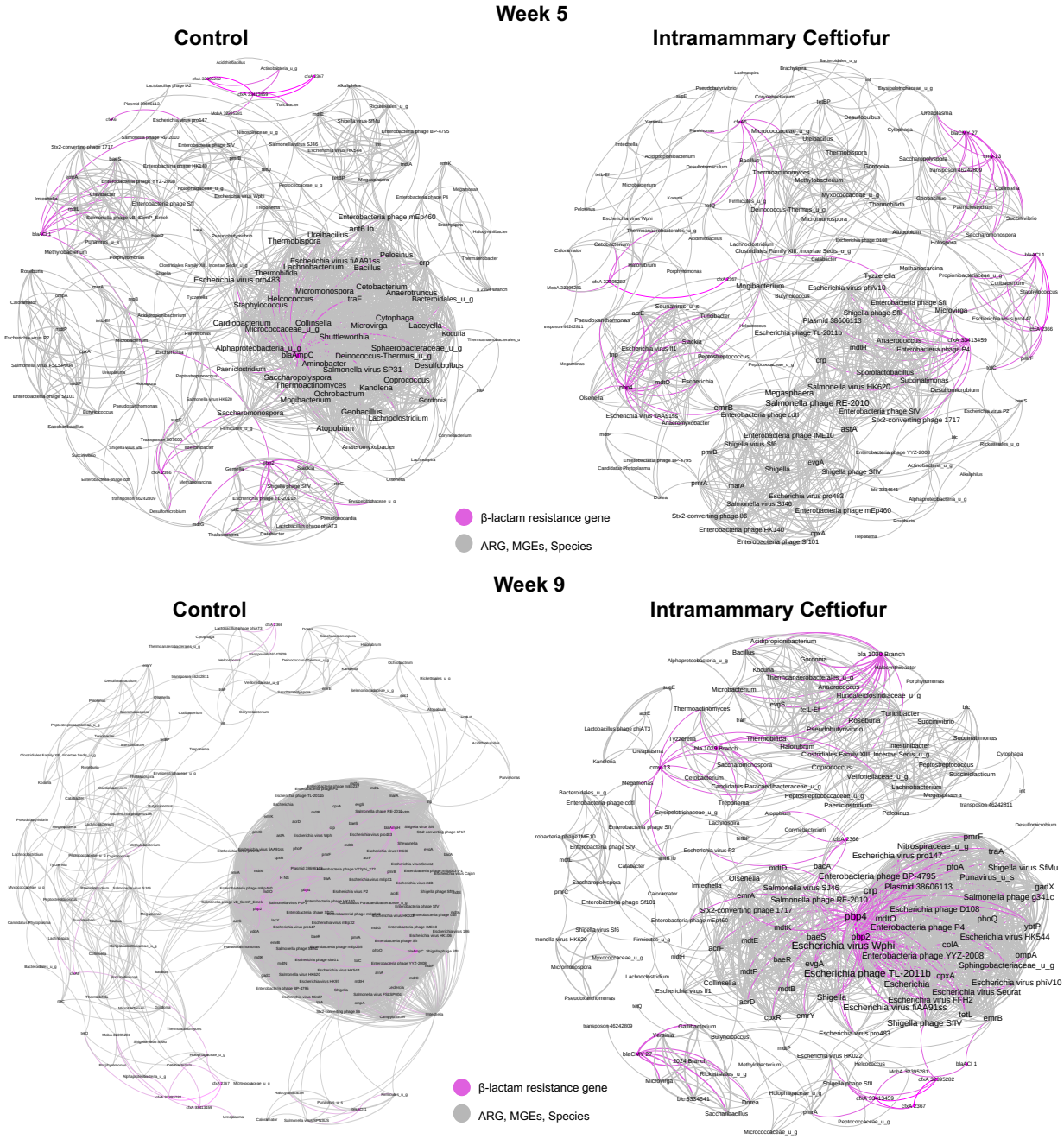


Figure B.2. Correlation networks between β-lactam resistant genes, MGEs, virulence genes, and bacteria genera shown by each sampling point and treatment group. Direct connections between a feature and a β-lactam resistance gene are highlighted in pink, while other correlations are shown in grey. Only significant correlations ($P < 0.01$) with a coefficient >0.75 were included in the network. The size of the edges represents the strength of the of correlation. The size of the nodes corresponds to the degree of centrality.

Figure B.2 (cont'd)



CHAPTER 4

Untargeted metabolomics and microbial-functional profiling of the cattle hindgut associated with antibiotic dry-cow therapy and lactation phase.

ABSTRACT

The gut microbiota in cattle is critical to produce protein, energy, and vitamins. As a result, changes in the microbiota induced by diet, physiology and antibiotic application can affect cattle performance. In this study, the effects of intramammary (IMM) ceftiofur on the functional microbiome and metabolome of feces were evaluated. A total of 40 dairy cows were enrolled; half of them were treated with IMM ceftiofur and a non-antibiotic teat sealant containing bismuth subnitrate (n = 20) and controls were treated only with the non-antibiotic teat sealant (n = 20). Fecal samples were taken a day prior to the IMM treatment, and in weeks 1, 5, and 9 post-treatment, which corresponded to three phases: late lactation, dry-off and fresh cows. Metagenomic sequences were used to predict microbial metabolic pathways with HUMAnN 3.0. Additionally, untargeted metabolomics was carried out to identify polar and nonpolar positive metabolites in feces. Long-term changes in the functional microbiome were observed in cows treated with IMM ceftiofur that included lower production of energy and higher activity of *Bifidobacterium*. The metabolome, however, was identical between treatment groups, though each lactation phase had a different microbial profile and metabolite composition. Cows in late lactation exhibited the highest number of metabolic pathways and the most distinct metabolome associated with a diet rich in metabolizable energy and protein. Multi-omics analyses showed higher levels of histamine-producing bacteria in the lactation phase. Furthermore, positive correlations were identified between natural antimicrobial compounds, pesticides, bacteriophages from enterobacteria, and the gene encoding the extended-spectrum β-lactamase *CFXA2*. These data demonstrate that IMM ceftiofur does not alter the metabolome of the gut, though slight differences were observed in microbiome functionality. Indeed, different lactation phases, which are associated with unique diets and physiology, exhibited important differences in the

metabolome and functionality of resident microbial communities. Importantly, the presence of bacterial stressors such as natural antimicrobial compounds and pesticides, can induce a higher activity of enterobacteria and the mobilization of resistance genes.

INTRODUCTION

Ruminant-gut microbiota produces proteins, vitamins, and over 70% of the energy necessary for their host through an obligatory symbiotic relationship. Rumen microorganisms ferment the plant biomass to generate energy in form of volatile fatty acids (VFAs) [1] and to convert nitrogen-containing compounds into protein [2]. Thus, multiple studies have sought to analyze the cattle microbiome composition to determine its relationship with animal production and methane emissions [3]. By contrast, the effects of antibiotics on the cattle fecal microbiome have been poorly studied [4, 5] and hence, little is known about the impact of antibiotics on the functional gut microbiome and host performance [6].

One of the most common classes of antibiotics used in dairy farms are β-lactams, which are applied to treat foot rot, metritis, mastitis, and respiratory infections [7]. These antibiotics have been shown to be excreted by the biliary system and gut where their active metabolites can alter the microbiota composition [8]. Ceftiofur, a third-generation cephalosporin used for intramammary (IMM) dry-cow therapy at the end of lactation to treat subclinical mastitis [9], can alter the gut microbiota of dairy cows due to activity against Gram-negative and Gram-positive bacteria. Indeed, our previous study showed that IMM ceftiofur treatment altered the abundance of some taxa in the short and long-term within the hindgut despite having no impact on microbiome diversity (Chapter 3). Specifically, we observed a higher abundance of Actinobacteria and Bacteroidetes and lower abundance of Proteobacteria and Firmicutes in ceftiofur-treated versus untreated cattle over a 9-week period. Understanding the function of

these taxa in the gut microbiota can ultimately help identify the effects of antibiotic therapy on cattle performance.

Metagenomics enables the prediction of microbial metabolic capacity based on the detection of genes encoding enzymes and mapping them into metabolic pathways [10]. The characterization of metabolites from host, dietary, and microbiome sources can also provide a better understanding of the functional interactions between the microbiome and environment. Untargeted metabolomics, for instance, is a tool based on liquid chromatography–mass spectrometry (LC–MS) that can simultaneously detect multiple compounds based on their retention time and tandem mass spectrometry fragmentation (MS/MS). Metabolomics of the rumen content from dairy cows has improved the understanding of diet-related metabolism, particularly influenced by the introduction of grain into the diet [11] and helped identify differences between fecal and rumen metabolites [12]. Furthermore, integrated approaches comparing metagenomics, metatranscriptomics, and metabolomics data have been used to analyze the functional microbiome in rumen and its association with feed efficiency [13].

In this study we sought to explore the effects of IMM ceftiofur on the function of the microbiome and metabolome in the hindgut of dairy cows. To identify short- and long-term changes due to antibiotic therapy, samples were taken a day prior to dry-off and in weeks one and nine after the treatment, which were selected based on data generated in our metagenomic analyses (Chapter 3). These three time points represent different phases during lactation including late lactation (day -1), dry-off (week 1), and fresh (week 9). Comparative analyses were carried out between the three stages, which also differ based on diet and physiology of the animals. Indeed, cows in late lactation need a maintenance diet containing high levels of metabolizable protein and energy. During the dry period, however, cows do not produce milk,

which allows the mammary gland and udder tissue to rest and renew before the next lactation. The dry period lasts approximately 60 days prior to calf birth or calving. As opposed to the lactation phase, cows require lower quantities of dry-matter intake and energy in their diet than during the dry-off period. Comparatively, the fresh phase lasts approximately 30 days post-calving and represents the start of the lactation period. Higher levels of energy, calcium, and metabolizable protein are needed in fresh cows as compared to dry cows to compensate for the energy imbalance induced by milk production and low dry-matter intake [14].

Here we applied multi-omics approaches to identify interactions between the microbiome and metabolites present in fecal samples from ceftiofur-treated and untreated dairy cows. Our goal was to understand the potential effects of antibiotics and diet-derived metabolites on the host and its microbiota. The use of metagenomics and untargeted metabolomics enabled the identification of functional microbiome profiles that varied between lactation phases as well as unique connections between metabolites and microbial taxa. Collectively, these findings highlight the potential impact of antibiotics on cattle health and the dispersion of resistant enterobacteria.

MATERIALS AND METHODS

Animals and treatments

Forty Holstein cows were enrolled to analyze the impact of the IMM cephalosporine application on the fecal metabolome and functional microbiome. Half of the cows (n =20) received 500 mg of IMM ceftiofur hydrochloride (CHCL) (SpectramastDC®; Zoetis Animal Health) in each udder quarter at the start of the dry-off period; the remaining 20 cows (controls) did not receive the CHCL IMM treatment. Both the ceftiofur-treated and control cows received a non-antibiotic internal IMM teat sealant containing bismuth subnitrate (Orbeseal®; Zoetis

Animal Health) to prevent mastitis. None of the cows received antibiotic treatment during the last 90 days of lactation and the somatic cell count (SCC) was <150,000 cells/mL at the most recent Dairy Herd Improvement Association (DHIA) test (**Figure 4.1**). Cows treated with IMM antibiotics and controls were matched based on parity and monthly milk production.

Diet analysis

A detailed diet ration report containing nutrient amounts and an analysis of nutrients was facilitated by the Dairy Cattle Teaching and Research Center at Michigan State University (MSU), where the study took place. The software Spartan Dairy 3 was used for diet formulation following the standards of the National Research Council's 2001 Nutrient Requirements of Dairy Cattle (hereafter NRC 01). Hierarchical clustering was applied to analyze patterns of change in the nutrient composition in each lactation phase.

Sample collection

Analyses were performed on fecal samples collected before treatment at the end of lactation when the cows received a maintenance diet (day -1, n = 40), a week after dry-off (week 1, n = 40) when they were given an early dry diet, and during calving (week 9, n = 39) when they were fed with the fresh formulation (**Figure 4.1**). Fecal grabs (n =119) were collected via the rectum using clean obstetric sleeves and were stored in whirl-pak bags for transport to MSU in a cooler as described previously (Chapter 3). Each sample was homogenized by hand and aliquots were stored for DNA and metabolite extractions consisting of 0.25 g of feces in 750 ul of 190 Proof ethanol and 0.25 g of pure feces, respectively. All fecal aliquots were subjected to flash freezing by liquid nitrogen for 1 minute and were stored at -80°C until further processing.

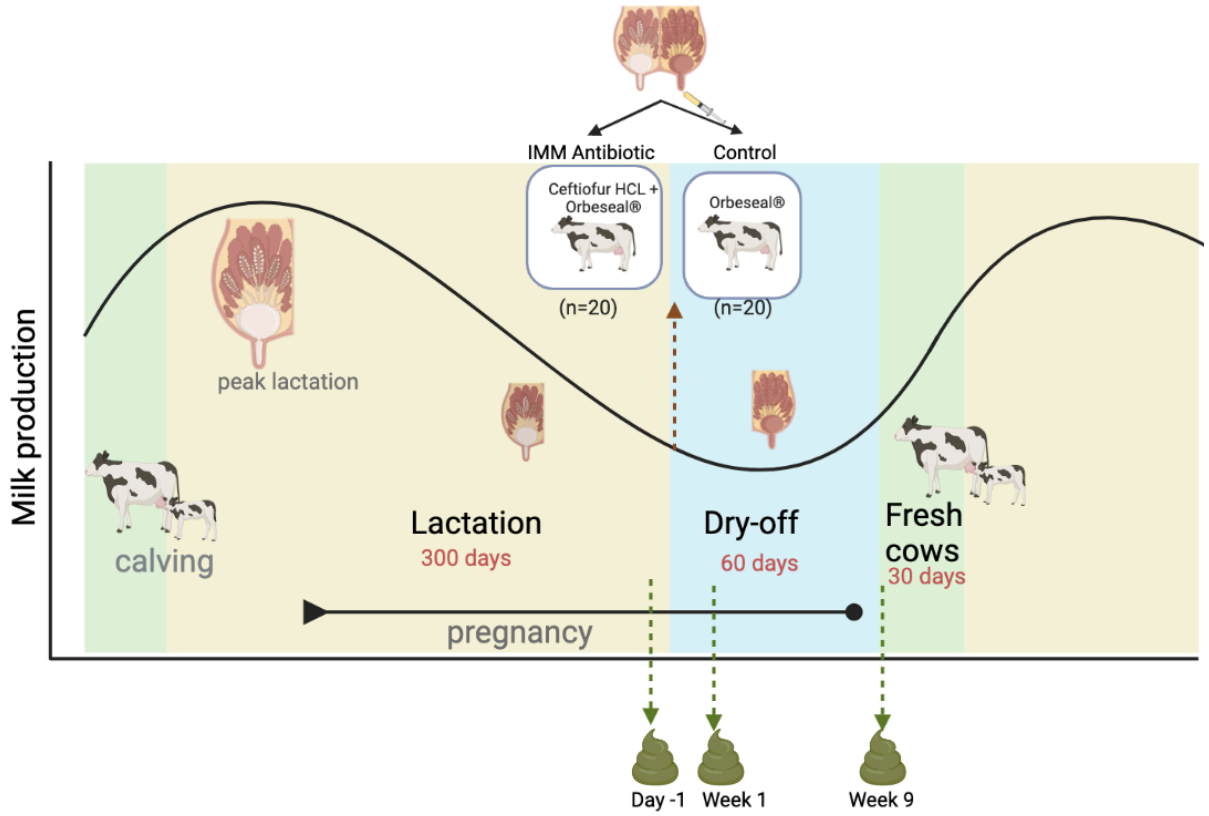


Figure 4.1. Scheme of animal treatment and sampling collection by lactation phase.

Metagenomic sequencing and analysis

Fecal DNA was extracted with the DNeasy PowerSoil Pro Kit (Qiagen, Germantown, MD, USA) as described in our prior study (Chapter 3). Briefly, the samples were centrifuged for 5 minutes at 16,000 rpm and 4°C to remove the supernatant containing ethanol used for preservation. To ensure that the remaining ethanol was removed, two washes with 1 ml of 1X PBS, which was removed as described in the prior step. The DNA-extraction kit protocol was followed with an additional wash step using the C3 solution to improve the DNA-quality ratio (260/230). An average of 1277.3 ng (+/- 310.5 ng) of dsDNA measured with a Qubit were sent to CosmosID (Rockville, MD, USA) for metagenomic Next-generation sequencing (mNGS) using a Nextera™ XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and an Illumina HiSeq X platform 2x150 bp.

The CosmosID bioinformatics software package was used for metagenomics analyses. After the sequences were screened and cleaned for bovine (*Bos taurus*) genome sequences, the reference GeneBook® database [15] was used to characterize the microbiome, resistome and virulome composition. These data are presented in Chapter 3. Abundance scores were determined based on genome size and the number of reads in order to calculate the relative abundance of taxa and genes.

Microbial metabolic profiling

The functional profiling of cattle microbiomes was determined using the HUMAnN 3.0 pipeline [10] that allows for the identification of metabolic pathways with their microbial species-level contributions. These analyses were carried out using the following databases: ChocoPhlAn 3 [10] for taxonomic identification, UniRef90 [16] for enzyme commission number screening, and MetaCyc v24.0 [17] for the assignation of pathways. First, paired-raw sequences were processed with Trimommatic v.0.39 [18] to remove low-quality reads and adapters used for Illumina sequencing. BWA v.0.7.15 [19] and Samtools v.1.4.1 [20] removed bovine DNA reads (*Bos taurus*, ARS-UCD1.2 [21]). Trimmed non-host paired FASTQ reads were merged with the UNIX command cat. Merged reads were used as input for HUMAnN 3.0 and the resulting pathway abundances, reported as reads-per-kilobase (RPK), were normalized as the relative abundance per sample. A joined matrix containing pathway relative abundances for all the samples was generated with the command humann_join_tables. While pathways of interest were depicted with the humann_barplot function stratifying the pathway contributions by bacterial taxa.

Fecal metabolite extraction

A protocol developed by Dr. Daniel Jones at the Mass Spectrometry and Metabolomics Core at MSU was utilized for metabolite extraction of cattle feces (n =119). First, internal standard solutions were prepared for quality control and normalization including: 1) labeled short-chain fatty acids (SCFAs) (10 uM each of [^{13}C]sodium formate, [$^{13}C_2$]sodium acetate, [$^{13}C_3$]sodium propionate, and [$^{13}C_4$]sodium butyrate) in 50:50 (v/v) methanol/water; 2) [$^{13}C_{16}$]palmitic acid (10 uM in 100% isopropanol); 3) phenylalanine-d_7 (10 uM in 50:50 methanol/water); 4) succinic acid-d_4 (10 uM in 50:50 methanol/water); and 5) labeled bile acids (10 uM each of glycocholic acid-d_4 and glycoursodeoxycholic acid-d_4 in 50:50 methanol/water). A total of 20 mg of feces were weighed under sterile conditions and 350 ul of ice-cold methanol containing 0.1% butylated hydroxytoluene (BHT) was added followed by homogenization and incubation on ice for 10 minutes. To sediment the feces, 10 ul of each standard was added, homogenized by agitation for 30 seconds and centrifuged at 10,000 x g and 4˚C for 10 minutes. The supernatant was pipette-transferred to a sterile microcentrifuge tube on ice, while ice-cold HPLC-grade isopropanol (200 ul) was added to the pellet, homogenized for 30 seconds, and centrifuged at 10,000 x g and 4°C for 10 minutes. Finally, the isopropanol supernatant was combined with the initial extract and 100 ul aliquots of the mixed extracts were stored into glass vials inserted in 2-mL Amber glass autosampler vials sealed with 9 mm screw septum caps. Metabolite extracts were preserved at -80°C until analyzed.

Untargeted metabolomics

Polar and nonpolar positive metabolites were analyzed through LC-MS in a Thermo Scientific Vanquish™ Ultra High-Performance Liquid Chromatography (UHPLC) coupled to a Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (MS). Along with the samples

(n =119), blanks and pools were included at the beginning of each run (polar and nonpolar) and every 20 samples. Three blanks were analyzed between polar and nonpolar runs. The Thermo Scientific™ Xcalibur™ software was used for method setup and data acquisition. Polar metabolites were detected with reversed-phase chromatography using 10 ul of each sample injected with a column Waters Acquity Ethylene Bridged Hybrid (BEH)-C18 UPLC (2.1x100mm) at 60°C. A 0.4 ml/min flow rate was used for a gradient analysis that consisted of 98% mobile phase A (water plus 0.1% formic acid) and 2% mobile phase B (acetonitrile plus 0.1% formic acid) for 1 minute; then, phase B was ramped to a 100% until the minute 8 when it was hold for 2 minutes. Finally, the phase B was returned to 2% at 10.01 minutes and hold at that concentration for 2 more minutes.

Nonpolar metabolites were analyzed through hydrophilic interaction liquid chromatography (HILIC). A Waters BEH-Amide UPLC column (2.1x100mm) held at 60°C was used to inject 10 uL of sample. The gradient analysis was carried out at a rate of 0.4 ml/min starting with 100% mobile phase B (10 mM ammonium formate/10 mM ammonium hydroxide in 95:5 acetonitrile/water (v/v)) and 0% mobile phase A (10 mM ammonium formate/10 mM ammonium hydroxide in water) for 1 min. Phase B was ramped to 40% until the minute 8 and hold at this concentration for 2 minutes. Finally, phase B was returned to a 100% at minute 10.01 and hold at this concentration for 2 minutes.

In addition, the MS settings for both methods included: electrospray ionization in positive mode with a capillary voltage of 3.5 kV, transfer capillary temperature at 262.5°C, sheath gas at 50, auxiliary gas at 12.5, probe heater at 425°C, and S-lens RF level at 50. For data acquisition the MS/MS method was set of 35,000 resolution, automatic gain control (AGC) target of 1E6, maximum inject time 100 ms, and m/z range 100-1500. Only the top 5 ions were kept with a

resolution setting of 17,500, AGC target of 1E5, minimum AGC of 5E3, maximum inject time 50 ms, isolation window of 1.5, fixed first mass at m/z 50, dynamic exclusion setting of 3 seconds and stepped normalized collision energy settings of 20, 40 and 60.

Mass-spectrometry (MS) data processing

The chromatographic component separation of fecal metabolites was exported as raw files (.RAW) for each sample with the Thermo Scientific™ Xcalibur™ software. These files were transformed to mzXML format with the Global Natural Product Social Molecular Networking (GNPS) vendor conversion software. Next, MS data processing was performed using MZmine v.2.53 [22] where polar and nonpolar files were analyzed separately. First, mzXML files were imported to MZmine for mass detection at the levels MS1 and MS2 (centroided spectrum type) using a noise level of 4E04 for MS1 and 3.5E03 for MS2, which was set based on visual analyses of chromatograms from pools and blanks. Then, chromatograms were built with the ADAP (Automated Data Analysis Pipeline) [23] module using a scan retention time of 1.00 – 10.00 min for MS level 1, minimum group size in number of scans equal to 4, group intensity threshold of 4.0E4, minimum highest intensity of 5.0E4, and scan to scan accuracy of 0.002 m/z or 10.00 ppm.

Chromatograms were smoothed with the Savitzky Golay algorithm with a filter width of 5 and deconvoluted with local minimum feature resolver. The deconvolution settings included MS/MS scan pairing with a retention time tolerance of 0.15 absolute min and MS1 to MS2 precursor tolerance of 0.002 Da. Additionally, the deconvolution algorithm was set up with a chromatographic threshold of 83.3999%, minimum search range RT/Mobility (absolute) of 0.05, minimum relative height of 0.0%, minimum absolute height of 5.0E4, min ratio of peak top/edge 1.80, and peak duration range (min/mobility) 0.00 – 1.51. Isotopes were grouped with a m/z

tolerance of 0.0015 m/z or 3.0 ppm, a retention time tolerance of 0.05 absolute mins, and a maximum charge of 2 while choosing the most intense representative isotopes. Next, an aligned feature list containing data from all samples was generated with module join aligner using a tolerance or 0.0015 m/z or 5.0 ppm, weight for m/z of 3, retention time (RT) tolerance of 0.1 absolute min, and weight for RT of 1. Gaps in the aligned list were filled with the module peak finder using an intensity tolerance of 20%, an m/z tolerance of 0.002 m/z or 10.0 ppm, and a retention time tolerance of 0.05 absolute min. Duplicate peaks generated during gap filling were removed at a m/z tolerance of 8.0E-4 m/z or 1.5 ppm and a RT tolerance of 0.035 absolute (min). To obtain a final feature list containing only features present in at least three samples, the module 'feature list rows filter' was used with at least 3 peaks in a row, keeping only peaks with MS2 scan, and resetting the peak number ID. Finally, the feature list was exported for analyses in GNPS for the Feature-Based Molecular Networking (FBMN) workflow using filter rows only with MS2. The exported files consisted of a feature quantification table (.CSV format) and a MS/MS spectral summary file (.MGF format) with a list of MS/MS spectra associated with the LC-MS ion features.

Metabolite classification

The FBMN workflow was carried out in GNPS [24, 25] by importing the MGF file and the feature quantification table generated in MZmine, as well as the metadata containing the sample attributes. Precursor ion mass and fragment ion mass tolerances were set at 0.02 Da. Additionally, default settings were used for the advanced options except for minimum matched fragment ions for networks and library search min matched peaks which were set at 4. All the spectra with IDs were downloaded; library ID and network component index were recorded for

each metabolite also referred to as ‘cluster’ for further analyses. Molecular networks were visualized in GNPS to explore metabolite components and identify clusters of interest.

Metabolome data analyses

The R package Phyloseq v.1.38 [26] was used to analyze metabolomics diversity and composition. First, a Phyloseq object was generated by merging metadata, feature table with cluster intensities, and cluster identifications which included three levels: network component, library ID, and cluster numbers. The package decontam v.1.14 [27] was used to remove contaminant clusters based on a combined method that uses the Fisher’s test to concatenate the probabilities of a cluster being present in a sample based on the amount of feces used for the metabolite extraction and the prevalence of a given cluster in negative controls vs. samples. Cluster intensities were normalized to their relative abundances per sample.

Diversity analyses

The alpha diversity was measured with Shannon and richness indexes. The paired, one-tailed Wilcoxon signed-rank test was used to compare the alpha diversity between treatments and time points, while the Friedman test examined the variability of the indexes per animal over time. Differences in the composition were evaluated using beta-diversity analyses based on Bray-Curtis dissimilarity distances, which were mapped with Principal Coordinate Analyses (PCoA) using the R packages Vegan v.2.5-7 [28] and ggplot2 v.3.3.5. The mean compositions, represented by the centroid of each group of samples (treatment and phase) in the PCoA, were compared with permutational multivariate analysis of variance (PERMANOVA) with 999 permutations, and the dispersion was compared with PERMDISP.

Biomarker detection

The identification of significantly different features between treatment groups and lactation phases (time points) was evaluated with three methods: 1) Linear Discriminant Analysis (LDA) Effect Size (LEfSe) [29]; 2) Analysis of compositions of microbiomes with bias correction (ANCOM-BC) [30]; and 3) Microbiome Multivariable Associations with Linear Models (MaAsLin2) [31], as suggested in a prior study [32]. Features that were significantly different (adjusted p-value ≤ 0.05) in at least two of the three tests were considered true biomarkers. In addition, Random Forest with 5000 decision trees was used to estimate the out-of-bag error (OOB) rate that allows for correctly classifying the sample groups based on the metabolite composition. Random Forest was also used to predict important features (clusters and components) based on their discriminatory levels between sample groups, which were ranked based on their mean decrease accuracy (MDA).

Multi-omics analyses

Associations between the fecal microbiome and metabolome were carried out by correlating the relative abundances of known metabolites (with library ID) with microbial taxa at the phylum and species levels, antimicrobial resistance genes (ARGs), virulence genes, and microbial metabolic pathways across samples. Spearman correlations were calculated with the R package Hmisc [33], and coefficients (ρ) greater than 0.75 with p-values lower than 0.01 were filtered to construct networks with Gephi v.0.9.2 [34].

To characterize patterns of change in the abundance of microbial and metabolic features, hierarchical clustering was carried out with the R package stats v4.1.2 [35]. Only features identified as biomarkers (n = 684) in the previous step were included in this analysis. First, a distance matrix was constructed with the euclidean metric using the fold-change (FC) relative to

each feature average per sample. The FC was calculated by computing the mean abundance of each feature across samples, and by dividing the abundance of a feature from a given sample by their corresponding mean. The distance matrix was used for hierarchical clustering with the Ward method (ward.D) and the resulting tree was conveniently cut into 30 clusters. The optimal number of clusters was identified with the NbClust v3.0.1 package [36], which resulted in 5 clusters; however, a finer analysis of each branch was biologically more meaningful. Finally, boxplots of each hierarchical clustering group were depicted to observe the patterns of change between lactation phases. All metagenomics and metabolomics methods have been summarized schematically in **Figure 4.2**.

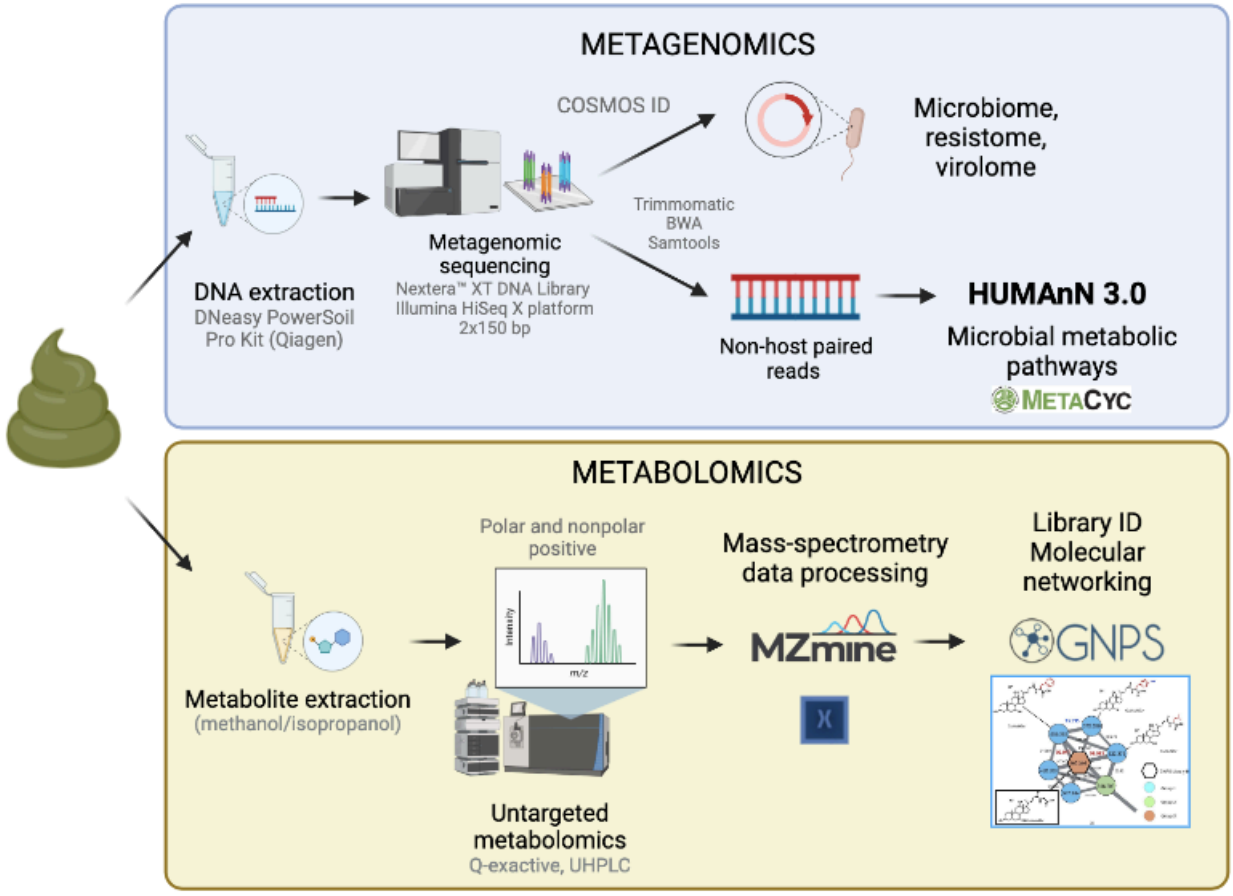


Figure 4.2. Summary of the methodology applied to analyze the functional microbiome and metabolome composition from cattle feces.

RESULTS

Overview of the study population

In this study, a total of 40 cows were enrolled at the end of lactation with average days in milk (DIM) of 266.24 (antibiotic group, *mean* = 262.69; control, *mean* = 269.59). Mastitis was rule out in these cows through the somatic cell count (SCC) in milk, which had an average of 34,8718 +/- 23,602 cells/mL (antibiotic group, *mean* = 35,300 cells/mL; control, *mean* = 34,4211 cells/mL). Fecal samples from all animals were collected through the 9-week period, except for one cow from the IMM ceftiofur-treated group that had a C-section in week 9, hence no sample was taken from this animal in that week. Metagenomic sequencing was carried out in samples collected the day prior to dry-off and on weeks 1, 5, and 9 after the treatment (n = 159). Since our prior study (Chapter 3) showed that the microbiome diversity and composition in weeks 1 and 5 were similar, untargeted metabolomics was performed on samples from day -1, week 1 and week 9 (n = 119).

Diet composition

Different diets were provided to the enrolled cows throughout the study in accordance with their production demands. During lactation, which corresponds to a day prior to the IMM treatment, cows received the maintenance diet with 14% more metabolizable energy and 2.5 times more metabolizable protein (g) than the dry-off diet (weeks 1 – 5) (**Table 4.1; Figure C.1**). In week 9 animals received a diet for fresh cows where they were given 64% of as-fed intake (lb) when compared to lactating cows (**Table 4.1**) but with transitioning levels of energy and protein that were 15% and 64% higher than during dry-off, respectively (**Figure C.1**). Animals in all phases received corn silage, soybean meal with 47.5% crude protein, CFE MSU dairy base, and haylage (**Table 4.1**). It was only during the lactation and fresh periods that the

ration included corn (ground fine and fed dry) and MSU fresh high supplement to increase the energy density and to provide essential nutrients such as calcium, magnesium, potassium and niacin to prevent metabolic disorders that can occur during the transition into lactation. Comparatively, soybean hull pellets, cottonseed, and long bulk brown midrib (BMR) corn silage (CS) were exclusively given to cows in late lactation. Moreover, alfalfa hay was only provided to fresh cows; while grass silage, MSU straw, grass pasture, and QLF ignite dry cow 25 were given only during the dry period (**Table 4.1**).

Table 4.1. Diet rations provided to dairy cows in four different lactation stages.

Ration component	Maintenance	Early dry	Close-up	Fresh
As-Fed lbs	107.51	71.82	43.82	69.08
DM Fed lbs	53.00	28.60	28.10	35.05
Corn grain ground fine (DM fed lbs)	9.50	0	0	5.00
Corn gluten feed dry (DM fed lbs)	4.00	0	0	1.00
Soybean Hulls Pellet (DM fed lbs)	4.50	0	0	0
Soybean meal 475 solvent (DM fed lbs)	2.50	2.10	6.00	3.00
Cottonseed Fuzzy (DM fed lbs)	3.00	0	0	0
MSU Corn silage (DM fed lbs)	9.00	9.00	8.38	12.30
MSU Haylage (DM fed lbs)	9.00	8.90	0	6.00
CFE MSU dairy base (DM fed lbs)	1.00	0.60	0.70	0.80
MSU Long bunk BMR CS (DM fed lbs)	8.00	0	0.00	0
MSU fresh high supplement (DM fed lbs)	2.50	0	0.00	2.35
MSU Purchased Alfalfa Hay (DM fed lbs)	0	0	0.00	4.60
CFE MSU PreFresh DE (DM fed lbs)	0	0	1.00	0
MSU Low K Grass Hay (DM fed lbs)	0	0	10.50	0
SoyChlor (DM fed lbs)	0	0	1.52	0
MSU grasslage (DM fed lbs)	0	6.00	0	0
MSU Straw (DM fed lbs)	0	2.00	0	0
Grass Pasture 16 CP 55 NDF 7 LNDF (DM fed lbs)	0	0.00*	0	0
QLF Ignite Dry Cow 25 (tub) (DM lb)	0	0.00*	0	0

DM = Dry matter; NDF = Neutral Detergent Fiber; LNDF = Lignin as a percent of the NDF; CP = Crude Protein; CS = Corn silage; BMR = brown midrib; *Provided only to dry cows.

Untargeted metabolomics data overview

Overall, mass-spectra analyzed through MZmine resulted in twice the amount of nonpolar (n = 11,199) than polar (n = 5,390) metabolite clusters (**Figure 4.3A**). Each cluster corresponds to consensus MS^2 spectra from identical compounds detected across samples

(**Figure 4.3B**). Similarly, molecular networks aggregating metabolites based on their MS^2 spectral similarity resulted in 2,604 nonpolar and 1,149 polar components (**Figure 4.3A**). Network components connect clusters (nodes) structurally related via edges that represent a modified cosine score calculated on ions that differ by the mass difference (**Figure 4.3B**). Only a small fraction of clusters had library identifications, corresponding to 2.48% of the total metabolites (polar, n =138; nonpolar, n =273), of which 68 were identified with both polar and nonpolar modes. FBMN data processed through GNPS is available online for polar and nonpolar metabolites (https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=d4a761f0a6be422c8b89db9408f57b0d and https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=1d6f7e95d2f04f96a94fede8c195702d, respectively).

Figure 4.3. Number of metabolite clusters, network components, and metabolites with identification. A) Bar plot showing the number of clusters identified with MZmine, components grouped with FBMN, and clusters with library ID assigned with GNPS pipelines. B) Scheme of a network component containing clusters with and without identification. A plot depicting MS/MS spectra shows how clusters are aggregated.

Number of microbial metabolic pathways identified with HUMAnN 3.0

Across samples, 262 metabolic pathways were identified. These pathways were assigned to bacterial taxa, producing 797 pathways with different bacterial contributions. Only nine bacterial genera were assigned to the pathways, including *Bifidobacterium* (pathways, $n = 75$), *Clostridium* ($n = 4$), *Escherichia* ($n = 25$), *Methanobrevibacter* ($n = 18$), *Olsenella* ($n = 2$), *Ruminococcaceae* unclassified ($n = 26$), *Sarcina* ($n = 12$), *Turicibacter* ($n = 12$), other ($n = 262$), and unclassified ($n = 200$). On average, 93% of the reads were classified as unmapped and 6% as unintegrated.

Alpha diversity of metabolome and microbial pathways

The within-sample diversity was measured with the number of observed features and the Shannon index. Significantly higher diversity of metabolites and microbial-metabolic pathways were detected during lactation than during the dry-off and fresh stages ($p < 0.05$) (**Figure 4.4**). Fresh cows exhibited a similar number of metabolites and microbial pathways as when they were dry (*Observed*, $p > 0.05$) but with lower evenness (*Shannon*, $p < 0.006$). Although the metabolite alpha diversity was similar between fresh and lactating cows, the Shannon index showed lower diversity in the fresh phase denoting a transition like the one detected in their diet composition (**Figure 4.4**). Curiously, the alpha diversity of metabolic pathways was more similar between dry and fresh phases than between fresh and lactating cows despite the similarity in diet between these last two phases.

No significant differences in alpha diversity were observed between treatment groups, except for the number of metabolic pathways on week 5 of dry-off, when controls had significantly higher diversity than cows given IMM ceftiofur ($p < 0.001$) (**Figure 4.4**). Because samples from week 5 were not analyzed with untargeted metabolomics, we could not compare

between treatment groups at this time point. Nonetheless, no long-term effects in the number of metabolites were observed on week 9 (fresh cows).

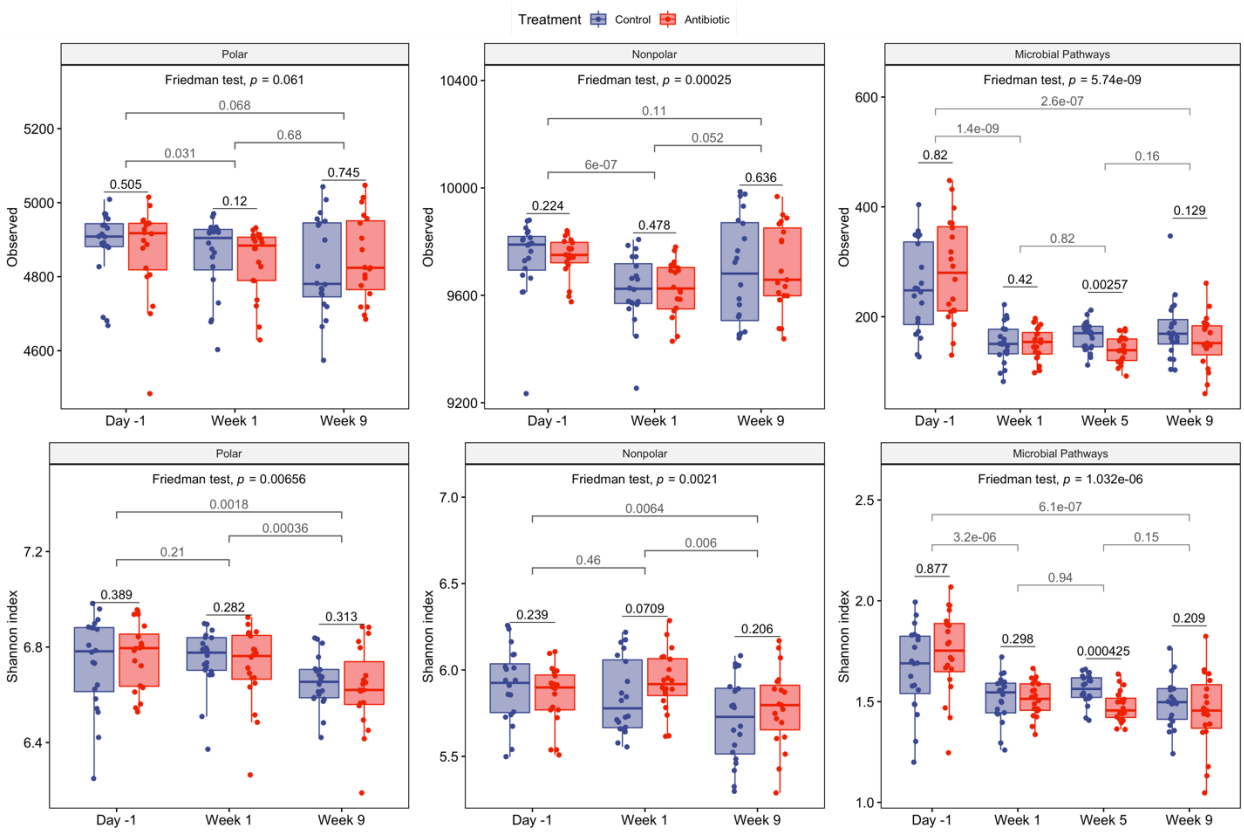


Figure 4.4. Alpha diversity of metabolites and microbial pathways.

Beta diversity of metabolome and microbial pathways

Bray-Curtis dissimilarity distances showed significant differences between lactation phases for polar and non-polar metabolome composition comprised of all metabolite clusters (PERMANOVA, $p < 0.001$) (**Figure 4.5A**). The microbial pathways had overlapping composition between dry and fresh phases (PERMANOVA, $p > 0.3$), however, samples from lactating cows showed a significantly higher dispersion in the PCoA (PERMDISP, $F = 53.32$, $p = 1.34e\text{-}11$) and different average composition (PERMANOVA, $F = 63.69$, $p = 0.001$) (**Figure 4.5B**). Thus, despite the metabolome composition differences associated with the lactation phase,

the microbial metabolic pathways were similar in dry and fresh cows. Furthermore, cows treated with IMM ceftiofur had an identical mean metabolite composition as the controls (PERMANOVA, $p > 0.38$), though differences in the composition of the microbial pathways were observed in weeks 5 (PERMANOVA, $F = 4.25$, $p = 0.007$) and 9 (PERMANOVA, $F = 2.67$, $p = 0.045$). These findings demonstrate that antibiotic dry-cow therapy does not significantly impact the metabolome at the time points examined but does result in some long-term changes in the mean composition of microbial pathways.

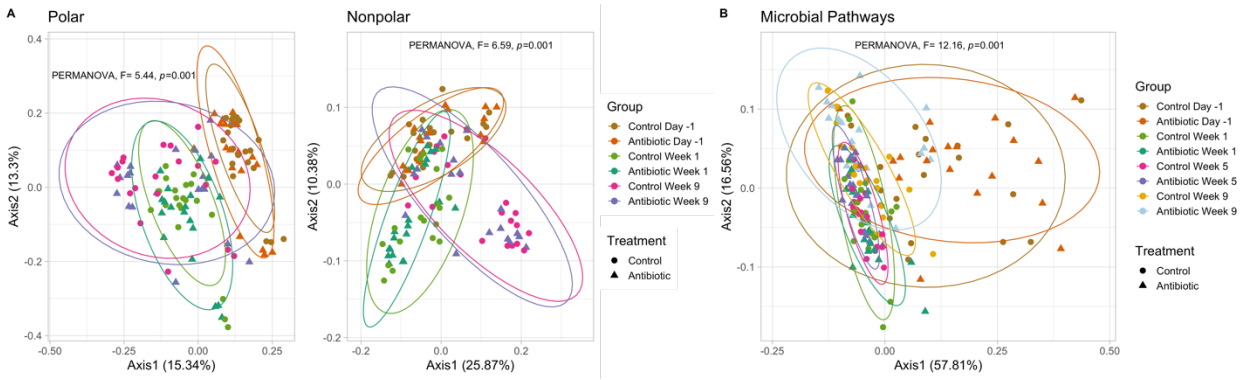


Figure 4.5. Beta diversity of metabolites and microbial pathways.

Biomarker identification

After comparing the abundance of a total of 16,589 metabolites, 3,753 components, and 797 microbial-metabolic pathways, only 1 biomarker was significantly different between controls and cows treated with intramammary ceftiofur in week 1 after the treatment. This corresponded to the metabolite cluster 6574 with a parent mass of 422.206 and a precursor charge of 1 (**Figure C.2**). This cluster is not identifiable and was not part of a network component, thereby limiting the understanding of its occurrence in ceftiofur-treated animals. Random-Forest (RF) was not able to correctly classify the metabolomic composition by treatment group at any time point, giving an out-of-bag (OOB) estimate of error rate higher than 55%. Likewise, the OOB error rate for the microbial pathways was 55% for day -1 and week 1,

47.5% for week 5, and 41.3% for week 9. Given the differences in composition in weeks 5 and 9 observed in the PCoA, a less restrictive analysis of metabolic pathways was performed. To do so, we included pathways that were significantly different with a non-corrected p-value less than 0.05 in two or more tests, and could explain differences between groups through RF. Use of these criteria identified 16 pathways to be significantly lower in week 5, including those involved in the biosynthesis of L-isoleucine, glycogen, isoprene, preQ0, chorismate, and coenzyme A, as well as in the degradation of D-galactose and L-arginine. The most significant biomarkers in week 5, however, were unintegrated pathways of *Ruminococcaceae bacterium P7* and methylerythritol phosphate pathway I, which were lower in ceftiofur-treated cows. In week 9, 38 pathways showed differences between treatments, of which 17 were assigned to *Bifidobacterium pseudolongum* that was higher in cows treated with antibiotics. The most important differential pathways identified in week 9 were a lower abundance of coenzyme A biosynthesis and glycogen degradation II in the ceftiofur-treated vs. untreated cows.

Among lactation phases, the OOB error was 1.68%, as the metabolomes of two fresh cows were misclassified as lactating cows. Hierarchical clustering of the 50 most important metabolite components identified with RF showed a transitional composition in fresh cows between the dry and lactation phases (**Figure 4.6**). Only four of these 50 components had known clusters representing long-chain fatty acids, which were higher in dry-off, and amino acids that were increased during lactation (**Figure C.3**).

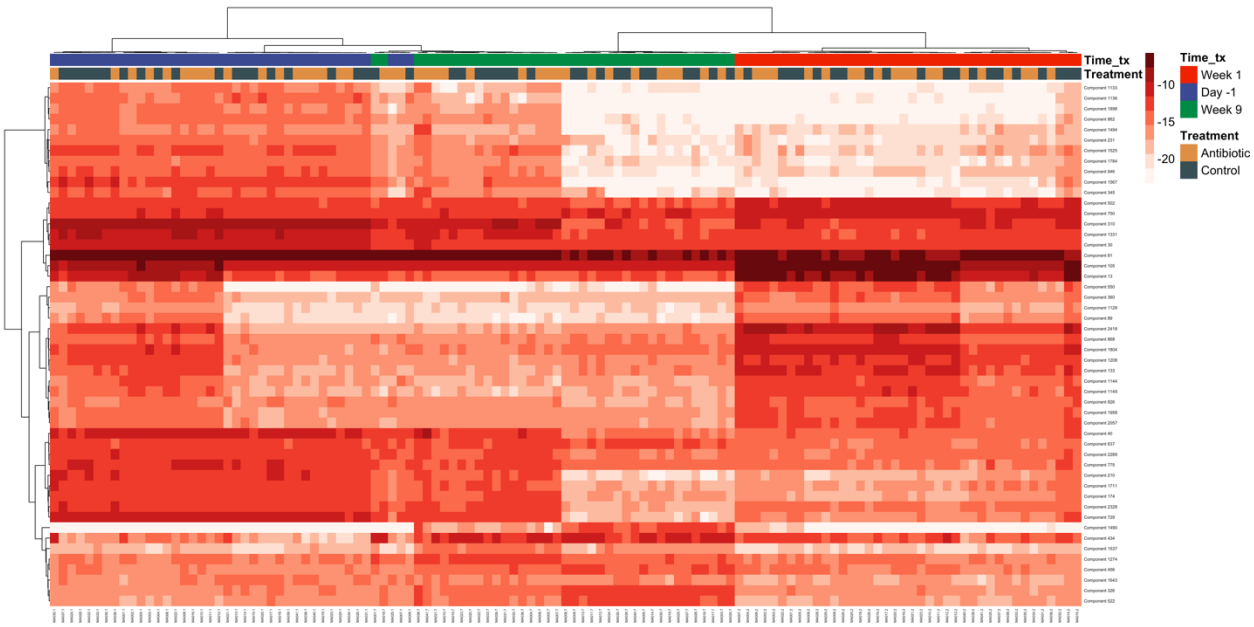


Figure 4.6. Heat map of the 50 most important nonpolar metabolite components for classifying samples by time point. Hierarchical clustering with the method Ward was used to aggregate samples and components.

Differential abundance tests identified 9,850 biomarkers between lactation phases corresponding to 46.59% of the total features (metabolites and microbial pathways) (**Figure 4.7**). Dry and fresh cows showed a lower number of different features (53.88% of the biomarkers) than dry and lactating cows (64.64%) or lactating and fresh cows (66.9%). In particular, dry and fresh cows differed in only a few microbial pathways, whereas approximately a third of the microbial metabolic pathways had a different abundance during lactation than in the other phases.

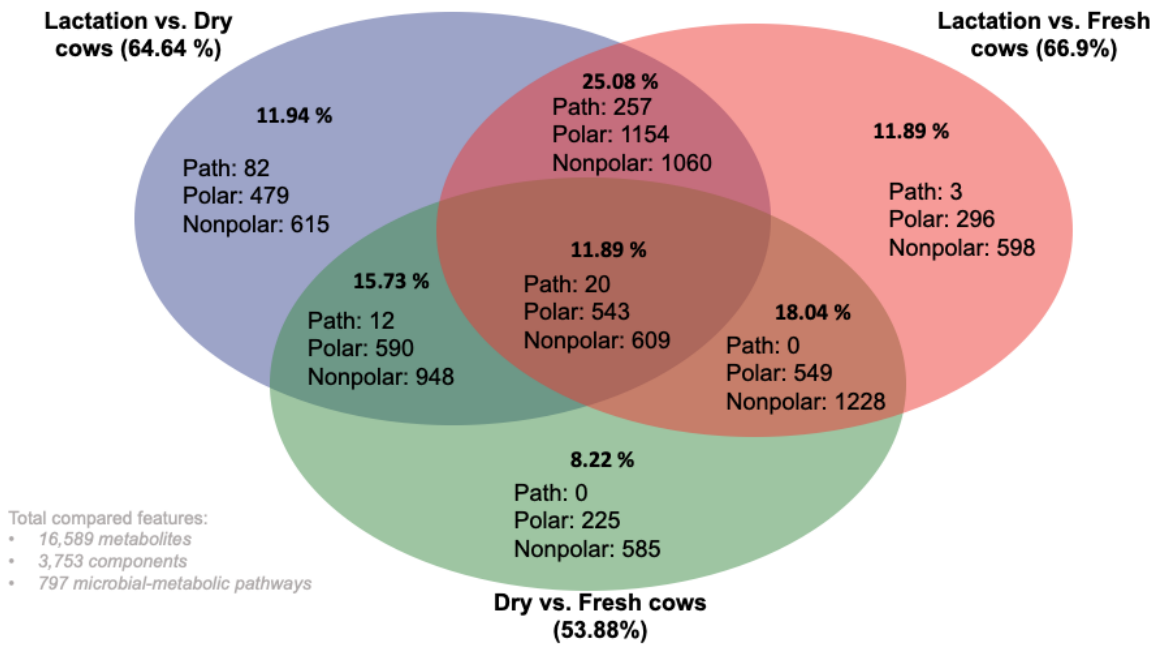


Figure 4.7. Venn diagram showing the number of biomarkers shared between lactation phases. Percentages represent the proportion of the total differential features.

The top 8 most important pathways that enabled lactation phase classification through RF corresponded to three categories: 1) cell division, 2) amino acid biosynthesis, and 3) carbohydrate biosynthesis. These pathways were significantly higher during lactation, particularly those related to cell division that were mostly absent during the dry-off period. Cell-division pathways included inosine-5'-phosphate biosynthesis III, pyrimidine deoxyribonucleotides de novo biosynthesis IV, UDP-N-acetyl-D-glucosamine biosynthesis I, and O-antigen building blocks biosynthesis (*E. coli*) which were assigned to *Bifidobacterium, Turicibacter, Olsenella, and Escherichia coli* (**Figure 4.8**). The three main pathways related to amino acid biosynthesis involved the superpathway of L-lysine, L-lysine biosynthesis VI, L-valine biosynthesis, and L-threonine and L-methionine biosynthesis II (**Figure 4.9**). During lactation, these amino acid biosynthesis pathways were mainly assigned to *Bifidobacterium* spp., while no taxa was assigned to them during the dry and fresh phases.

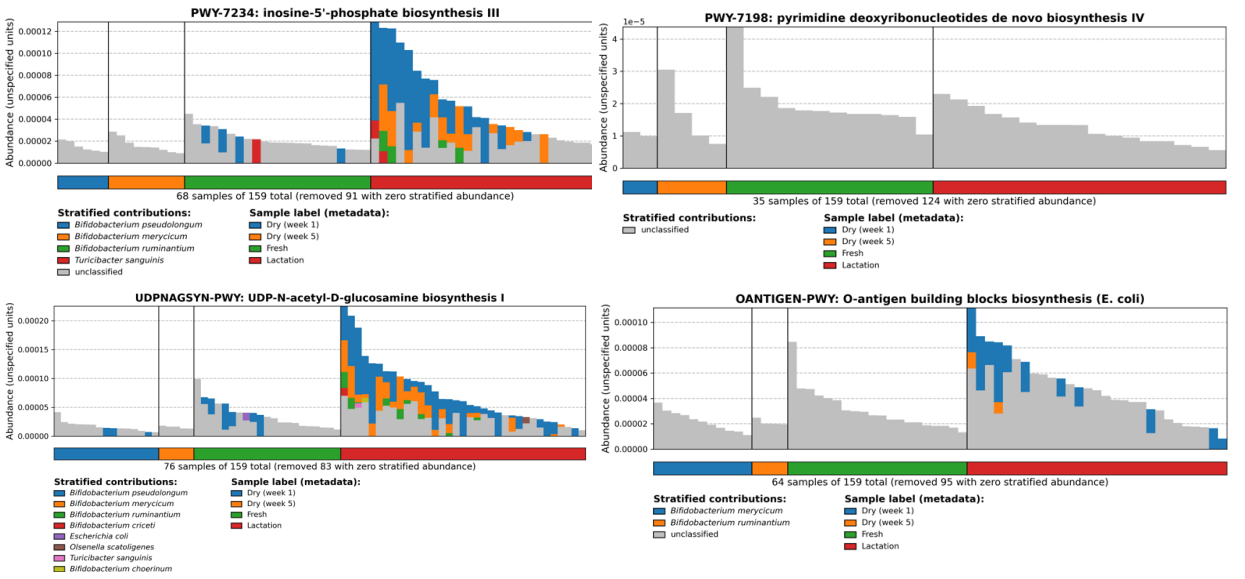


Figure 4.8. Cell-division-related pathways significantly different between lactation phases.

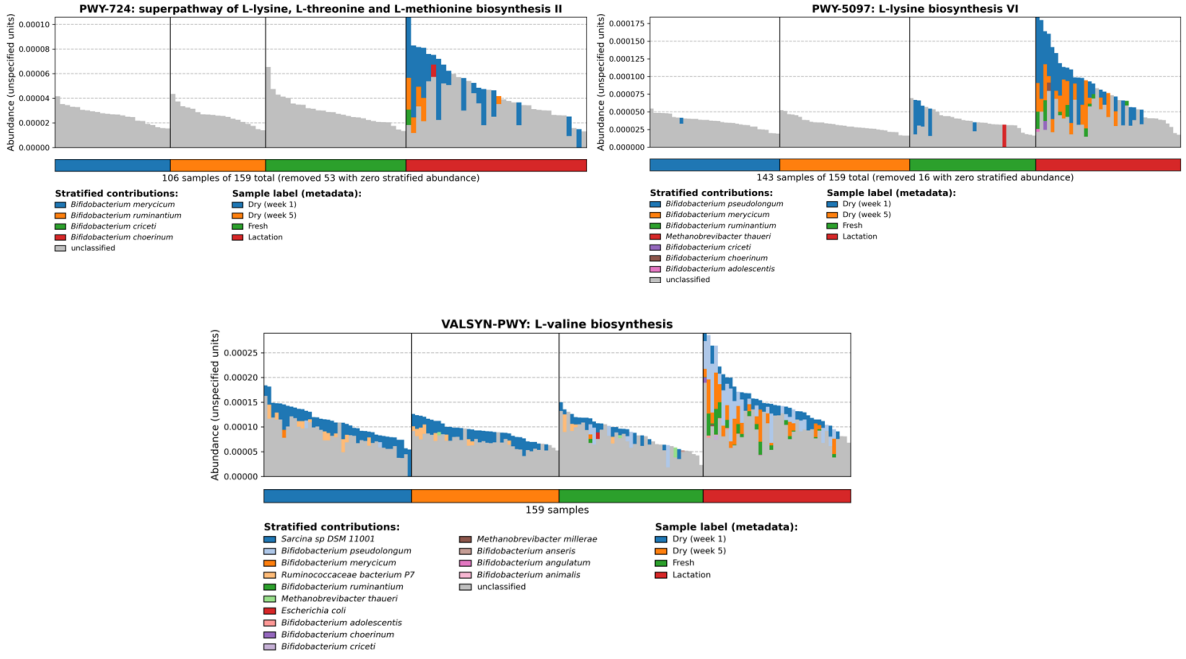


Figure 4.9. Amino acid biosynthesis pathways significantly different between lactation phases.

Finally, carbohydrate biosynthesis pathways included glycogen biosynthesis I (from ADP-D-Glucose), sucrose biosynthesis II, and chorismate biosynthesis I (**Figure 4.10**). Although *Sarcina* was the main taxa assigned to glycogen and sucrose biosynthesis, during the dry-off and fresh periods, *Bifidobacterium* was mainly associated with carbohydrate biosynthesis during lactation.

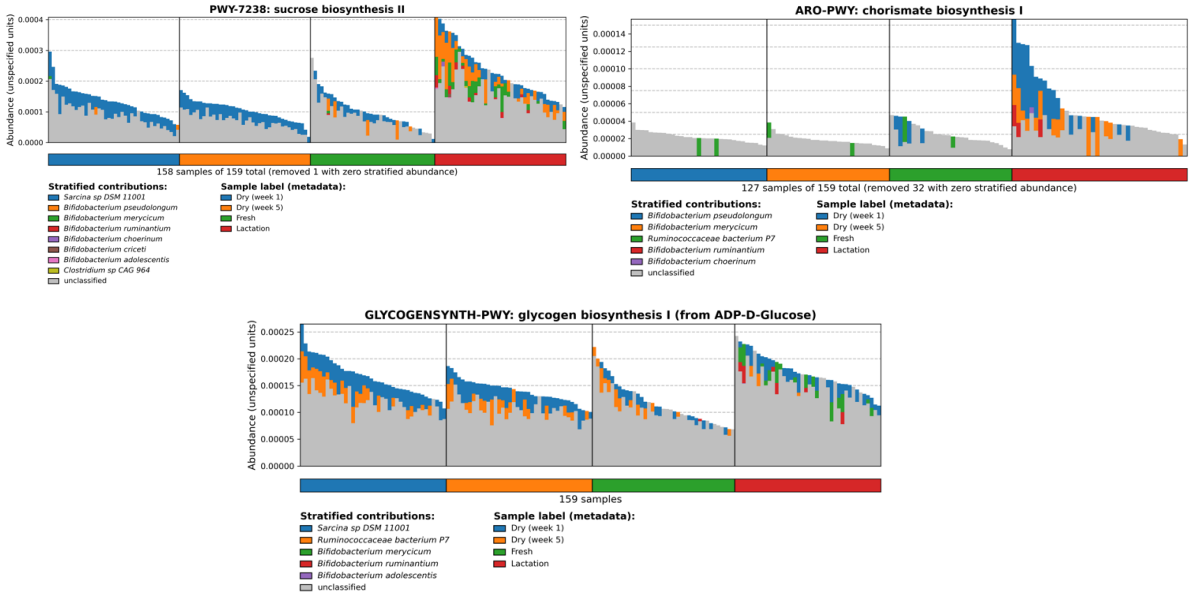


Figure 4.10. Carbohydrate biosynthesis pathways significantly different between lactation phases.

Multi-omics correlations

Positive Spearman's correlations among metabolites, microbial pathways, microbial species, and genes encoding antimicrobial resistance and virulence factors were analyzed. Interestingly, three nonpolar metabolites with antibiotic properties, monolinolenin, beauvericin [+NH4+], and neobavaisoflavone, were directly connected to bacteriophages from enterobacteria including *Escherichia*, *Salmonella*, and the extended-spectrum β-lactamase (ESBL) gene *CFXA2* (**Figure 4.11**). Monolinolenin and beauvericin are produced by fungi and have activity against Gram-positive bacteria, while neobavaisoflavone is produced by plants and has activity against

Gram-negatives. Two fungal species, *Byssochlamys nivea* and *Trichoderma atroviride* were correlated with these compounds suggesting a potential role in the occurrence of beauvericin and monolinolenin. The lowest levels of these two compounds were observed during the dry-off period (**Figure 4.12**), whereas the level of monolinolenin was steady over time. Furthermore, these three antimicrobial metabolites were correlated with metabolic pathways for amino acid, nucleotide, and sucrose biosynthesis from *Clostridium* and *E. coli* (**Figure C.4**). Similarly, the pesticide piperonyl butoxide was related to three *Salmonella* and two *E. coli* bacteriophages (**Figure 4.13A**). This compound was significantly higher in fresh cows as compared to both the lactating and dry cows (**Figure 4.13B**). Fruchterman Reingold networks showing other correlations between microbiome and polar metabolites as well as nonpolar metabolites are shown in **Appendices 5** and **6**, respectively. Finally, a module network containing multiple ARGs, virulence factors, plasmids, bacteriophages, and microbial pathways suggest the co-occurrence of these features in a multi-resistant *E. coli* (**Figure C.7**).

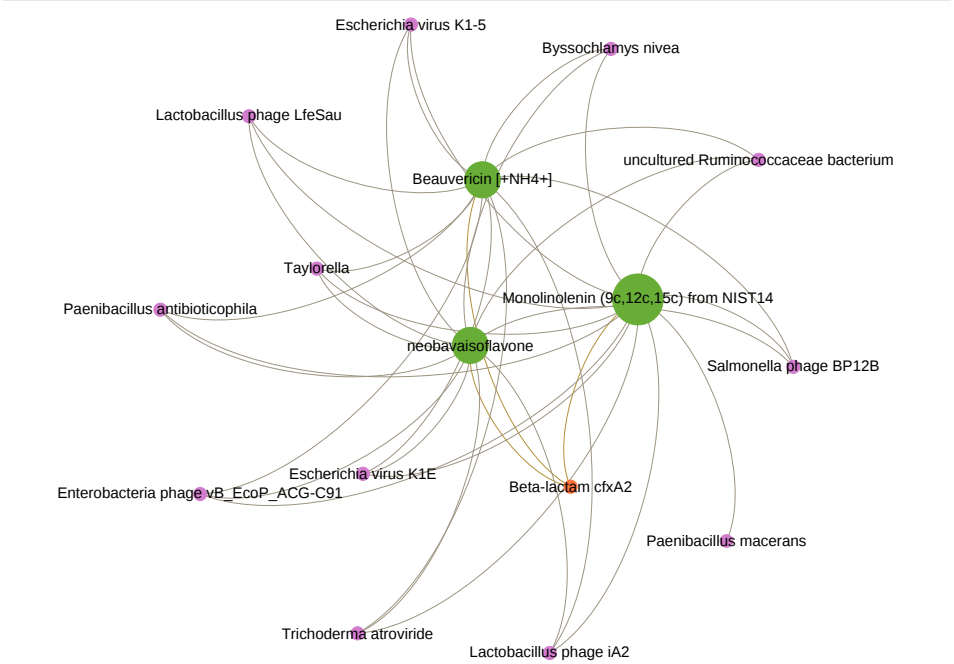


Figure 4.11. Correlation network of a module containing three antimicrobial compounds. The size of the nodes corresponds to the degree of centrality.

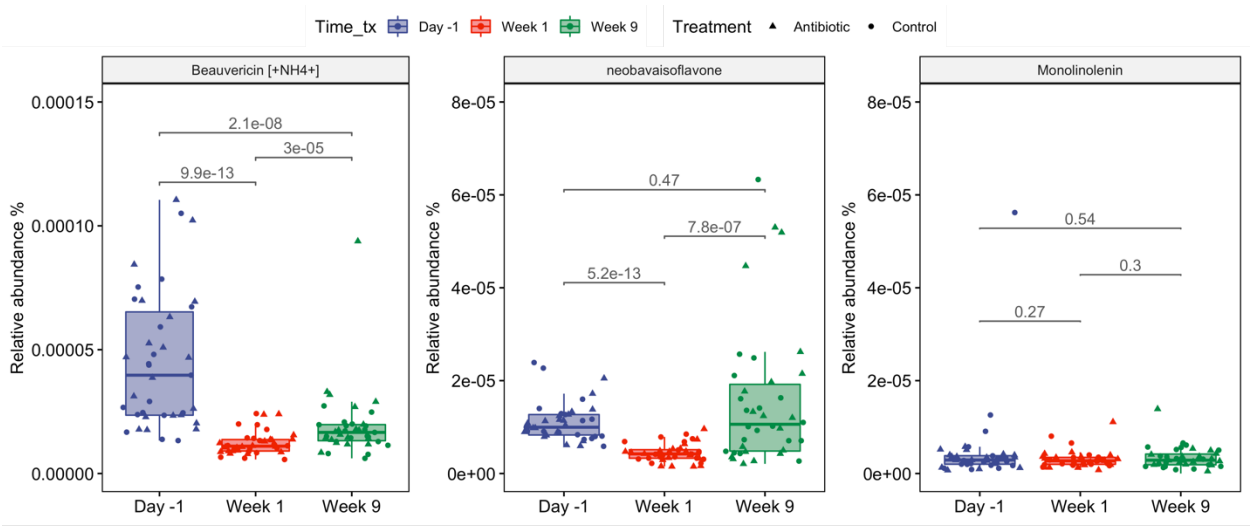


Figure 4.12. Boxplots of the three antimicrobial compounds between time points.

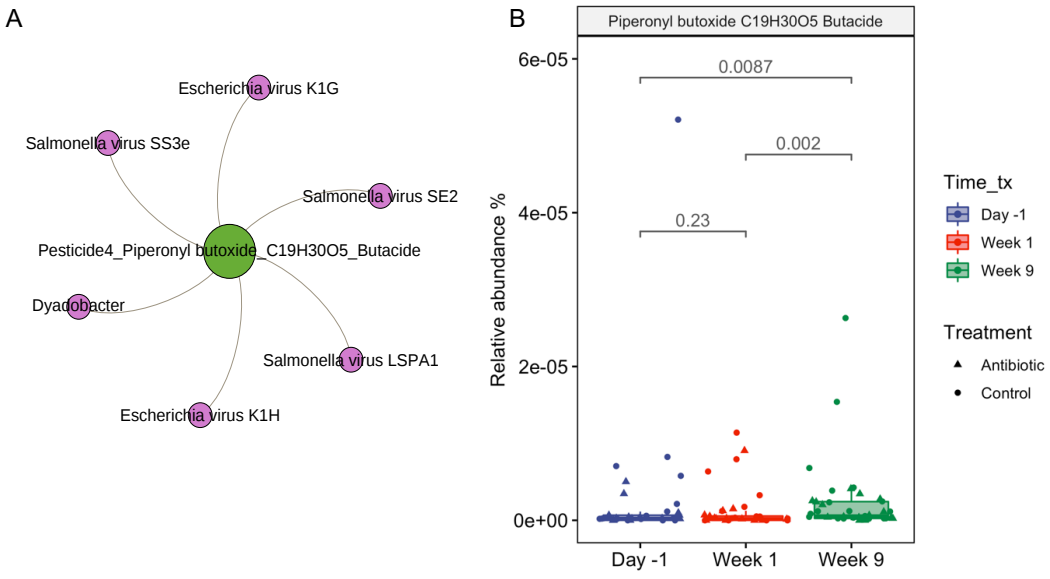


Figure 4.13. Piperonyl butoxide correlations and abundance in cattle feces. A) Correlation network. B) Boxplot showing the abundance of the pesticide by timepoint.

Changing metabolome and microbiome patterns among lactation phases

To better explore the functional associations of differentially abundant features, hierarchical clustering was carried out only with known metabolic clusters that were significantly different. A hierarchical clustering tree was constructed based on a distance matrix with the fold-change of metabolites and metagenomic features identified as biomarkers between lactation

phases. One relevant group showed concomitant higher levels of Actinobacteria, Proteobacteria, and three forms of histamine including C16:0, C18:1, and C18:2 during lactation (**Figure 4.14A**), suggesting a role of these taxa in the production of some pro-inflammatory compounds. Interestingly, correlation networks also identified associations between the microbiome and histamine, including histamine C18:0 that correlated with *Clostridium* and histamine C18:1 that was associated with *Prevotella*, *Atopobium*, Asteriusvirus, and *Lactobacillus* virus LP65 (**Figure C.4**).

During the dry-off period, traces of compounds found in pesticides and detergents such as nopol, aleuretic acid, and comphene, were identified at higher levels relative to the other lactation phases (**Figure 4.14B**). Similarly, the unsaturated fatty acids elaidic acid and petroselinic acid, and the diterpenes karuane and manool, were higher in dry cows, which was the only time when straw and grass silage were given (**Figure 4.14B**). Fresh cows showed higher levels of urate and nonpolar plant-derived compounds related to a diet rich in alfalfa hay, which was exclusively supplemented in this phase (**Figure 4.14C**). Monensin [M+NH4]+ also was detected at high levels in fresh cows although it was also detected during the lactating phase (**Figure 4.14C, Figure C.8**). This ionophore is used as a coccidiostat and growth promoter, though it was also shown to have activity against some Gram-positive bacteria [37]. No direct associations were observed between monensin and the microbiome composition. Other clusters also showed patterns with lower contrast across the lactation phases, with the most relevant HC groups displayed in **Appendices 9 and 10**. For instance, higher quantities of amino acids and dipeptides were detected with the polar mode on samples from the lactation phase, which was related to a higher abundance of bacterial amino acid synthesis pathways mentioned priorly

(**Figure C.9**). Not surprisingly, higher levels of androstane were also detected in fresh cows, which are expected to have peak levels of estrogens at this time (**Figure C.10**).

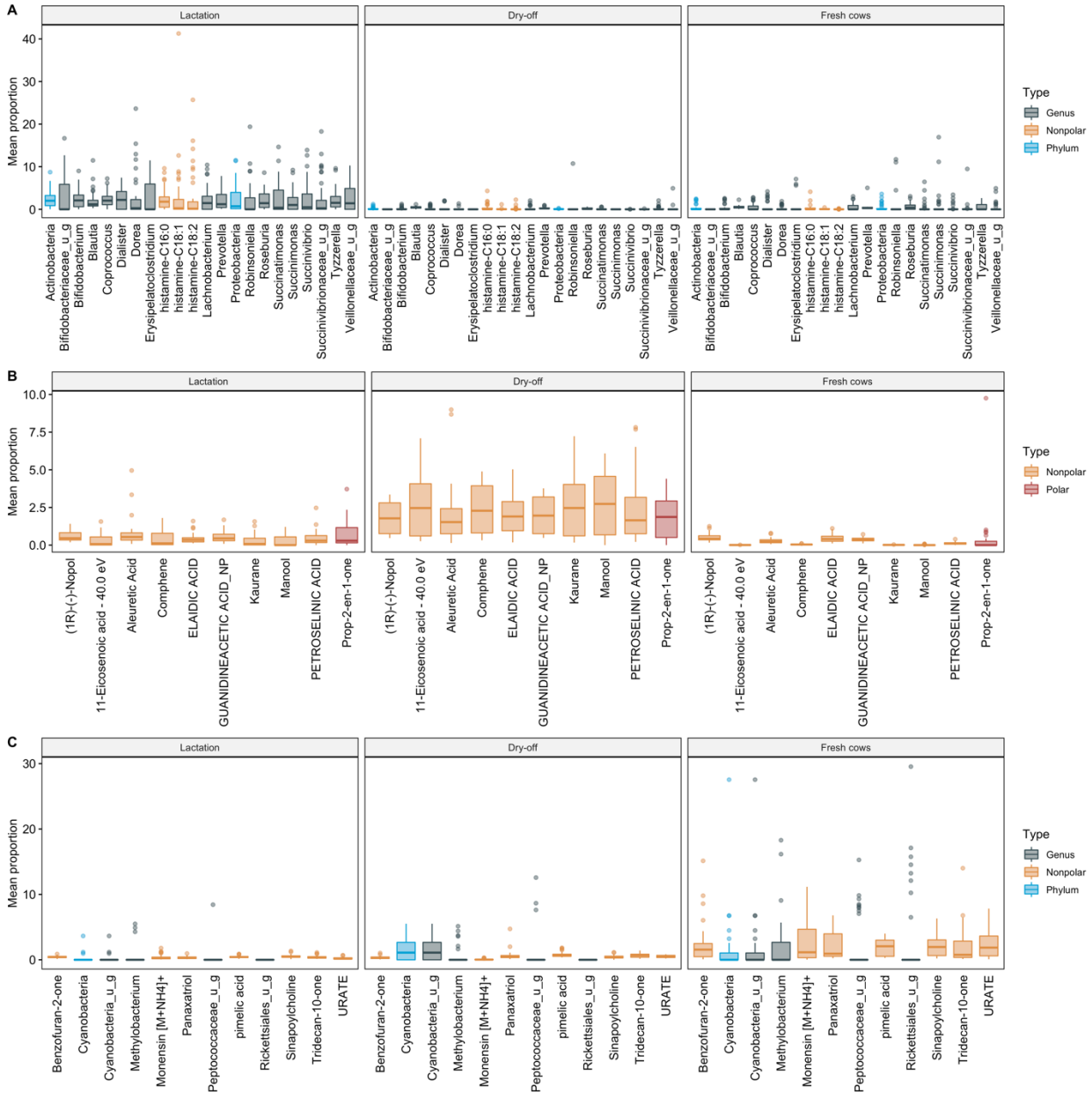


Figure 4.14. Relevant patterns of FC of metagenome and metabolome features in three lactation phases. Features higher in each phase are shown A) Lactation; B) Dry-off; C) Fresh cows.

DISCUSSION

In this study, we aimed to identify short and long-term changes in the gut metabolome of dairy cows due to intramammary treatment with a third-generation cephalosporin applied at dry-off. Although we observed no major changes in the metabolome associated with the IMM antibiotic treatment, the metabolome and microbial-metabolic pathways were significantly impacted by lactation phase. Furthermore, integrative analyses of metagenomics and untargeted metabolomics data revealed fingerprints unique to each lactation phase that help define the metabolite-microorganism interactions in the cattle gut.

IMM ceftiofur treatment had no short- or persistent effects on the gut metabolome diversity or composition, which is consistent with data from our prior study of the same animals showing no significant differences in microbiome diversity (Chapter 3). Importantly, ceftiofur metabolites were not detected in feces through untargeted metabolomics, demonstrating that these compounds did not persist in high levels one week following treatment. This finding was expected since a prior study found the excretion of ceftiofur to occur as quickly as 24 hours after intramuscular application and that most of the residues were found in the urine (60-80%) [38]. No prior studies had examined the IMM application of ceftiofur, which likely results in the excretion of lower levels than parenteral administration, thereby contributing to fewer functional changes in the gut. One unidentified metabolite, however, was detected only in cows that received IMM ceftiofur a week post-treatment. This compound could belong to a constituent of Spectramast® DC, which not only contains ceftiofur hydrochloride but also microcrystalline wax, oleoyl oilyoxyglyceride, and cottonseed oil. Future characterization of this compound is therefore warranted.

Furthermore, IMM ceftiofur treatment had long-term effects on the functional microbiome composition. A lower abundance of coenzyme A biosynthesis and glycogen degradation was observed in weeks 5 and 9, suggesting lower bacterial production of energy. However, several pathways linked to *Bifidobacterium* were higher in ceftiofur-treated cows in week 9; this genus belongs to phylum Actinobacteria, which was significantly higher in antibiotic-treated cows in our prior study (Chapter 3). Similarly, at week 5 lower abundance of genes that did not contribute to pathways (unintegrated) of *Ruminococcaceae* were observed in ceftiofur-treated cows. This genus is part of the phylum Firmicutes and was also less abundant in our prior study (Chapter 3). Unfortunately, most pathways were unassigned or unmapped, thereby complicating the understanding of differences between groups.

Each lactation phase, characterized by unique physiology and diet, determined the fecal metabolome composition. Even though the individual impact of these two factors cannot be explored based on our study design, diet seems to greatly impact the fecal metabolome and microbiome composition in cows as demonstrated previously [39–42]. For instance, increasing the grain-forage ratios are associated with a higher abundance of Proteobacteria and a lower abundance of Bacteroidetes in feces [41, 42]. Moreover, diets with over 30% grain given to cows in early lactation significantly changed the ruminal metabolome, increasing the abundance of short-chain fatty acids as well as toxins, inflammatory compounds, putrescine, methylamines, and ethanolamine [11]. In our study, cows in late lactation received the highest amount of grain in the diet, constituting about 39% of the dry matter intake vs. 26% in fresh cows and 7% in dry cows. Comparatively, those cows sampled during late lactation showed enhanced diversity of microbial pathways and metabolites, along with greater levels of histamine produced by Actinobacteria and Proteobacteria. Higher amounts of histamine-producing bacteria have been

associated with asthma in humans [43] and visceral hyperalgesia in mice colonized with human-fecal microbiota from patients with irritable bowel syndrome [44]. Bacterial-derived histamine can enhance cell-mediated (Th1) immune responses and suppress antibody-mediated immune responses (Th2), though their role in chronic inflammatory disorders is poorly studied [45].

A higher diversity of microbial pathways, as was observed during late lactation, has also been associated with enteric infection in a case-control clinical study performed by our group [46]. Despite the similarity in diet and the metabolome between the fresh and lactating cows, the microbial metabolic pathway diversity and composition were significantly different. In fact, the pathway profiles of fresh cows were similar to those observed in the dry phase suggesting a slow adaptation to a high grain diet. Although it only took a week on a forage-based diet at dry-off to identify changes in the functional gut microbiome, this was accompanied by lower levels of histamine-producing bacteria compared to the lactation phase. Since the core microbiome composition is unique to each farm due to factors that include housing, breed, and age [40, 47], changes in the diet are the most impactful on the cattle metabolome and microbial diversity. Consequently, manipulation of the functional microbiome through dietary changes is plausible; however, functional changes can take longer time to develop in a new environment as was observed herein.

Interestingly, integrated analyses between untargeted metabolomics and metagenomics exposed natural antimicrobial compounds from plant and fungi sources to be associated with the expansion of lytic bacteriophages of enterobacteria and genes encoding ESBL. For instance, beauvericin is a mycotoxin commonly found in animal feed, particularly corn, wheat, and barley, contaminated with fungi representing *Fusarium* spp. or *Beaveria bassiana* [48]. Similarly, monolinolenin has been isolated from ryegrass and *Rhizobium* [49, 50], while neobavaisoflavone

is derived from plants [51]. These compounds are being studied as alternatives to treat multidrug resistant infections [48, 51, 52]. Thus, identifying levels of these compounds in different food sources as well as associations with the microbiome and resistome could enhance understanding of their role in the abundance of resistant bacteria and pathogens. Importantly, the positive correlation between these natural antimicrobials and bacteriophages of *E. coli* and *Salmonella* indicates their indirect effect on the reduction of some enterobacterial populations by inducing the lytic phase of these phages. However, bacterial stress could also promote the mobilization of other genetic elements [53, 54], which could explain their association with the β-lactam resistant gene *CFXA2.* Beavericin, monolinolenin and neobavaisoflavone were also related to a higher abundance of metabolic pathways from *Clostridium* and *E. coli* suggesting their role in the activation and growth of these taxa.

Untargeted metabolomics also showed traces of pesticides in feces, like piperonyl butoxide. The chronic exposure to pesticides can induce dysbiosis, toxicity, metabolic diseases and reproductive disturbances [55], which are conditions that are critical to prevent in dairy farm production. Furthermore, the association between piperonyl butoxide with lytic phages from *Salmonella* and *E. coli* indicates the role of pesticides as bacterial stressors. Despite a higher abundance of compounds associated with lytic bacteriophages of enterobacterial populations in fresh cows, our prior analyses (Chapter 3) showed an increased number of total and β-lactam resistant Gram-negatives at the same time point/phase. Hence, these phages are either strain specific or in the lysogenic stage, where their expansion is linked to the multiplication of their bacterial hosts. Fresh cows are immunocompromised and under a higher level of stress as compared to dry cows, which can also contribute to differences in the microbiota. The higher amounts of monensin added in the diet of fresh cows, for instance, could be related to bacterial

stress and the higher abundance of resistant microorganisms during peripartum observed in our prior study (Chapter 3). Even though no direct associations were detected between monensin and the microbiome, a prior metagenomic investigation of steers found that the administration of monensin in the diet contributed to reduced levels of some Gram-positive genera [6].

Not surprisingly, the vast majority of metabolites and microbial-metabolic pathways was unknown in this study, which has also been observed in prior metabolomic studies [56, 57]. Despite this limitation, biologically important compounds and metabolic pathways enabled the interpretation of some associations that were observed between the microbiome and metabolome. Future studies should include GC/MS to promote the identification of short-chain fatty acids (SCFAs) since they have been previously linked to health outcomes in humans [58] and production in cattle [59]. Moreover, associations between metagenome, metabolome and milk production could guide improvements to diet formulations, health, and probiotic development. Serum metabolome analyses could also help identify associations between microbiome functionality and host factors such as hormonal levels (i.e., estrogens, cortisol, progesterone) or metabolic disorders in cattle. Finally, future studies should also explore the use of fecal proteomics to define and target biomarkers of immunity and inflammation for classifying host responses, as well as metaproteomics to characterize the functional microbiome and resistome [60].

CONCLUSIONS

IMM ceftiofur treatment of dairy cattle at dry-off did not alter the fecal metabolome, though a lower production of energy and higher activity of *Bifidobacterium* was observed in the long term among the ceftiofur-treated cows. The presence of natural antimicrobial compounds and pesticides in feces was also correlated with Enterobacteria stress and ESBL genes.

Moreover, each lactation phase was characterized by a distinct metabolome composition that was related to the feed ration and physiology. During lactation, a higher level of microbial activity was observed as compared to dry and fresh cows; however, proinflammatory bacteria were more abundant during lactation that can negatively affect the performance of the animals. In all, these data highlight how integrative analyses of metagenomics and untargeted metabolomics data can be used to define the metabolite-microorganism interactions in the cattle gut. Comprehending the role of the gut environment in the microbial profile is key to identify factors related with health and production in cattle farms.

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APPENDIX

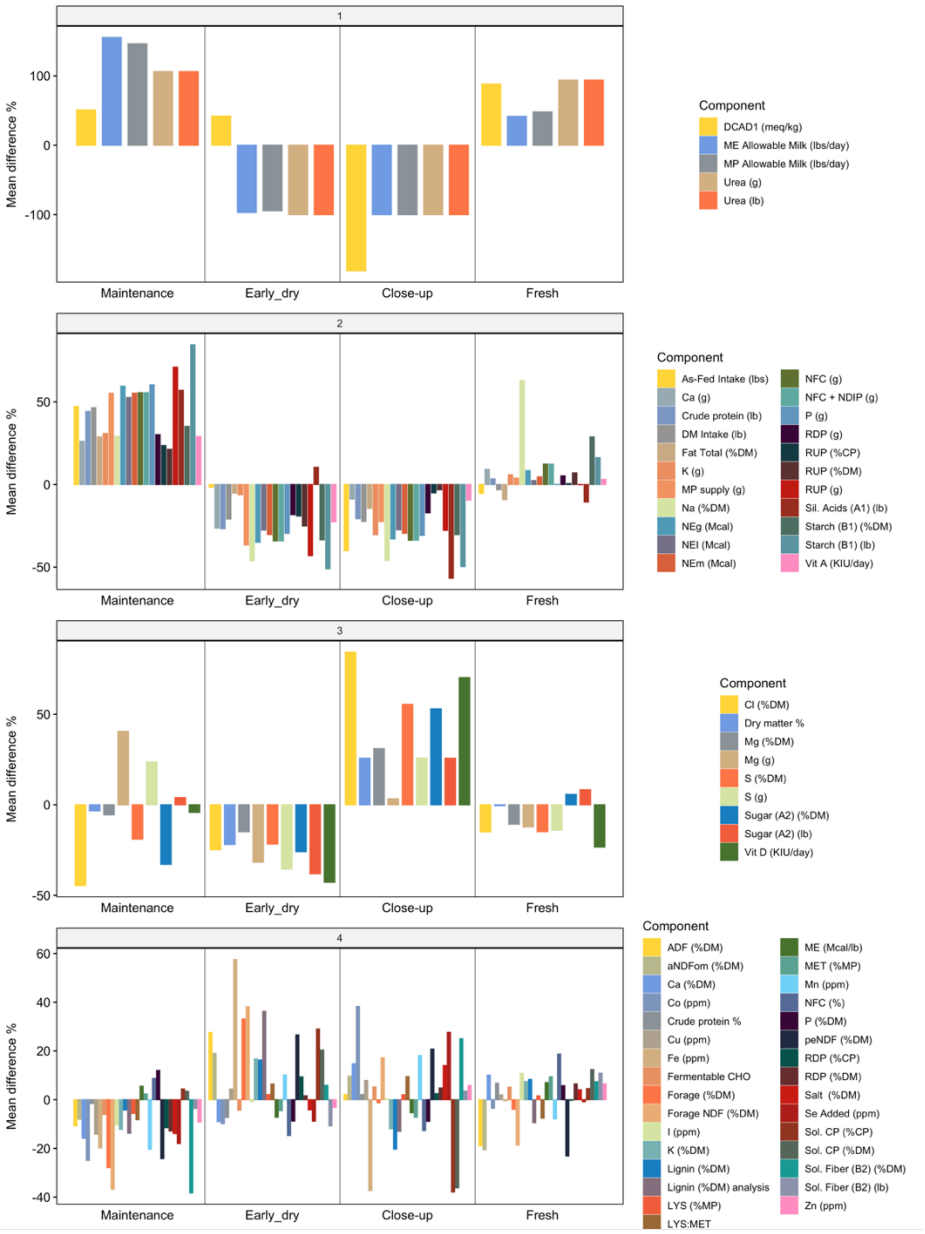

Figure C.1. Bar plot showing four clusters of diet components given to cows in four lactation phases. The abundance is shown as fold change relative to the mean for each feature. Hierarchical clustering with the Ward method was used to classify nutrients into four groups based on their abundance in each lactation stage. Hierarchical cluster 1 contains nutrient components with the most important differences between diets provided to lactating (maintenance and fresh diets) and dry cows (early dry and close-up diets). Cluster 2 groups nutrients and amount of feed intake significantly higher in the maintenance diet compared to the other ones. Similarly, cluster 3 has nutrients given in higher amounts in the close-up phase. Group 4 shows the rest of the nutrients given at different levels in each diet.

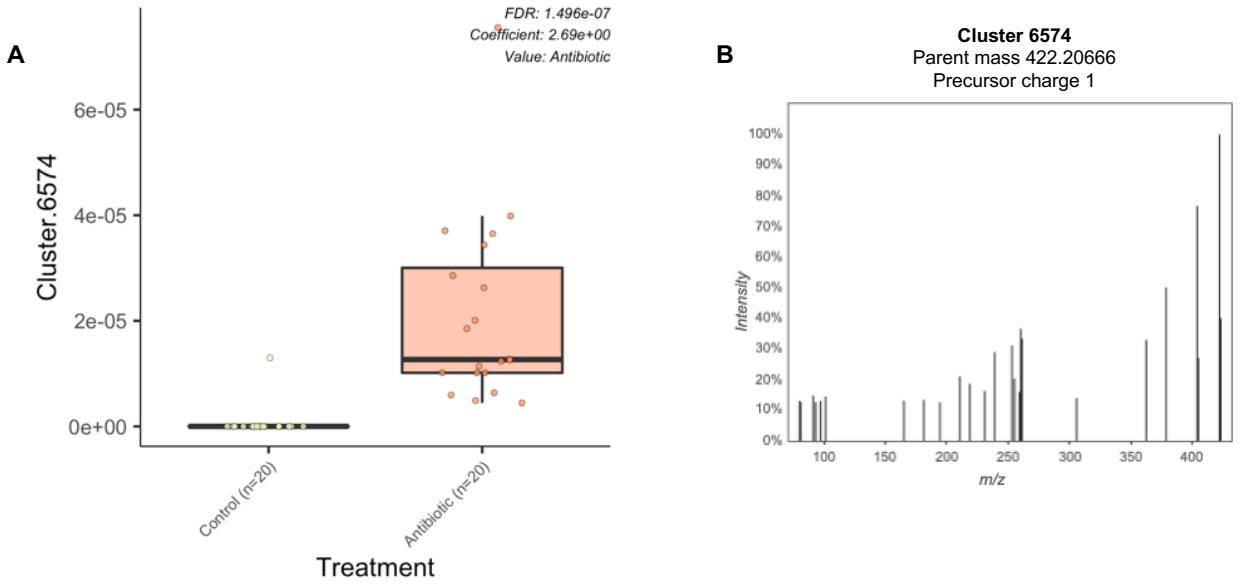

Figure C.2. Cluster 6574 significantly different between treatments in week 1. A) Box plot based on the relative abundance per sample. B) Mass spectra of the unknown cluster**.**

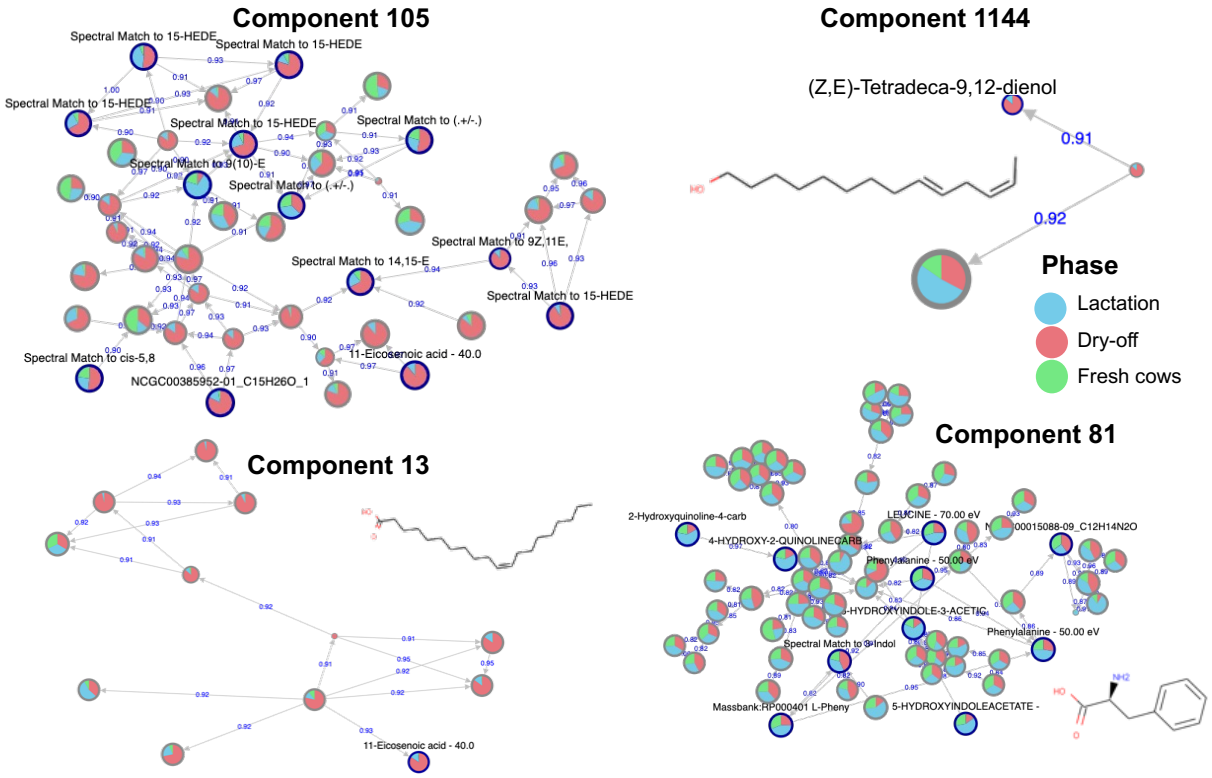

Figure C.3. Components containing clusters with library ID that were among the 50 most important features for classifying the metabolome by lactation phase with RF. Edge numbers represent the cosine score of spectral similarity between two fragmentation spectra. The size of the clusters represents the number of files.

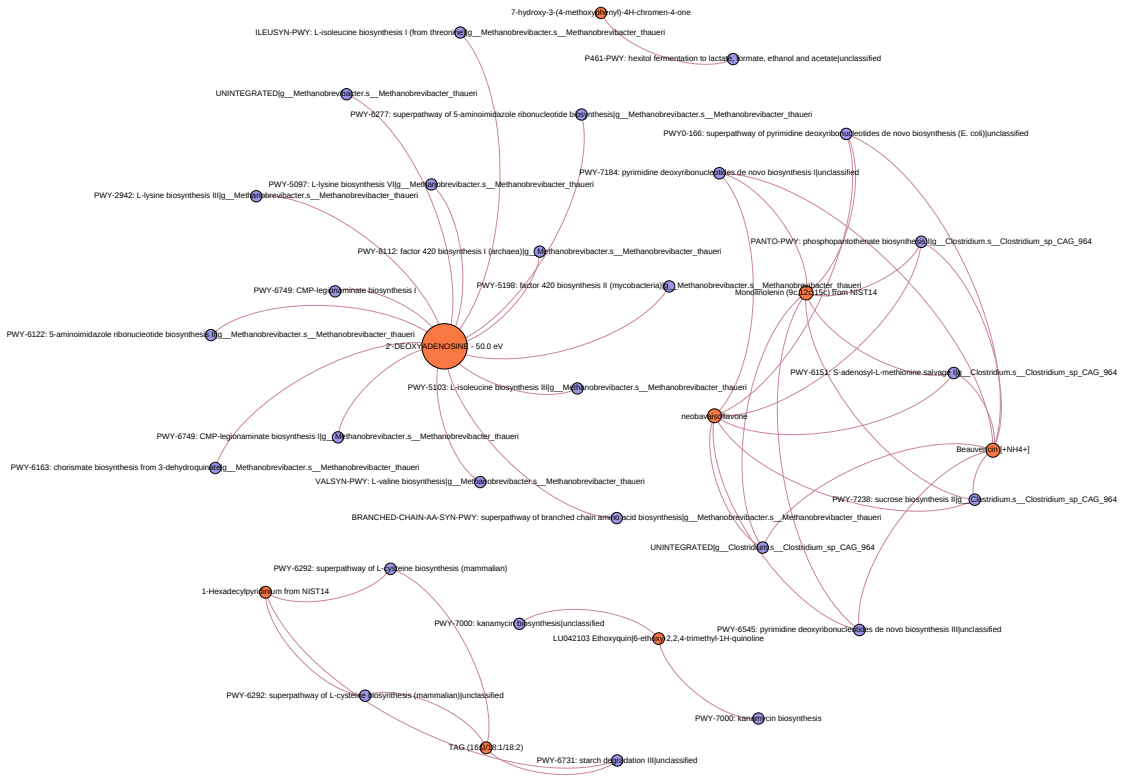

Figure C.4. Correlation network between nonpolar metabolites and microbial-metabolic pathways.

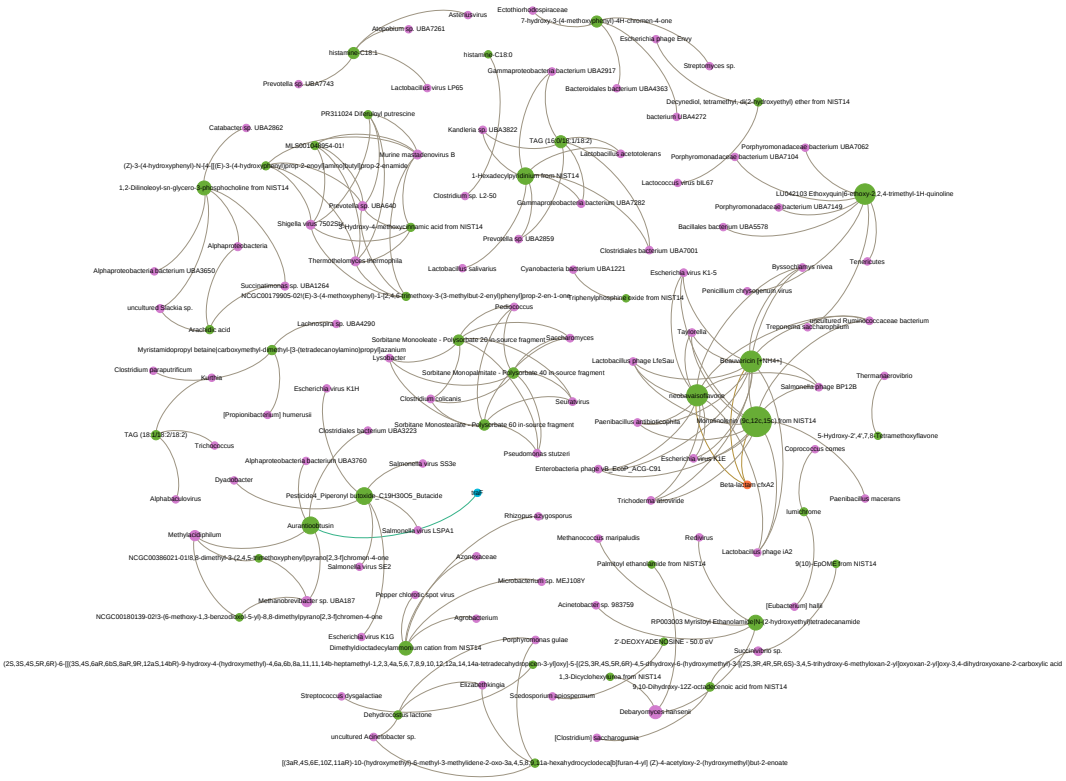

Figure C.5. Correlation network between nonpolar metabolites, microbial taxa, and genes.

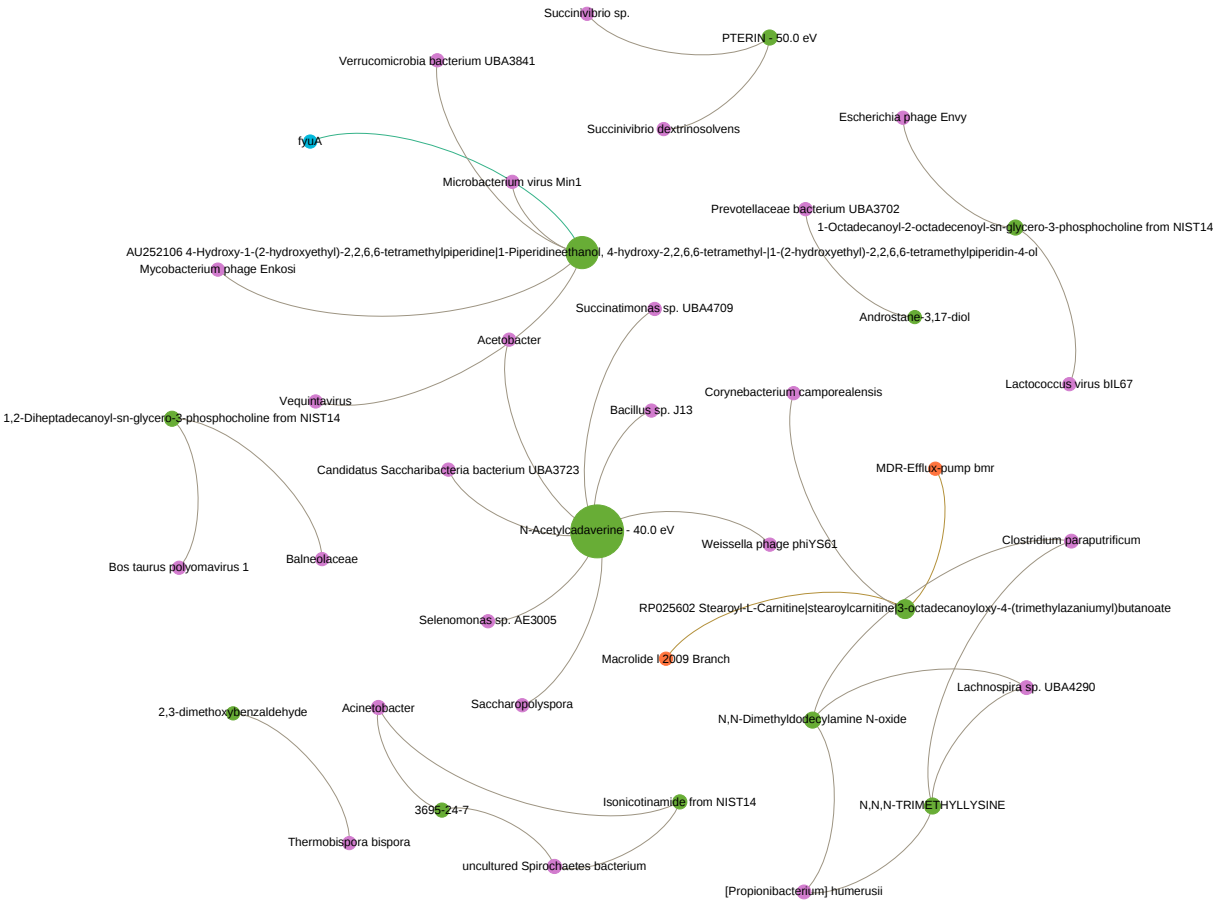

Figure C.6. Correlation network between polar metabolites, microbial taxa, and genes.

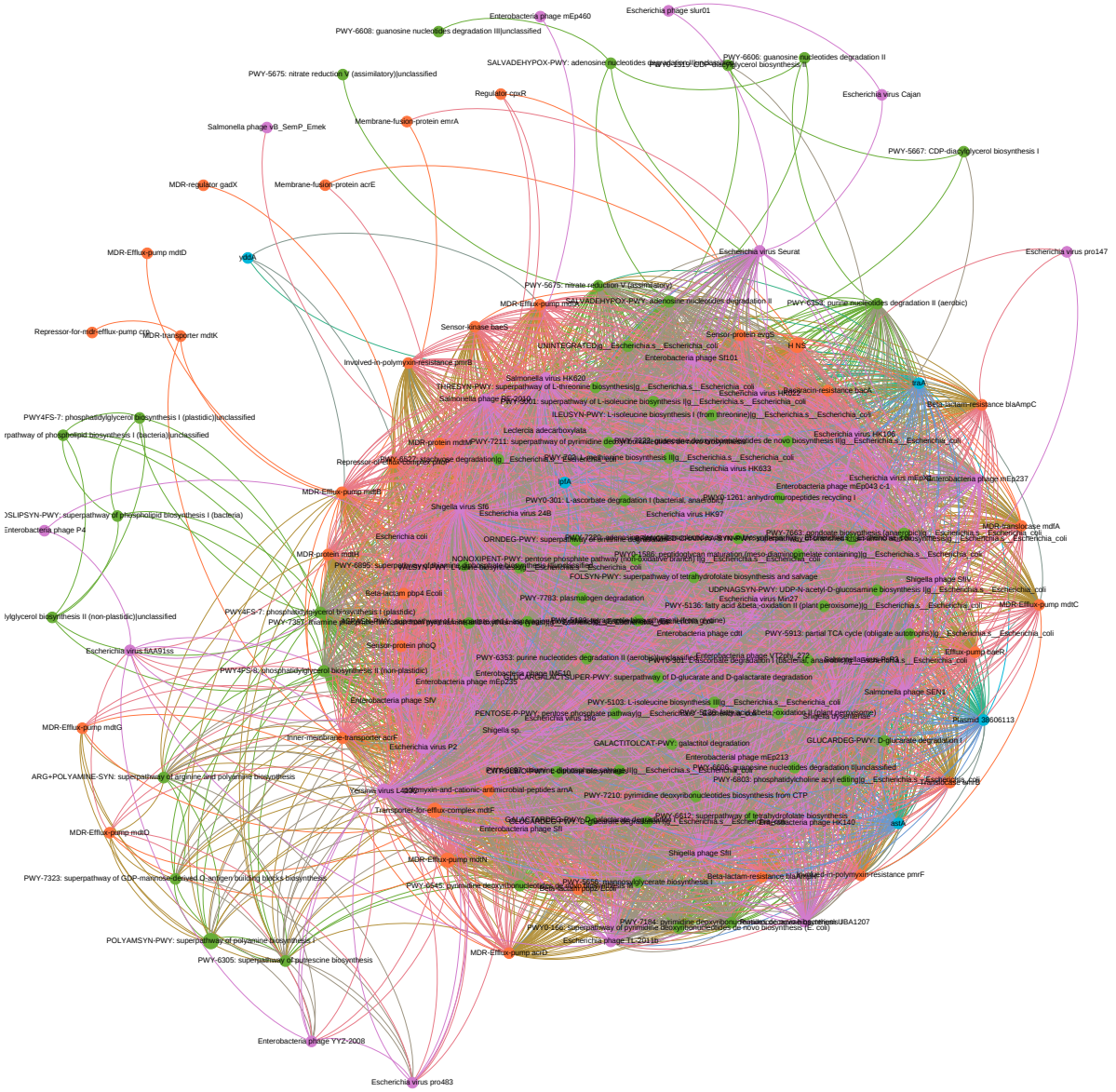

Figure C.7. Network module with the highest number of connections including microbial pathways, microbial taxa, and genes.

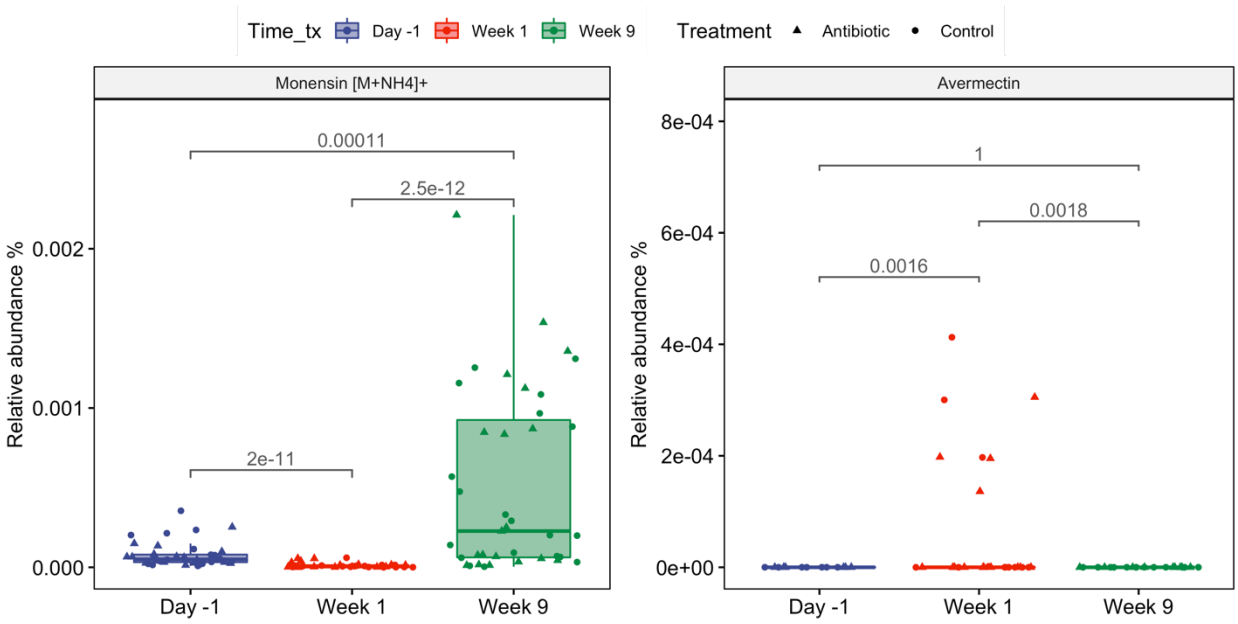

Figure C.8. Relative abundance of Monensin and Avermectin detected with the nonpolar mode.

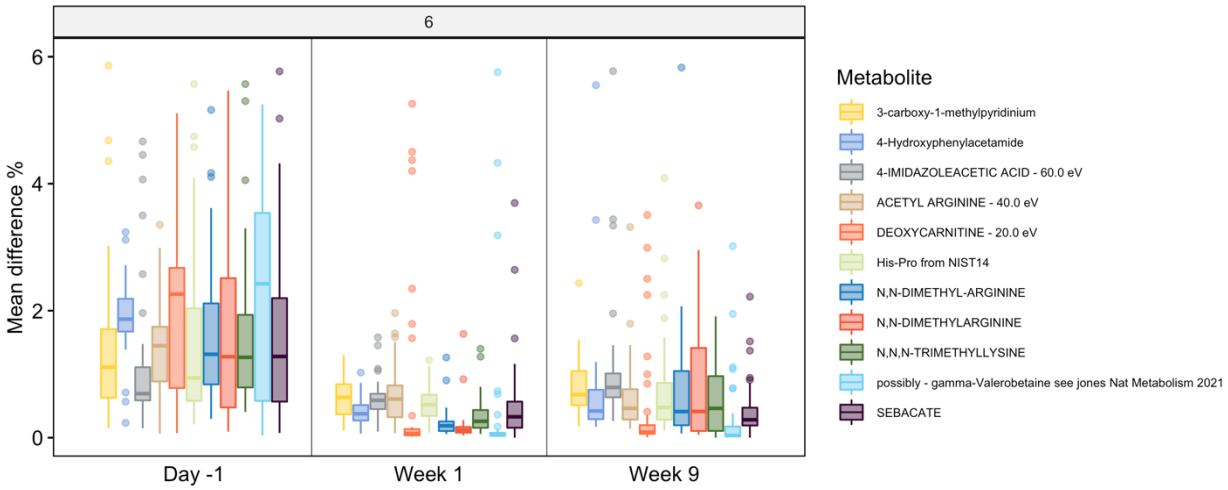

Figure C.9. Group of polar metabolites significantly higher on lactation phase

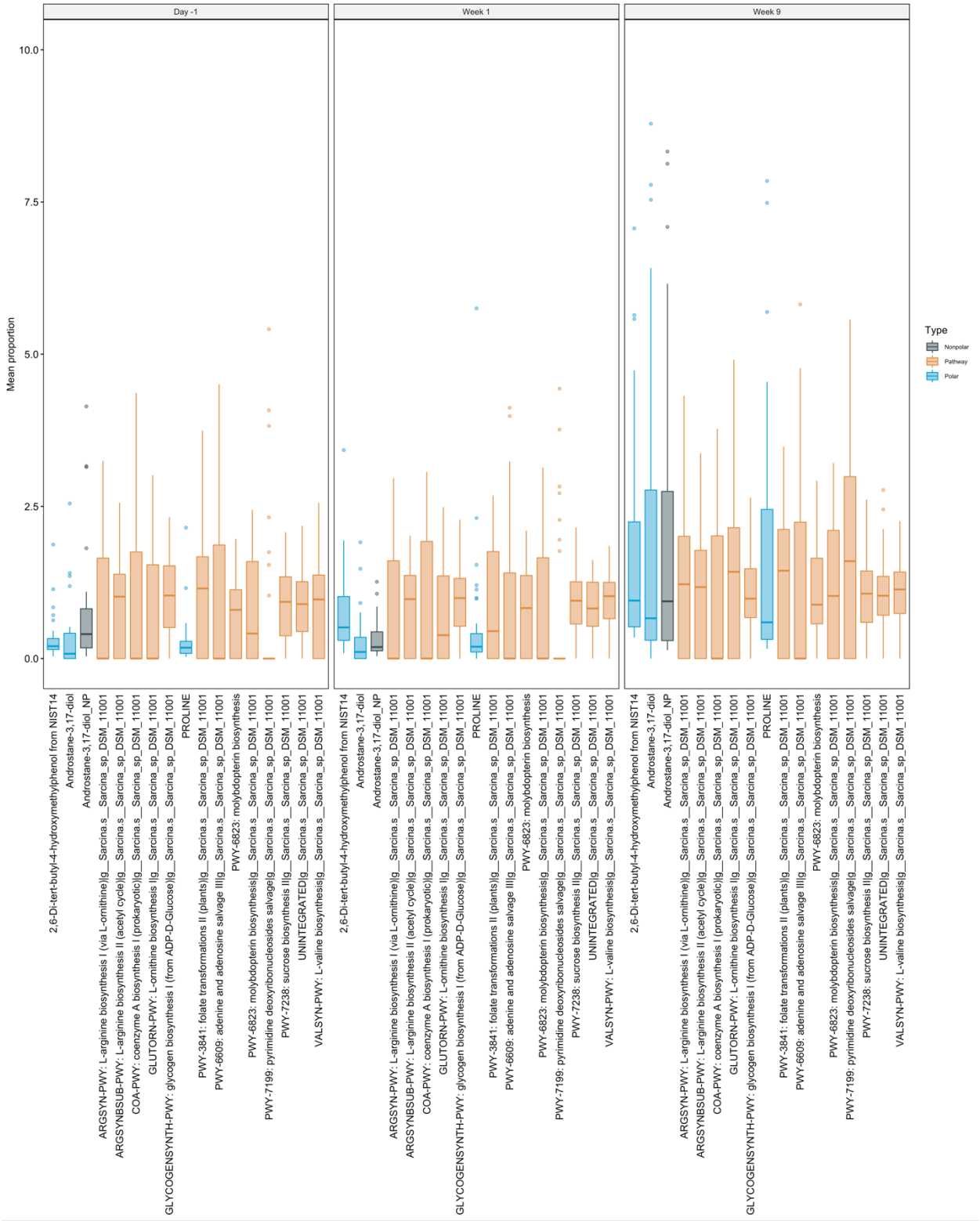

Figure C.10. Cluster of microbial pathways and polar and nonpolar metabolites significantly higher in fresh cows.

CHAPTER 5

Conclusions and Future Directions

Bovines are the most important source of protein for humans, and their demand is expected to increase abreast with the human population [1]. The symbiotic relationship with their complex gastrointestinal (GI) microbiome makes ruminants the most efficient food-producing animals [2, 3]. Despite the benefits of bovine production, the intensification and expansion of cattle farms will escalate, and potentially their negative footprints such as the transmission of foodborne pathogens [4] and antibiotic-resistant bacteria [5–14]. The main public health concerns associated with bovines are their role as primary carriers of STEC [15, 16] and the use of critically important antibiotics for human health applied in these animals [17]. Thus, we sought to identify ecological associations between the fecal microbiome of bovines with both STEC shedding and intramammary (IMM) use of third-generation cephalosporins.

While EHEC O157:H7 is the most studied STEC serotype in cattle [18, 19], prior studies failed to characterize the microbiome of animals carrying non-O157 STEC serotypes, even though they cause about two-thirds of STEC infections in humans [20]. Hence, in Chapter 2 we used 16s *rRNA* sequencing to characterize the hindgut microbiome of cattle carrying non-O157 STEC at two levels: 1) between farms with a high and low prevalence of STEC; and 2) between animals carrying STEC and controls. At the farm level, significant differences were observed in the microbiome composition between facilities with a low and high prevalence of STEC, also referred to as LSP and HSP, independently of breed (i.e., beef and dairy). Though, these differences in the microbiome were associated with the diet composition. HSP farms had forage-dominant diets characterized by a higher abundance of Firmicutes and Bacteroidetes and a lower abundance of Proteobacteria. Whereas LSP farms had diets with higher levels of grains and presented a lower microbiome diversity. When comparing the microbiome composition of shedders and non-shedders of STEC, differences were observed in the abundance of some taxa

but not in the microbiome richness. Yet, longitudinal approaches of STEC carriage over time revealed different patterns of microbiome alpha diversity during STEC shedding related to the pathogen prevalence in the farms. For instance, STEC shedding was associated with a higher richness in animals from LSP but not in HSP farms, suggesting that preexposure to STEC plays a role in the microbiome richness during its shedding which can potentially be associated with immunotolerance. Finally, factors priorly identified with a higher prevalence of STEC in dairy farms, such as days in milk and warm temperatures affected the abundance of taxa associated with STEC shedding.

In summary, Chapter 2 identified that STEC shedding by cattle is multifactorial, where diet independently of the breed is the main force shaping the hindgut microbiome of cattle carrying the pathogen. Despite the identification of distinctive microbiome diversity and bacterial taxa associated with STEC shedding at the farm and individual levels, different dynamics were observed among animals with varying levels of exposure. Through our approach, however, we cannot distinguish if STEC carriage affects the microbiome composition or if a rich microbiome enables better colonization of STEC. Therefore, this investigation calls for the identification of other factors that may affect STEC colonization in the bovine's gut. Examples of factors that can alter the gut environment with consequences in the microbiome composition and STEC establishment include quorum sensing signals [21], antimicrobial peptides [22], diet-associated metabolites [23, 24], immunoglobulin A [25, 26], mobile genetic elements (MGEs) [27] and intestinal pH [28]. Future approaches should include metabolomics, metaproteomics, and MGE (i.e., bacteriophage) analyses of the intestinal content and mucosa where STEC colonizes [29, 30]. Identifying molecular factors *in-vivo* that influence the establishment of

STEC could help design alternatives to decrease the levels of this foodborne pathogen in cattle, such as diet modifications and probiotics.

Furthermore, the use of medically important antibiotics in cattle farms is a global concern, particularly for the potential emergence of resistant bacteria to last-resort drugs [17, 31]. Third-generation cephalosporins are critically important antibiotics of highest priority given the number of rising infections with ESBL *Enterobacteriaceae*, which are the main cause of deaths by multi-resistant bacteria worldwide [31, 32]. Ceftiofur is a third-generation cephalosporin widely used in dairy farms to treat mastitis, metritis, respiratory infections, and foot rot [33–35]. Studies demonstrated the increase of ESBL genes in the resistome of cattle after its parenteral application [36, 37], but there is no evidence of the effect of intramammary (IMM) ceftiofur in the abundance of resistant bacteria in cattle feces.

Therefore, in Chapter 3 we studied the effects of a single application of 2 g of IMM ceftiofur in dairy cows at dry off in the fecal resistome and microbiome over a 9-week time frame. Plate counting of Gram-negative and Gram-positive bacteria showed no effect of the antibiotic IMM treatment in the number of β-lactam (i.e., ceftiofur and ampicillin) resistant bacteria found in feces. Low levels of Gram-negative bacteria resistant to ceftiofur were detected in dairy cows and were not associated with IMM treatment. Metagenomics sequencing did not identify associations between the IMM ceftiofur and the diversity of the fecal microbiome and resistome. However, a persistent increment of β-lactam ARGs was observed in cows treated with IMM ceftiofur, particularly of genes encoding ESBLs (i.e., *bla-CFX-A*, *bla-CMY,* and *bla-ACI*). Correlation networks revealed the association of ESBL genes with bacteriophages and plasmids that can play a role in the mobilization of these genes across different taxa. Contig analysis and *in-vitro* identification showed that β-lactam resistance was carried by three phyla: Bacteroidetes,

Firmicutes, and Proteobacteria. Enhanced β-lactam ARG dispersion in the microbial communities was influenced by IMM ceftiofur treatment, evidenced a week after the treatment by a higher number of correlations between ARGs, different bacterial species, and MGEs. In short, this study revealed that a single application of IMM ceftiofur in dairy cows at dry-off persistently increased the levels of ESBL genes in cattle feces. Moreover, third-generation cephalosporin application enhanced the mobilization of ARGs across different bacterial phyla in the intestinal microbiome. However, the detection of ESBL *Enterobacteriaceae* by culture-based methods showed low levels of shedding by dairy cattle with no significant differences between treatments.

Future studies should focus on the characterization of taxa carrying ESBLs to identify with better resolution the MGEs implicated in their dispersion, as well as the co-selection of other ARGs. Furthermore, molecular identification of ESBL bacterial hosts can enable the comparison of isolates recovered from cattle with the ones from human infections to understand their genetic relationship and zoonotic potential. Enrichment of ESBL-resistant bacteria with subsequent long-read sequencing has been identified as an effective method for the characterization of plasmids and bacterial hosts that could constitute minorities in the microbiota of fresh feces [38, 39]. Whole-genome sequencing (WGS) of ceftiofur-resistant isolates can also help identify virulence genes and the evolution events associated with MGEs and ARG acquisition. Finally, identifying the evolution and dispersion of ESBL *Enterobacteriaceae* in manure is necessary to measure the risks of dispersion of these resistant threats into the environment; particularly, given that ceftiofur is mainly eliminated by urine [35, 40] and that *Enterobacteriaceae* can increment their abundance in open environments [41].

Cattle rely on their microbiota to produce cofactors, 80% of their protein [42], and three-quarters of their energy [3]. However, different factors including diet, pathogens, and antibiotics can induce dysbiosis in the cattle microbiota with negative consequences on the bovine's health and production [43]. Thus, in Chapter 4 we explored the potential effects of IMM ceftiofur treatment in the functional microbiome and metabolome of cattle feces from Chapter 3. Our results identified slight changes in the functional microbiome induced by the third-generation cephalosporin. Differences in the functional microbial profiles were only observed in weeks 5 and 9 when cows treated with IMM ceftiofur had a lower abundance of energy-related pathways and higher metabolic activity of *Bifidobacterium*. Although differences were identified in the microbial pathway abundance, the metabolome analyzed through LC/MS did not differ between treatment groups. Unique metabolome composition was observed between time points that corresponded to three phases of lactation (i.e., lactating, dry, and fresh cows) characterized by unique diets and physiology. Multi-omics analyses combining metagenomics and metabolomics data allowed the identification of interactions between microorganisms and the gut environment. For instance, high levels of histamine were associated with a greater abundance of Actinobacteria and Proteobacteria in the lactation phase. Moreover, correlations between lytic bacteriophages, ESBL genes, natural antimicrobial compounds and pesticides indicate the role of diet-associated compounds in the selection of MGEs and ARGs. In sum, Chapter 4 revealed that IMM ceftiofur has minor impacts on the functional microbiome that were observed at the end of the dry-off period. Furthermore, the multi-omics approach exposed the role of natural antimicrobial compounds in the activation of lytic bacteriophages of *Enterobacteriaceae* which can also enhance the mobilization of ARGs. Given the identification of differences in the microbial profiles induced by the IMM ceftiofur treatment in fresh cows, information regarding

the milk performance efficiency of these bovines can help associate the impact of changes in microbial pathways in the cow's productivity. In addition, future studies can include the identification of short-chain fatty acids (SCFAs) given their relevance in production in cattle [58], along with analysis of host-derived metabolites (i.e., hormones and biomarkers of inflammation) to determine interactions between cattle and their microbiome.

Altogether, this study identified that changes in the gut microbiome associated with the shedding of non-O157 STEC are multifactorial, with diet and level of exposure as the most relevant factors shaping changes in the microbiome composition of STEC shedders. Despite identifying differences in the abundance of certain taxa associated with the carriage of STEC, more research is needed to understand the influence of metabolites derived from the host, diet, and microbiota in the colonization of this pathogen to help design alternative methods for STEC reduction in cattle farms. Furthermore, the use of IMM third-generation cephalosporins in dairy cows at dry-off selectively and persistently increased the abundance of ESBL genes with no repercussions in the microbiome or resistome diversity. ESBL genes were distributed across different phyla and their associations with MGEs may aid their mobilization in the GI microbial communities. Nevertheless, ESBL Enterobacteriaceae were minorities in the cattle feces and their abundance was not associated with the antibiotic treatment. Finally, metabolic profiling of the microbial communities showed minor effects of the IMM treatment with ceftiofur and no changes in the metabolic profile. Multi-omics approaches revealed the presence of pesticides and natural antibiotics associated with bacteriophages and ESBL genes, highlighting the importance of metabolites present in the diet in ARG mobility. These studies revealed complex interactions between microbial communities, pathogens, and antibiotics in cattle. Future research should

include the influence of these interactions with the host as well as environmental factors that may risk the persistence of pathogens and resistance in cattle farms.

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