DISSERTATION

IMPACT OF ANTIBIOTIC USE ON RESISTANCE IN BEEF FEEDLOT AND DAIRY CATTLE

Submitted by

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ABSTRACT

IMPACT OF ANTIBIOTIC USE ON RESISTANCE IN BEEF FEEDLOT AND DAIRY CATTLE

In recent years, consumer demand for natural and organic foods has increased, partly due to concerns about the use of antimicrobials in food producing animals. The aim of this study was to evaluate antimicrobial resistance (AMR) in beef feedlot and dairy cattle raised without use of antibiotics compared to cattle raised in conventional (CONV) production. Three research projects were conducted to accomplish that general goal. In the first study, a conventional feedlot, natural feedlot, conventional dairy and organic dairy were visited to collect cattle feces, wastewater from lagoons and soil where the wastewater was applied. After DNA extraction, sequencing, and processing, metagenomic reads were aligned to reference databases for identification of antibiotic resistance genes (ARGs; i.e. the resistome) and bacteria (microbiome). Resistome composition was influenced by rearing method, cattle type, and type of sample. Most mechanisms of resistance affected by rearing method were enriched ($P < 0.05$) in conventional samples. Resistome differences were greatest for wastewater samples by rearing method but with contradictory results that suggested an impact of effluent management on wastewater resistome. Resistance to tetracycline and macrolide-lincosamide-streptogramin classes were more abundant in feces of feedlot cattle than in dairy cattle ($P < 0.05$); whereas resistance to beta-lactams was greatest in feces of dairy cattle ($P < 0.05$). Resistome and microbiome of feces differed ($P < 0.05$) between wastewater and soil samples. Results indicated that ARGs are widespread in beef feedlot and dairy cattle farms even in those with restricted antibiotic use.

In the second study, feces from RWA (n=36) and CONV (n=36) cattle lots were recovered from colons at a commercial beef processing plant. Samples were equally distributed by month and production protocol over one year (3 samples/production protocol/month). After extracting DNA from individual samples, composite samples were prepared by mixing DNA from each lot into a single composite sample (N = 72) and sequencing the composites on an Illumina platform. Metagenomic reads were processed similarly to those in experiment 1for identification of ARGs and bacteria. Resistomes of CONV and RWA cattle were significantly different by season. In general, mechanisms conferring resistance to beta-lactams, tetracyclines, multi-drug and macrolides were more prevalent ($P < 0.05$) in feces from CONV colons than in RWA colons.

In the third study, a systematic review and meta-analysis was performed to assess the relationship between antimicrobial use (AMU) and antimicrobial resistance (AMR) in feedlot cattle. After conducting a literature search and screening reported studies, 32 studies were selected for use that addressed AMR in *Escherichia coli*, *Enterococcus*, *Salmonella*, *Campylobacter*, and *Mannheimia haemolytica*. Overall, 60% (95% CI: 26% to 88%) of the observational studies and 50% (95% CI: 30% to 70%) of the controlled trials reported a positive association between AMU and AMR. Meta-analysis provided evidence for an increase in average relative risk (RR) associated with antibiotic use. Isolates recovered from treated cattle were 2.5 times (95% confidence interval: 1.7 – 3.5) as likely to display antibiotic resistance compared to isolates recovered from unexposed animals. Risk of resistance increases with animal defined daily doses (DDDs). More comprehensive studies that consider the relationship between antibiotic use in cattle and antibiotic resistant bacteria in humans are needed as a part of a farm to fork approach to tackle antimicrobial resistance.

ACKNONWLEDGMENTS

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CHAPTER I

INTRODUCTION

Antibiotic resistance is a global concern threatening human health. In Europe, 25,000 people die per year as a result of multidrug-resistant bacteria costing around € 1.5 billion per year (ECDC/EMEA Joint Technical Report, 2009). Similarly, over 2 million people are infected in the U.S. annually with bacteria resistant to antibiotics; these cause 23,000 deaths each year (CDC, 2015). As development of new antibiotic molecules is challenging, time-consuming, and costly, increased use and resistance to current antibiotics has led to questions regarding efficacy, predicting a return to the pre-antimicrobial era (Baker, 2015). To accommodate increased consumer concerns, some conventional livestock operations are adopting organic or "natural" production practices that include cessation of all antibiotic use (Fox et al., 2008). Consumers perceive meat derived from those systems as "safer" in terms of the presence of antibiotic resistance (Brennan et al., 2003); however, that expectation is controversial according to scientific studies (Luangtongkum et al., 2006; Cho et al., 2007; Kazimierczak et al., 2009; Reinstein et al., 2009; Morley et al., 2011; Santamaria et al., 2011).

Traditional approaches to study antibiotic resistance bacteria include culture-based (i.e., susceptibility testing) and molecular (i.e., polymerase chain reaction) techniques. An example of this approach is the National Antibiotic Resistance Monitoring System for enteric bacteria (NARMS) which tests for susceptibility to 15 antimicrobials in *Salmonella* and *E. coli*, 9 in *Campylobacter*, and 16 in *Enterococcus* (CDC, 2013). While traditional approaches have focused on antibiotic resistance only in pathogens that are clinically important in human health,

commensal bacteria are probably the most important reservoir of resistance genes because they serve as the main component of the microbiome, thereby increasing the chance of contact and genetic exchange through horizontal gene transfer with pathogenic bacteria (Penders et al., 2013). Next-generation sequencing and shotgun metagenomics analysis is an alternative approach to study microbial composition ("microbiome") and resistance genes ("resistome") in complex environments (Thanner et al., 2016). Briefly, DNA is extracted from the microbial community found in an environmental sample, sheared into small fragments, and sequenced, generating millions of DNA sequences per sample ("reads") (Sharpton, 2014). Metagenomic reads are aligned to reference microbial genomes and resistance genes for taxonomic classification, and identification of microbial or resistance genes are based on sequence similarity (Peabody et al., 2015).

The main objective of the present series of projects was to evaluate the impact of different rearing methods (conventional, natural and organic) on the resistome and microbiome of feces and environmental samples in beef feedlot and dairy cattle production systems using a shotgun metagenomic approach. In the first two observational studies (Chapters III and IV), we provide a characterization of the resistomes and associated microbiomes of differing farms (beef feedlots and dairy farms) and ecological niches (feces, wastewater and soil). In the third study (Chapter V), we performed a systematic review of published literature and meta-analysis to assess the relationship between antimicrobial use and antimicrobial resistance in feedlot cattle.

CHAPTER II

REVIEW OF LITERATURE

Origin of antimicrobial resistance

Many antibiotic resistance genes (ARGs) have existed long before the clinical use of the corresponding antibiotics, thereby suggesting that antibiotic resistance (AMR) is a natural and ancient phenomenon (D'Costa et al., 2011; Wright and Poinar, 2012). For example, the *Actinomycete* class of bacteria is responsible for synthesis of the vast majority of natural β-lactam antibiotics (Gibson et al., 2015). This not only generates resistance mechanisms in the antibiotic-producer organism itself, but also exposes other environmental bacteria to antimicrobial molecules promoting selection of resistance in other species (Blair et al., 2015). In addition, resistant bacteria carrying antibiotic resistance genes (ARGs) have been found in areas with little to no anthropogenic influence (Singer et al., 2006; Hernandez et al. 2012).

There is concern that antimicrobial use in food producing animals and humans may promote development and dissemination of resistant bacteria and ARGs in environments impacted by livestock and human activity (Agga et al., 2015; Woolhouse et al., 2015). In particular, use of antibiotics for growth promotion in animals is a long standing controversial issue. Social, academic and political debates continue regarding the benefits and detriments of growth promoting uses of antibiotics (i.e., increase food production for a "hungry" world and development of antibiotic resistant bacteria, respectively) (Angulo et al., 2005; Sorensen et al., 2014).

Biocides and metals have the ability to co-select for antibiotic-resistant bacteria (Pal et al., 2015). In this case, antimicrobial resistant determinants can be disseminated even in the absence of primary selective pressure associated with use of the corresponding antibiotic. After an extensive review, Pal et al. (2015) concluded that copper, silver, arsenic, antimony, cobalt, nickel, cadmium, iron, zinc, mercury and quaternary ammonium compounds are potential co-selectors for bacteria resistant to sulfonamides, β-lactams, amphenicols, tetracyclines and aminoglycosides.

Mechanisms of antimicrobial resistance

Antimicrobials have different mechanisms of action (Table 1, Tenover et al. 2006). Bacteria can have resistance to antimicrobial compounds because naturally occurring (intrinsic) or acquired mechanisms of resistance. One example of intrinsic resistance is given by gram-negative bacteria that are resistant to effects of certain antibiotics molecules due to their inability to cross the outer membrane and reach targets inside the cell (Blair et al., 2015). Such intrinsic resistance occurs independent of antibiotic usage, chromosome mutations and/or acquisition of mobile genetic elements conferring resistance (Cox and Wright, 2013).

Table 2.1. Mechanisms of action of antibacterial agents (Adapted from Tenover, 2006).

Antimicrobial	**Mechanism**
β-Lactams, glycopeptides	Interference with cell wall synthesis
Macrolides, tetracyclines, aminoglycosides	Protein synthesis inhibition
Fluorquinolones, rifampin	Interference with nucleic acid synthesis
Sulfonamides	Inhibition of metabolic pathway
Polymyxins, daptomycin	Disruption of bacterial membrane structure

Mechanisms of antimicrobial resistance that are acquired traits include enzymatic modification of the antibiotic or target molecule, export of the antibiotic from the cell (efflux pumps) and permeability changes in the cell wall that reduce antibiotic uptake (McDermott et al., 2003; van Hoek et al., 2011; Blair et al. 2015). Acquired mechanisms of resistance are the result of chromosomal DNA mutations and/or acquisition of mobile genetic elements (MGEs) carrying resistance genes, such as plasmids, transposons and integrons (Ochman et al. 2000; Clewell, 2014). An example of chromosomal mutation is quinolone resistance in gram-negative bacteria. Quinolones are antibiotics that prevent bacterial DNA replication, but a single mutation in the *gyrA* gene in the quinolone resistance determining region protects the bacteria from lethal action of quinolones (Gruger et al., 2004). Lee et al. (2012) found a rate of spontaneous mutation in *E. coli* (1×10^{-3} per genome per generation) lower than previously expected. An example MGE acquisition is most *Salmonella* resistant to ceftiofur and isolated from U.S. food-producing animals are due to the $bla_{\text{CMY-2}}$ gene encoded in a plasmid mobilized by conjugation (Frye and Fedorka-Crey, 2007). Conjugation is a mechanism of lateral gene transfer which requires physical contact between the donor and recipient bacteria, and the formation of a pore through which DNA harbored by a plasmid can pass (Thomas and Nielsen, 2005). In addition to conjugation, transformation (uptake of naked DNA from the environment) and transduction (DNA introduced into bacteria by bacteriophages) are additional ways of horizontal gene transfer between bacteria (Ochman et al., 2000). Ochman et al. (2000) questioned the magnitude of gene acquisition through HGT from an evolutionary perspective as individual bacterial genomes have remained relatively small over millions of years. They hypothesized that bacterial genomes tend to delete non-essential DNA coming from horizontally transferred genes or ancestral resident sequences, thereby counterbalancing gene acquisition with gene loss.

Dissemination of antimicrobial resistance

Antimicrobial resistance can spread from food animals to humans and from humans to food animals through different mechanisms of transmission (Figure 2.1). Transmission of resistant bacteria between livestock farms and humans may occur via ingestion of contaminated meat (Leverstein-van Hall et al., 2011), via direct contact with animals (Woolhouse and Ward, 2013), or via an intermediary vehicle of transmission such as air, manure, soil or water which may contaminate fruits and vegetables for human consumption (Lazarus et al., 2015). One of the most likely sources of environmental dissemination of antibiotic resistance genes is through cattle manure. Cattle manure carries resistance genes from antibiotic-treated animals which are candidates for horizontal transfer and dissemination to other bacterial species in the environment (Wichmann et al., 2014). Additionally, manure from animals produces a bloom of resident antibiotic-resistant bacteria and genes already present in soils, even if the manure comes from animals unexposed to antibiotics (Udikovic-Kolic et al., 2014).

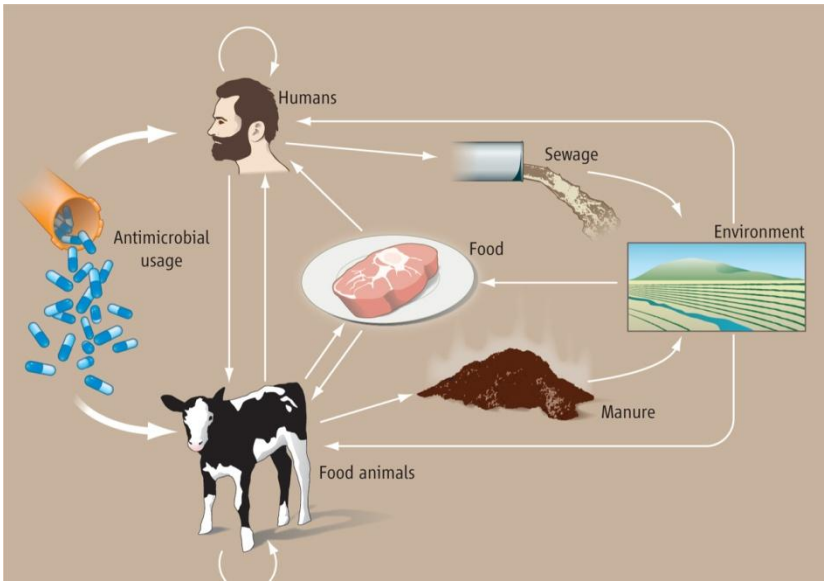


Figure 2.1. Antimicrobial resistance spread through different ecological niches. Reprinted from Woolhouse and Ward (2013) (Credit: P. Huey/Science).

Transmission of resistant bacteria also is possible from humans to animals, and may be responsible for the incursion of human-related antibiotic resistance genes into livestock operations (Lazarus et al., 2005). Waste-water treatment plant effluents located in cities usually contain high concentration of antibiotic resistance genes from hospitals and individual households (Brown et al., 2006; Harris et al., 2014). That pool of genes can be disseminated to farms located closer to the source of the effluent by runoff (Singer et al., 2006). The recent discovery of carbapenem-resistant bacteria from the feces of dairy cattle reported by Webb et al. (2016) raised questions about the origin of the resistance genes. Carbapenems are not approved for use in livestock (OIE, 2015) so antibiotic-resistant bacteria found in animals most probably originated from a human source (Woolhouse et al., 2015).

Evidence of exchange of resistance genes among animals and humans was found by Ma et al. (2015) who reported ARGs that were shared by chicken, pig, and human feces, showing a significant preponderance of ARGs in chicken feces. Coincidently, Lazarus et al. (2015) affirmed that poultry is the most probable source of specific human infections, like extra-intestinal extended-spectrum cephalosporin resistant *E. coli*. However, Gibson et al. (2104) and Forsberg et al. (2014) reported that environmental and human-associated microbial communities have distinct ARGs, suggesting that the resistome is determined by bacterial community composition in each ecological niche. In summary, there is evidence of ARG exchange among animal and human environments, but we do not have a clear understanding of all types of transmission, including a lack of understanding regarding the direction and frequency of the transmission events.

Antibiotic resistance in organic and natural animal production systems

In order to meet natural and organic standards, livestock must be managed without antibiotics, among other requirements (USDA National Organic Program, 2013). For that reason, Gerzova et al. (2015) suggested that ARGs would be less common in microbial communities of organically raised animals compared with those raised conventionally due to lower antibiotic exposure. Several studies have compared ARGs in conventional and organic or natural livestock farms.

In pigs, Kazimierczak et al. (2009) found tetracycline resistance genes in pigs raised on organic farms. Even though they did not track source of those genes, they hypothesized that ARGs came from environmental sources as they were located in mobile genetic elements. Similarly, Zwonitzer et al. (2016) found antibiotic resistant *E. coli* isolates in organic swine manure; however, more predominant resistance was found in *E. coli* isolates recovered from conventional swine manure. Thakur and Gebreyes (2005) reported a high prevalence of antimicrobial-resistant *Campylobacter* coli in swine raised in conventional and antimicrobial-free farms, but the frequency of resistance was greater among conventional herds than among antimicrobial-free herds for the two most common resistance genes (tetracycline and erythromycin).

In chickens, Luangtongkum et al. (2006) found a greater prevalence of antimicrobial resistance *Campylobacter* spp. isolates from conventional farms than from organic farms. However, even in the absence of antibiotic exposure, a high prevalence of tetracycline resistance was observed in organically raised broilers and turkeys, suggesting that resistant strains exist in the absence of anthropogenic selection pressure. Similar results were reported by Price et al. (2005) who found fluoroquinolone-resistant *Campylobacter* in conventional and antibiotic-free

chicken products. Sapkota et al. (2014) reported fewer antibiotic-resistant *Salmonella* after antimicrobials were withdrawn from a poultry operation transitioning from conventional to organic production practices.

In cattle, Santamaria et al. (2011) found several tetracycline resistance genes in feces, soil and water from grassland-based dairy farms, where use of antibiotics in animals was restricted to only treating disease. Dissemination of tetracycline genes was attributed to runoff water. Presence of ARGs in cattle with no previous exposure to antibiotics has been extensively reported, with the largest resistance category being to tetracyclines (Durso et al., 2011; Chambers et al., 2015). Morley et al. (2011) did not find a consistent increase in prevalence of antimicrobial resistant *E. coli* in conventional feedlot cattle compared to rearing methods that limit exposure to antibiotics. Similarly, Johnston (2002) did not find differences in resistance to penicillin between bacteria isolated from conventionally versus organically raised cows. Reinstein et al. (2009) found similar prevalence and antibiotic susceptibility patterns among *E. coli* O157:H7 recovered from organically and naturally raised beef cattle. For many of the antibiotics tested, minimum inhibitory concentrations (MIC) for isolates from organically or naturally raised cattle were greater than those isolates from conventionally raised cattle. The authors suggested that high concentration of heavy metals in the diet of organic or natural feedlot cattle, such as copper and zinc to replace conventional antibiotics, may result in emergence of bacterial populations resistant to metal and antibiotics due to co-selection mechanisms (Reinstein et al., 2009; Pal et al., 2015). In dairy farms, Sato et al. (2005) reported that *E. coli* isolates from organic farms showed lower prevalence of resistance to 7 antimicrobials compared to those from conventional farms. However, Cho et al. (2007) did not observe a significant difference in percent resistance

in Shiga toxin-producing *E. coli* between organic and conventional dairy farms, even though numerically, a greater proportion of isolates from conventional farms were resistant.

Microbiome and antibiotic resistance genes

The microbiome can be defined as the collection of genes and genomes of members of a microbiota, the assemblage of microorganisms present in a defined environment (Marchesi and Ravel, 2015). Most of the microbiome is comprised of commensal or indigenous microorganisms, which are known to be symbiotic and beneficial for the host (Zhang and He, 2015). Commensal microbiome plays an important role in promotion of health and prevention of pathogens from colonizing the host not only by competing for nutrients and spaces, but also by producing antimicrobial substances such as organic acids and bacteriocins (Hanning and Diaz-Sanchez, 2015; Buffie and Pamer, 2013). On the other hand, the microbiome warrants special attention as the most important reservoir of resistance genes due to the potential for genetic exchange of ARGs between commensal bacteria and pathogens (Penders et al., 2013). In narrow and specific environmental or animal niches, high rates of horizontal gene transfer, including those carrying antibiotic resistance determinants, can occur among bacteria with similar taxonomy (Thomas and Nielsen, 2005; Popa and Dagan, 2011). Jernberg et al. (2010) reported that the intestine is an ideal location for transmission of resistance genes between the commensal and pathogenic microbiota (moist, warm, nutrients, slow passage rate, high concentration of bacteria). Stanton et al. (2008) found that in-feed antibiotics (carbadox) increased bacteriophages or phage-like elements in swine, which can transfer ARGs by transduction between bacteria. Therefore, it is crucial to study the antimicrobial resistance potential from a broader ecological perspective, including the commensal bacteria, and not restricting studies to known clinical pathogens (Gillings, 2013; Penders et al., 2013). Su et al (2016) found a significant correlation

between ARGs profiles and bacterial community composition during sewage sludge composting suggesting that the shift in microbiome due to changes in physicochemical properties during composting was the main driver shaping the resistome. Similar results were found by Noyes et al. (2016) and Forsberg et al. (2014), who also found a correlation between microbiome and ARGs in beef cattle environments and soil, respectively. According to Pitta et al. (2016) and Gerzova et al. (2014), some bacteria are more relevant than others in carrying ARGs; for example members of the phylum *Proteobacteria*.

Use of antimicrobials is one of the main factors that can alter the host microbiome. Several studies reported that use of antibiotics can result in decreased richness (number of species) and/or diversity (number of species and abundance of each taxon) in the microbiome (Flanagan et al., 2007; Francino, 2015; Raymond et al., 2016). As a result, host susceptibility for infections can increase in the short-term due to more nutrients and space for opportunistic pathogenic bacteria (Bailey et al., 2010). Recovery to the background or normal microbiome depends on the antibiotic used, dose, duration of treatment, route of administration, pharmacokinetic and pharmacodynamic properties of the antibiotic (Jernberg et al., 2010; Looft and Allen, 2012).

In addition to antimicrobials, diet, age and gut maturation has been shown to cause shifts in the microbial community (Danzeisen et al., 2011). For example, the amount of fiber and starch in the diet, as well as the main source of nitrogen (ammonia or amino acids), can promote growth of different bacterial groups (cellulolytic or amylolytic) within the rumen of cattle (Thoetkiattikul et al., 2013; Petri et al. 2012; Pitta et al., 2010; Russell et al. 1992). In chicken, Danzeisen et al. (2011) reported a greater complexity and stability of the cecum microbiome in older birds compared to younger birds. They also suggested that the core microbiome shared

across diets, age or different antibiotic regimes is not determined by "who" is present in the microbiome, but more so by "what they are doing" because different microbial communities (in terms of membership) can have the same functionality (Danzeisen et al., 2011).

Overview of shotgun metagenomics

Traditional approaches to detect antibiotic resistant bacteria and their resistance genes have been culture-based methods and quantitative PCR (qPCR) (Figure 2.2). The culture-based approach is limited by the fact that most bacteria are unculturable in laboratory conditions (Handelsman, 2004; Wichmann et al., 2014). In addition, bacterial isolates can lose plasmids carrying ARGs or the resistance genes may not be expressed due to cell stress during culturing (Smith and Bidochka, 1998). Shotgun metagenomics (referred as "deep sequencing" in Figure 2.2) can be defined as the study of all genomes present in an environmental sample, answering the question of who is present in an environmental community (Oulas et al., 2015; Zepeda-Mendoza et al., 2015). This involves determining not only which microbes or genes are present, but also at what relative or absolute abundance when comparing different samples (Sharpton, 2014). In shotgun metagenomics, after DNA is directly extracted from an environmental sample, random DNA is sheared into smaller fragments for library preparation (Sharpton, 2014). This is one of the main differences when comparing metagenomic Next Generation Sequencing with target PCR, which amplifies specific genes within the bacterial community for taxonomic classification (i.e., 16S ribosomal RNA). In addition to shearing DNA, metagenomic library preparation includes fragmentation and end-repair of DNA, phosphorylation of the 5' ends, Adenosine-tailing of the 3' ends, ligation of adapters, and PCR amplification of fragments with adapters so sufficient DNA is loaded into the sequencer (Head et al., 2014). The main concern

about library preparation is that it can cause bias, mainly during the PCR amplification of DNA fragments with adapters, as longer fragments or fragments with unusual Guanine-Cytosine (GC) content are amplified less efficiently than shorter or GC neutral DNA fragments (Head et al., 2014; van Dijk et al. 2014).

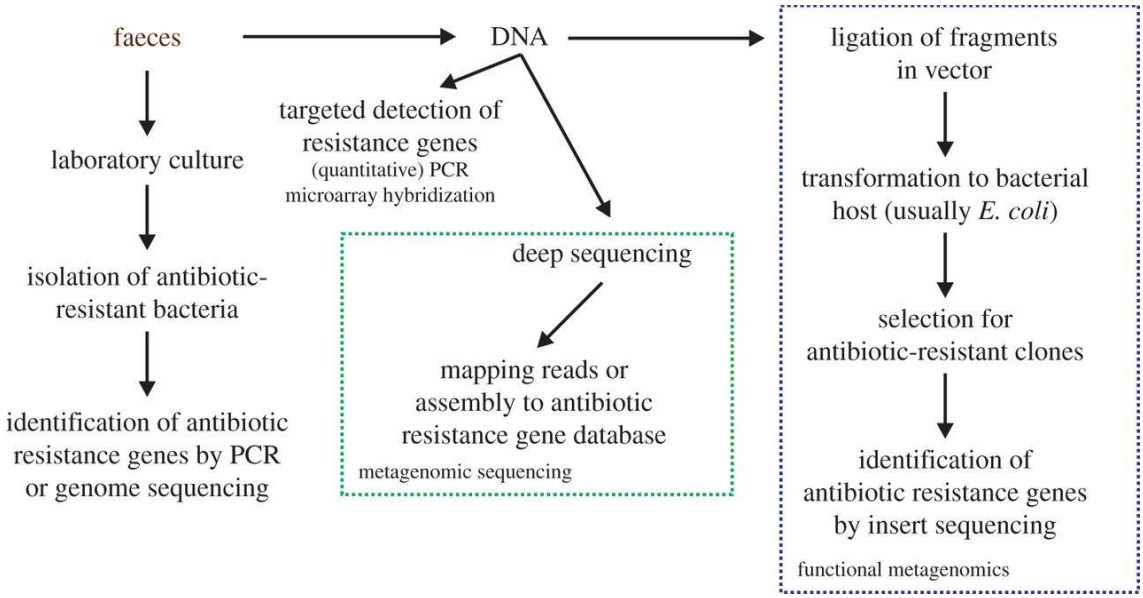


Figure 2.2. Different approaches for analysis of resistance genes. Reprinted from van Schaik (2015).

After DNA extraction and library preparation, DNA is ready for sequencing. There are several next-generation sequencing platforms, including Roche 454 Pyrosequencer, Illumina, SOLiD, PacBio, and Ion Torrent, among others (Liu et al., 2012; Quail et al. 2012). They differ in terms of their sequencing method (i.e., sequencing by synthesis or by ligation), chemistry, cost, error rate, length and number of reads (single or pair-end reads), and output (Mb/run). Selection of the proper platform depends on objectives of each study; Illumina is one of the most common platforms today. After immobilization and clonal cluster of DNA fragments in a flow cell, Illumina sequencing is carried out by synthesis, using primers complementary to adaptors

present in the DNA fragment. Then, DNA polymerase adds one reversibly-blocked nucleotide at a time (Mayo et al., 2014).

Bioinformatic analysis in shotgun metagenomics

The first step in treatment of raw sequencing data from the sequencer is to assess overall quality, including base quality, GC content, length distribution, number of duplicate reads, and adapter content. This can be easily obtained using softwares that determines whether data contain any serious problems that researcher should be aware before performing further analysis (Ju and Zhang, 2015). Secondly, low quality bases and technical sequences, such as library adapters, are removed in a process known as trimming, as they can result in inaccurate downstream analyses (Bolger et al., 2014). Each nucleotide is associated to a Phred score (Q), which is the probability that the corresponding base call is wrong (Del Fabbro et al., 2013). For example, a Q score of 30, which is generally used as a cut-off for base quality, is equivalent to the probability of an incorrect base call 1 in 1,000 nucleotide assignments. After trimming low quality bases, the next step is filtering. When filtering a sample obtained from an animal (i.e., rumen, feces, meat), to study its bacterial composition or resistance genes, the goal is to filter out genomic sequences corresponding to the host. The most common approach to remove host DNA is by aligning metagenomic sequences to the reference genome of the host if available (Wooley et al., 2010). Host DNA can be removed before sequencing if it corresponds to the vast majority of the sample using methods that selectively enrich bacterial DNA based on different methylation patterns in eukaryotes and prokaryotes (Barnes et al., 2014). After trimming and filtering, shotgun metagenomic reads are aligned against a database of a reference set of sequences searching for similarities. For taxonomic classification of reads, Kraken is a fast and accurate software that contains a database with unique *k*-mers (usually 31 bases long) for the bacterial genomes

contained in the National Center for Biotechnology Information database (Wood and Salzberg, 2014). For identification of ARGs in metagenomic sequences, trimmed and filtered reads are aligned to reference databases containing the known sequence of ARGs, some using Resfinder (Zankari et al., 2012), ARG-ANNOT (Gupta et al., 2013) and CARD (McArthur et al., 2013) databases. Either for taxonomic classification or identification of antibiotic resistance genes, the number of reads that map to each reference gene or genome are used as a proxy for abundance in the sample. However, resulting read counts are highly dependent on sequencing depth or number of reads in each sample, and normalization is required to allow comparison across samples (Manor and Borenstein, 2015). Several methods have been developed to normalize metagenomic reads before proceeding with downstream analysis, including normalization based on average genome size (Frank and Sorensen, 2011), universal single-copy genes (Manor and Borenstein, 2015), or simply dividing each gene count by the total number of reads in each sample (Dillies et al., 2013).

Binning and assembling are alternative strategies for data analysis in shotgun metagenomics. Binning clusters metagenomic reads into groups of sequences with high similarity that represents taxonomic groups (i.e., GC content) (Sharpton, 2014). This approach cannot answer the question "who is in the community", but by placing sequences in different groups, gives an idea of how many different "members" exist in a community as sequences within the same cluster are expected to come from the same taxonomic group (Wooley et al., 2010; Zepeda-Mendoza et al., 2015). Assembly merges metagenomic sequences based on overlapping reads, thus generating longer sequences known as contigs. It simplifies downstream bioinformatic analysis, but increases the rate of error as sequences from different genomes can be assembled, abundance of the genome being assembled cannot be quantified, and assembly

tends to be restricted to the most abundant taxa in the community (Sharpton, 2014). *De novo* assembly is an alternative metagenomic approach that allows recovery of unknown genomes without relying on reference databases containing previously described genomes (Zepeda-Mendoza et al., 2015).

Whole genome sequencing and antimicrobial resistance

Identification of ARGs through whole genome sequencing (WGS) accurately predicted antimicrobial resistance phenotypic expression in *Escherichia coli* isolates (Tyson et al., 2015). Similarly, McDermott et al. (2016) reported a high correlation between WGS and phenotypic methods (minimum inhibitory concentration breakpoints) to predict antimicrobial resistance in more than 600 strains of *Salmonella*. Gordon et al. (2014) reported that WGS was as sensitive and specific as traditional antimicrobial susceptibility testing methods for resistance prediction in *Staphylococcus aureus*, but they acknowledged that it is unlikely that WGS will be able to replace phenotypic methods entirely as resistance genes identified by WGS will be expressed or not depending on complex interactions between promoters and repressors (Gordon et al., 2014). McDermott et al. (2016) reported discrepancies in several *Salmonella* isolates that carried streptomycin resistance genes, but that were phenotypically susceptible, probably due to lack of standard minimum inhibitory concentration breakpoints.

Whole-genome sequencing not only is able to identify ARGs, but also can identify mobile genetic elements carrying them, such as integrons, transposons, and plasmids (Baquero, 2012). For example, Leekitcharoenphon et al. (2016) used WGS to study epidemiology of *Salmonella Typhimurium* DT104 and found an antibiotic resistance gene cassette in a multidrug resistance region within a genomic island which is mobilizable by IncA/C plasmids. In addition,

bacteriophages and phage-related particles can act as mobile genetic elements able to transfer antibiotic resistance based on their high invasion rate (Brown-Jaque, 2015; Lekunberri et al., 2017). Metagenomics and whole genome sequencing allow the option to combine ARGs with the microbiome and, eventually, metabolic pathway information to provide a more complete profile of specific samples (Schmieder and Edwards, 2012). Currently, regulatory authorities are evaluating use of WGS as one simple workflow to replace traditional pulsed-field gel electrophoresis, phenotypic tests, agglutination assays, PCR for virulence profiling, and culture-based antimicrobial susceptibility tests for the main pathogens of concern (Lindsey et al., 2016). Whole genome sequencing has also been successfully applied to detect pathogens in different environments (Köser et al., 2012; Yang et al., 2016).

CHAPTER III

IMPACT OF NATURAL AND ORGANIC REARING METHODS ON THE RESISTOME OF FECES AND ENVIRONMENTAL SAMPLES IN BEEF FEEDLOT AND DAIRY CATTLE PRODUCTION SYSTEMS

Summary

The objective of this study was to evaluate the impact of animal rearing methods on antibiotic resistance genes (ARGs) in feedlot and dairy farms. Samples of feces, wastewater from lagoons, and soil where wastewater was applied were collected from a conventional feedlot, natural feedlot, conventional dairy and organic dairy. After DNA extraction, sequencing, and processing, metagenomic reads were aligned to reference databases for identification of ARGs (resistome) and bacteria (microbiome). Resistome composition was influenced by production practices, cattle type, and type of sample. Most mechanisms of resistance affected by production practices were more prevalent ($P < 0.05$) in samples collected from conventional production systems. The greatest separation of the resistomes was observed in wastewater samples (conventional vs. natural or organic), but differences in effluent management among farms was a confounding factor. Tetracycline and macrolide-lincosamide-streptogramin (MLS) resistance was more abundant in feces of feedlot cattle than in dairy cattle ($P < 0.05$); whereas beta-lactam ARGs were more prevalent in feces of dairy cattle ($P < 0.05$). Fecal resistomes and microbiomes differed ($P < 0.05$) from those for wastewater and soil. Our results indicate that ARGs are widespread in beef feedlot and dairy cattle farms irrespective of restricted antibiotic use.

Introduction

Emergence of antimicrobial resistance (AMR) has become an important public health issue worldwide (Penders et al., 2013; WHO, 2014). Because of human health concerns regarding AMR (specifically, the consequence of treatment failure when treatment is required for a human infection), there is interest in producing food animals without using antibiotics. In the dairy sector, the number of certified organic milk cows on U.S. farms increased from 38,000 in 2000 to 255,000 in 2011(USDA-ERS, 2013). A recent consumer survey, conducted by the Food Marketing Institute and the North American Meat Institute (2016) found that 40% of all respondents had purchased meat produced using organic or "natural" production practices within 3 months of the survey, increased from 20% in 2007. Nevertheless, several scientific studies have detected significant AMR bacteria in organic and natural animal production systems where use of antibiotics was prohibited or restricted (Price et al., 2005; Luangtongkum et al., 2006; Cho et al., 2007; Kazimierczak et al., 2009; Reinstein et al., 2009; Morley et al., 2011; Santamaría et al. 2011; Zwonitzer et al., 2016).

Traditionally, factors affecting AMR have been investigated using cultures of indicator bacteria or pathogens (Luangtongkum et al., 2006; Morley et al., 2011; Sapkota et al. 2014; Zwonitzer et al., 2016), or by PCR amplification of a limited number of specific antibiotic resistance genes (Johnston, 2002; Cohen Stuart et al., 2012, Guarddon et al., 2014) detected via polymerase chain reaction (PCR) methods. These approaches limited findings and conclusions because they select for only certain antibiotics or bacterial species, and it is uncertain whether presence of AMR genes in specific pathogens is representative of the entire microbial and antibiotic resistance gene (ARG) population or not (Gerzova et al., 2015). Recently, metagenomic approaches have been used to characterize microbial communities ("microbiome")

and their associated ARGs ("resistome") in different agricultural environments (Forsberg et al., 2014; Agga et al., 2015; Gerzova et al., 2015; Noyes et al., 2016a; Noyes et al., 2016b; Yang et al., 2016), i.e., by evaluating the ecological impact of antimicrobial use. The main advantage of using metagenomic investigations is the ability to look at the whole microbiome community and resistome in environmental samples, improving our understanding of microbial communities and their associated ARGs (Noyes et al., 2016a). In the present study, we characterized the resistome and associated microbiome in feces and environmental samples in conventional, natural and organic beef feedlot and dairy cattle systems of production. We hypothesized that different production practices, types of cattle and types of samples create differences in the resistomes of microbial communities.

Materials and Methods

Study overview

Samples of cattle feces, wastewater from lagoons, and soil where wastewater was applied were collected from a conventional feedlot, natural feedlot, conventional dairy, and organic dairy. After DNA extraction, sequencing, and processing, metagenomic reads were aligned to reference databases for identification of antibiotic resistance genes (resistome) and bacteria (microbiome). The objective was to characterize the variation of the microbial resistome associated with production practices (i.e., feces from conventional farms vs. feces from natural farms), type of cattle (i.e., feces from beef cattle vs. feces from dairy cows), and type of sample (i.e., feces vs. soil samples). Primary comparisons were conducted using ordination plots and differences in abundance of individual resistance features at class, mechanism and group levels.

Study sites

One each of a conventional feedlot (CONV-F), natural feedlot (NAT-F), conventional dairy (CONV-D), and organic dairy (ORG-D) participated in the study. Feedlots were located in Alberta, Canada, with a capacity of 38,000 and 22,000 heads in NAT-F and CONV-F, respectively. In the NAT-F, approximately half of the cattle were managed for natural beef production, while the other half were raised using conventional methods. Both areas, conventional and natural, were physically separated in the facility, including separate waste water drainage and catchment basins. However, some conventional pens were located in the natural area. Cattle raised in the natural feedlot had a branded program in compliance with the Canadian Food Inspection Agency (CFIA) guidelines for the label claims raised without added hormones, raised without antibiotics and not fed animal by-products. Both dairy farms, CONV-D and ORG-D, were located in northern Colorado (USA) and milked approximately 1,200 cows each at the time of sampling. Lactating cows were housed in free-stalls (CONV-D) or dry lots (ORG-D). The ORG-D farm was certified organic by USDA National Organic Program certifiers meeting requirements such as not administering antibiotics or artificial growth hormones to the animals.

Antimicrobial use data

Early feeding pens (mean ± s.d.: 13 ± 11 days after placement) and late feeding pens (mean ± s.d.: 243 ± 38 days after placement) in CONV-F were exposed to ionophores, tetracyclines, macrolides, phenicols, sulfonamides, and beta-lactam classes of antimicrobials (Appendix 1 and 2). In CONV-D, cows in the high and low producing pens were treated with beta-lactams for clinical illness. For every cow that was present at the time of sampling in each

pen (371 and 171 cows in low and high producing pens, respectively), individual antimicrobial use data was obtained for the previous 365 days (Appendix 3).

Sample collection

Collection of soil and feces occurred in September 2015. For feces, 16 pens were sampled in each feedlot, corresponding to 8 pens that were on feed the shortest amount of time ("feces early") and eight pens that were on feed for the longest amount of time ("feces late"). For fecal samples collected at dairies, 2 pens were sampled in each facility, one with high producing milking cows ("feces high") and the other with low producing milking cows ("feces low"). Composite pen floor fecal samples (~400 g/sample) were collected from the floor of each feedlot pen (one sample/pen) and dairy pen (8 samples/pen) by pooling feces from 20 fresh fecal pats using sterile tongue depressors, resulting in a total of 64 composite samples. Soil samples were collected from land where wastewater from the main catchment basins was used for irrigation. In the case of NAT-F, wastewater from the lagoon receiving the effluents from the natural pens usually overflows and drains into a bigger lagoon containing effluents from both natural and conventional pens. Wastewater from this main lagoon, containing mixed effluents from conventional and natural pens, was applied to fields in NAT-F. In all cases, wastewater stored in lagoons was applied to the land using pivot irrigation equipments between 1 day and ~ 6 months before soil sampling. In each production field (range: 2 - 65 ha.), eight composite soil samples (~ 400g/sample) were collected walking in a zigzag pattern with a standard soil auger at a depth of 5-10 cm; 20 soil cores per composite sample were used for a total of 32 composite soil samples (8 samples/farm). Composite fecal and soil samples were placed in Whirl-Pak bags (Nasco, Fort

Atkinson, WI) refrigerated in the field, transported to the laboratory, and frozen at - 80°C until DNA extraction.

Wastewater lagoons were sampled in September 2016. Eight water samples (500 ml each) were collected at each production system by walking the side of the lagoons with the easiest access. Samples were collected 15-20 cm below the water surface using a sampling pole (Nasco, Fort Atkinson, WI) and 500 ml sterile plastic containers (Thomas Scientific, Swedesboro, NJ). Bottles were transported to the laboratory, and within the same day of collection, the entire 500 ml volume from each sample was centrifuged at 10,000 g for 20 min at 4° C. The resultant pellet was stored at -80°C for further DNA extraction.

DNA extraction and metagenomic sequencing

Ten grams of feces from each sample was weighed and allowed to sediment to separate bacterial cells from heavy particles and debris as described by Noyes et al. (2016a). For soil samples, 10 grams was weighed from each sample with no further sedimentation step. DNA was extracted directly from soil samples and from the fecal and water pellets (1.5 – 8.0 g) using the PowerSoil® DNA isolation kit (Mo Bio Laboratories, Inc., CA) according to the manufacturer's protocol.

After DNA extraction and precipitation, DNA concentration and quality was measured using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, DE) and a Qubit™ assay (Thermo Fisher Scientific, DE). Two µg of DNA (40-50 µl) from each sample was transported on ice to the Genomics and Microarray Core at the University of Colorado Denver (Denver, CO). Genomic libraries were prepared with the Illumina TruSeq DNA PCR-Free LT Library Kit (Illumina, Inc., San Diego, CA). Paired-end sequencing (2 x 150 bp) was performed

on the Illumina HiSeq 4000 HT sequencing platform (Illumina, Inc., San Diego, CA) with 5.6 soil, 8 feces, and 16 wastewater samples per lane. Raw sequencing data for all 128 samples in the present study are publicly available at the NCBI BioProject database with accession number PRJNA379303.

Data processing

After sequencing, fastq files were analyzed using the AmrPlusPlus pipeline (available at https://megares.meglab.org/amrplusplus/latest/html/). In brief, raw sequencing reads were trimmed using Trimmomatic version 0.36 (Bolger et al., 2014) and bovine DNA sequences were removed by aligning trimmed sequences to reference cattle genomes (Zimin et al., 2009; Canavez et al., 2012) using Burrows-Wheeler Aligner (BWA) version 0.6.2 (Li and Durbin, 2009). For detection of ARGs, remaining trimmed sequences were aligned using BWA to approximately 4,000 hand-curated, non-redundant ARGs contained in the MEGARes database (Lakin et al., 2016). Only ARGs that were covered > 80% in length by sample reads, and those where resistance was not conferred by single nucleotide polymorphism were considered for downstream statistical analysis. Individual ARGs were defined as genes with published sequences and unique accession numbers in public databases; these ARGs were aggregated and classified hierarchically. The MEGARes annotation scheme consists of three hierarchal levels regarding ARGs: class (i.e., tetracycline resistance), mechanism (i.e., tetracycline ribosomal protection protein), and group (i.e., tetQ) (Lakin et al., 2016). In addition, reads were aligned to a database of genes conferring resistance to metal and biocides (MBRGs) (Pal et al., 2014) following similar data processing described for ARGs. Phylogenetic classification were assigned to trimmed microbial sequences using Kraken (version 0.10.6-beta), which utilized the National

Center for Biotechnology Information (NCBI) reference nucleotide database (RefSeq) to classify bacteria at different taxonomic levels (Wood and Salzberg, 2014). In this study, results are presented at the phylum level. Antibiotic resistance and microbial count tables were normalized using a cumulative sum scaling (CSS) method (Paulson et al., 2013) and features below the 15^{th} quantile were removed from count tables to avoid misclassification related to potential sequencing errors.

Statistical analysis of sequencing data

A zero-inflated Gaussian distribution mixture model native to the metagenomeSeq R package (Paulson et al. 2013) was used to perform analysis of differential abundance in features of the microbiome and resistome. Primary comparisons included resistome and microbiome differences between feedlot and dairy farms; between conventional and natural/organic samples; and differences among type of samples (feces, wastewater and soil). Statistical inference for each feature occurred after $\log_2$ transformation followed by a multiple-comparison Benjamini-Hochberg adjustment using a critical $\alpha = 0.05$. Data were visualized in non-metric multidimensional scaling (NMDS) ordination plots and statistical inference ($\alpha = 0.05$) was made using the analysis of similarity (ANOSIM) included in the vegan package (version 2.2-2) (Oksanen et al., 2014). ANOSIM *R*-value ranges from 0 to 1 indicating the extent of differences between groups (0 = total similarity; 1 = total dissimilarity). Procrustes (Peres-Neto and Jackson, 2001) and Protest (Jackson, 1995) included in vegan R package were used to compare congruence of the microbiome and resistome ordinations based on $\alpha = 0.05$, correlation coefficient (r) and measure of fit (m^2). Richness and Shannon's diversity index were calculated using vegan package (version 2.2-2) (Oksanen et al., 2014).

Results

Sequencing results

Shotgun metagenomic sequencing yielded 5.45 billion reads corresponding 52%, 35%, and 13% of those sequences to feces, soil and wastewater, respectively. The mean quality (Phred score) of reads was 35.3 in feces (n = 64; min. 33.8; max. 37.2), 35.2 in soil (n = 32; min. 31.2; max. 37.8), and 31.3 in wastewater samples (n = 32; min. 28.9; max. 33.2). A total of 440 unique ARGs were identified and assigned to 16 classes, 44 mechanisms and 192 groups of antimicrobial resistance, accounting for 3.96 million reads (~ 0.07% of the total raw reads). Between production practices, samples collected from CONV farms yielded more ($P < 0.05$) normalized reads with aligned ARGs than samples collected from farms with restricted antibiotic use (46,300 and 34,483 reads, respectively), mainly driven by differences between dairy farms (CONV-D: 20,725 and ORG-D: 11,939 reads, $P < 0.05$), especially in wastewater samples (Figure 3.1). The total number of normalized reads aligned to ARGs ("hits") was greater ($P < 0.05$) in samples obtained from feedlots (48,119 reads) vs. dairies (32,664 reads), a difference principally related to numbers of reads obtained from fecal samples ($P < 0.05$).

Composition of the resistome on farms using conventional vs. restricted production practices

The average number of unique ARGs identified per sample was greater ($P < 0.05$) in samples obtained from CONV production systems (ARGs = 77, min. 2, max. 260) than in samples obtained from farms with restricted antibiotic use (ARGs = 51, min. 0, max. 126). This was a consequence of differences in feces (118 and 79 ARGs per sample in CONV and NAT+ORG, respectively; $P < 0.05$) and wastewater (47 and 22 ARGs per sample in CONV and NAT+ORG, respectively; $P < 0.05$).

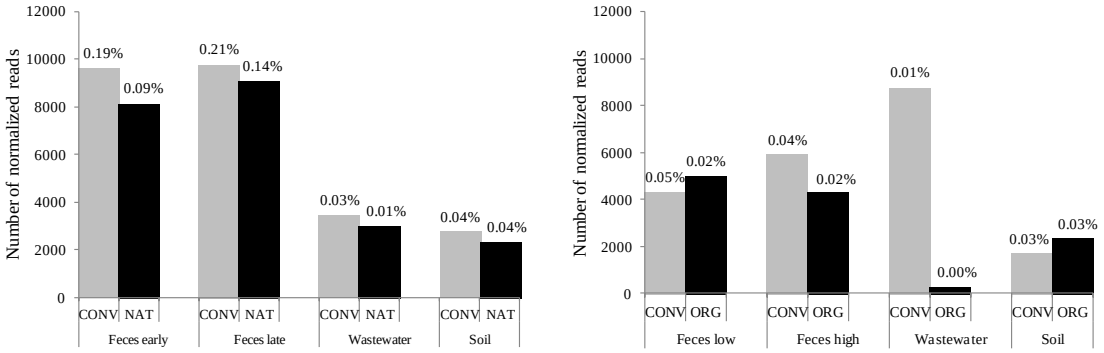


Figure 3.1. Number of normalized antimicrobial resistance reads (ARR) aligned to antibiotic resistance genes in (a) feedlot and (b) dairy samples. Above each bar appears the raw relative abundance (%) calculated as the ratio between the number of raw ARR and the number of total raw reads for each type of sample.

From all pairwise comparisons between CONV and natural or organic resistomes segregated by type of sample, only feces early (Figure 3.2a) and soil (Figure 3.2d) collected from CONV and NAT feedlots did not cluster apart ($P > 0.05$) at any level of resistance in ordination plots. Wastewater samples showed the greatest separation ($P < 0.05$) (Figure 3.2c and 3.3c) at all levels of resistance (ANOSIM $R > 0.75$). Among fecal samples, separation of CONV-F and NAT-F resistomes was greater in feces late (Figure 3.2b; ANOSIM $R = 0.18$, 0.37, and 0.68 at class, mechanism, and group level, respectively) than feces early (Figure 3.2a; ANOSIM $R =$ 0.00, 0.09, and 0.25 at class, mechanism, and group level, respectively).

Separation of CONV-D and ORG-D resistomes was greater in feces obtained from high producing dairy cattle (Figure 3.3b; ANOSIM $R = 0.63$, 0.81, and 0.84 at class, mechanism, and group level, respectively) than in feces collected from low producing dairy cattle (Figure 3.3a; ANOSIM $R = 0.30$, 0.45, and 0.49 at class, mechanism, and group level, respectively). Twenty-nine different classification groups of resistance genes were uniquely identified in samples collected from feedlot or dairy CONV farms (i.e., not on farms with restricted antibiotic use), but these were relatively low abundance genes accounting for ~ 1% of the total reads aligned to

ARGs (Appendix 4). Those unique groups were found in few CONV samples (< 9 out of 64 total samples) except where OXA (D beta-lactamase), ERM (23S rRNA methyltransferase) and LNUF (lincosamide nucleotidyltransferase), which were identified in 23, 17 and 16 conventional samples, respectively.

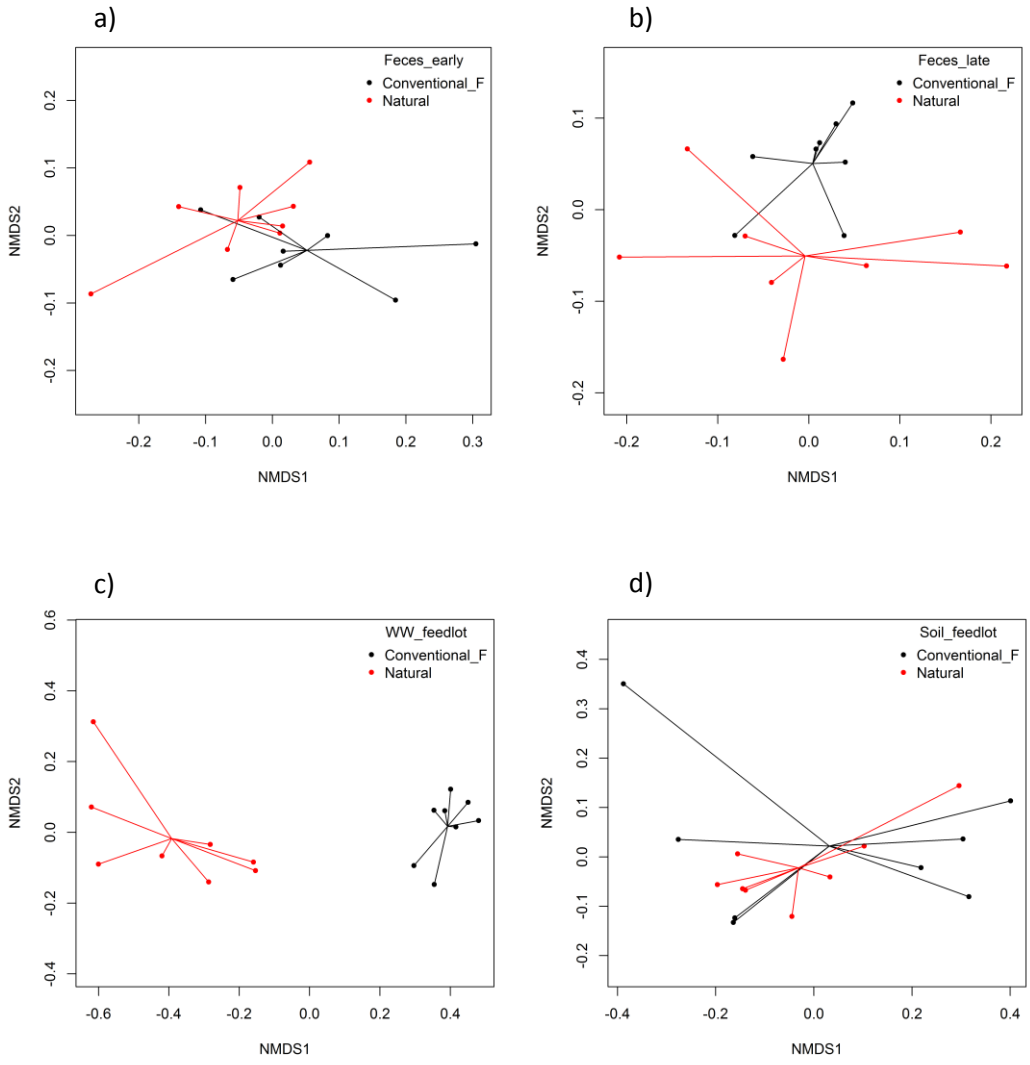


Figure 3.2. Non-metric multidimensional scaling (NMDS) ordination of the resistomes at the mechanism level for (a) feces early, (b) feces late, (c) wastewater and (d) soil samples separated by protocol of production (black: conventional, CONV; red: natural, NAT) in feedlots.

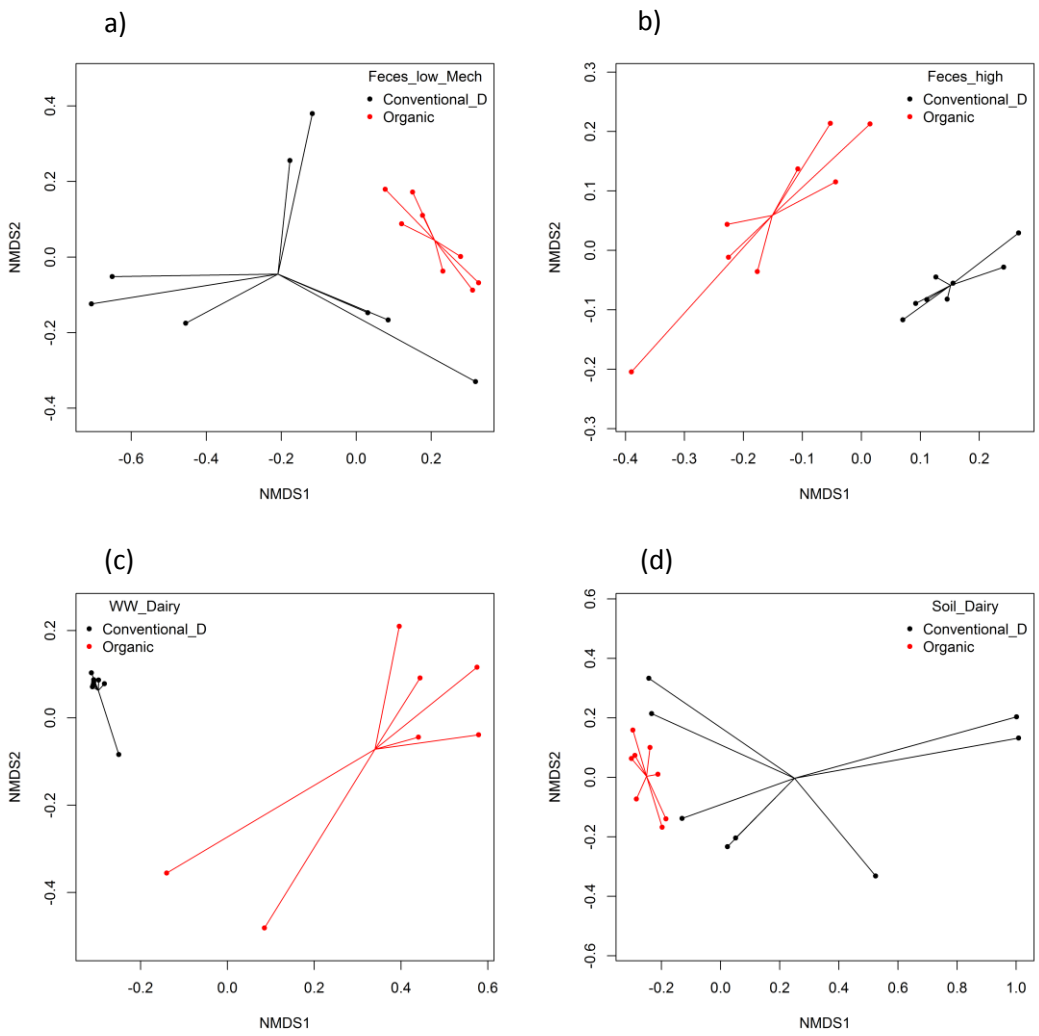


Figure 3.3. Non-metric multidimensional scaling (NMDS) ordination of the resistomes at the mechanism level for (a) feces low, (b) feces high, (c) wastewater and (d) soil samples separated by protocol of production (black: conventional, CONV; red: organic, ORG) in dairy farms.

Across all samples, 24 out of 44 mechanisms of resistance were significantly associated by production practices (i.e., CONV vs. NAT) (Figure 3.4). In general, macrolide phosphotransferases, 23S rRNA methyltransferases and aminoglycosides mechanisms of resistance were consistently more abundant in CONV samples. Among sequences obtained from feces, abundance of mechanisms of resistance was greater in CONV samples than in NAT samples, but with a few exceptions.

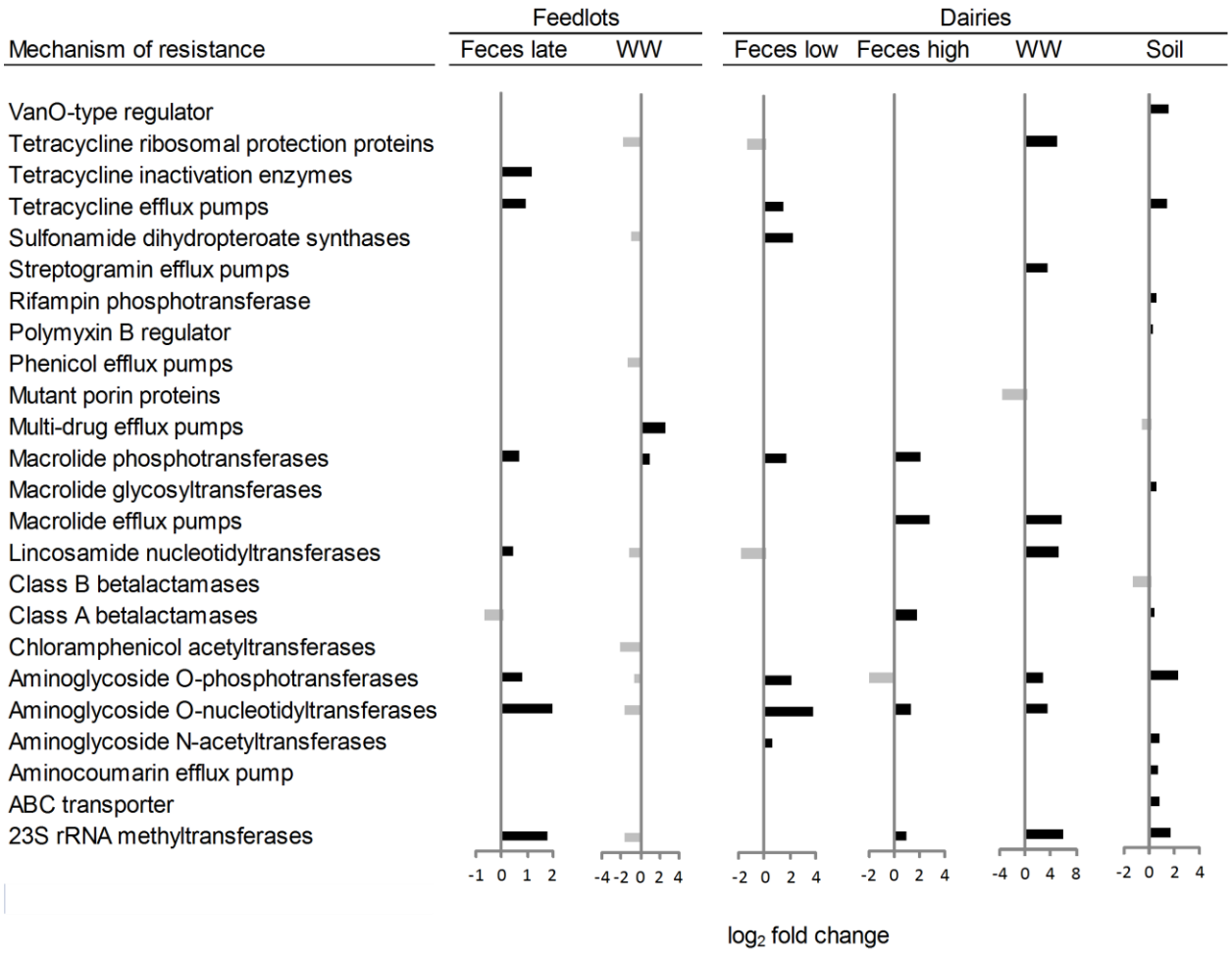


Figure 3.4. Mechanisms of resistance significantly (a) more abundant or (b) low-abundant in conventional feedlot and dairy farms compared to natural or organic farms separated by niche.

Contradictory results were observed in wastewater samples. In feedlots, most of the significantly affected mechanisms of resistance were more abundant ($P < 0.05$) in NAT compared to CONV lagoons, while in dairies most mechanisms were more abundant ($P < 0.05$) in CONV than in ORG lagoons. Among soil samples, mechanisms of resistance were greater in soil samples from CONV-D compared to ORG-D; with the exception of multi-drug efflux pumps which were greater ($P < 0.05$) in ORG-D. In general, the number of different resistance determinants per sample (richness) was greater ($P < 0.05$) in CONV samples than in NAT samples. This was observed at the class (feedlot feces early, dairy feces low and feedlot

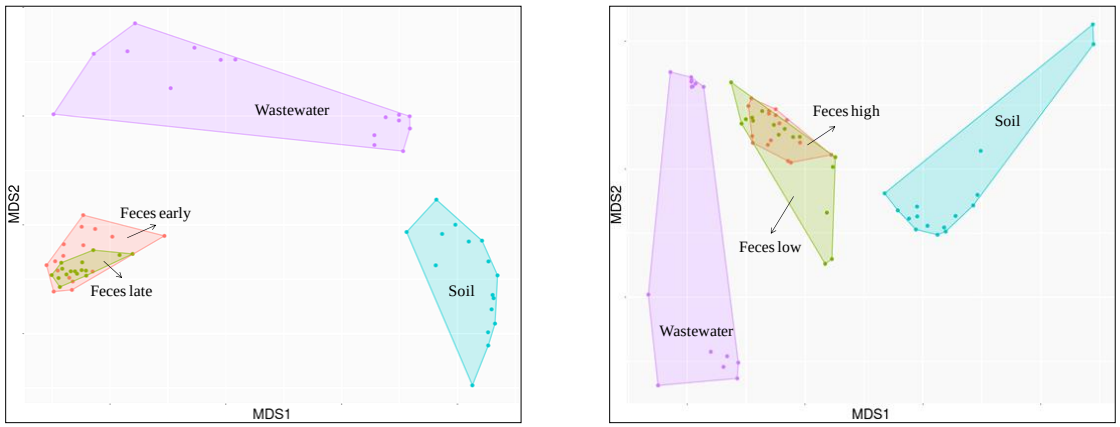
wastewater), mechanism (all feces types, feedlot and dairy wastewater) and group level of resistance (feedlot feces early, dairy feces late, feedlot and dairy wastewater). Shannon's diversity index, which accounts for both abundance and evenness (similarity of frequencies) of resistance determinants, was significantly greater in wastewater samples from CONV-D compared to ORG-D at the mechanism and group level of resistance, and in CONV-D feces low at the mechanism level.

Comparison of the resistomes between fecal and environmental samples

Fecal samples contained more ($P < 0.05$) ARGs (99 per sample, min. 20, max. 260) compared to soil (24 per sample, min. 2, max. 89) and wastewater (35 per sample, min. 0, max. 92). Sixteen groups of resistances (8%) were shared by feces, wastewater and soil corresponding to multi-drug (MEXK, HNS, MSR, SME, EMRE), tetracycline (TETA, TETL, TETX, TETZ), macrolide-lincosamide-streptogramin or MLS (MPHE, MYRA, ERM), aminoglycoside (APH6, APH3-DPRIME), phenicol (CAT) and beta-lactam (OMPR) classes of resistance.

Wastewater samples shared 62 (83%) and 5 (28%) groups of resistance with feces and soil, respectively. Feces, wastewater and soil had 49, 8 and 60 unique groups of resistance (Appendix 5). Clustering pattern was influenced by the type of sample (Figure 3.5a, 3.5b). Resistance to tetracyclines and MLS was more likely ($P < 0.05$) in feces than in environmental samples. Soil samples showed a greater abundance ($P < 0.05$) of classes conferring resistance to multiple-drugs, rifampin, aminocoumarins and glycopeptides than feces and wastewater samples. Tetracycline, MLS and sulfonamide were the most prevalent classes of resistance in wastewater samples. Within feedlots, Shannon's diversity for the resistome was greater ($P < 0.05$) in soil samples than in fecal samples at class (1.25 and 0.83, respectively) and mechanism (1.48 and

1.08, respectively) levels, while wastewater had a diversity index of 0.89 at the class level and 1.33 at the mechanism level. Conversely, fecal resistome diversity was greatest ($P < 0.05$) on dairy farms at the class, mechanism and group level (1.38, 1.31 and 2.73 respectively) compared to soil (1.06, 1.12 and 2.11, respectively) and wastewater samples (0.79, 0.74 and 1.56, respectively).



(a) (b)

Figure 3.5. Non-metric multidimensional scaling (NMDS) ordination plot showing resistome differences at the mechanism level between fecal and environmental samples in (a) feedlots and (b) dairy farms.

Comparison of the resistomes between feedlot and dairy farms

The average number of ARGs per sample was greater ($P < 0.05$) in feedlots (76 ARGs; min. 6, max. 260) than in dairy samples (52 ARGs; min. 0, max. 151). This tendency was observed for feces (112 and 86 ARGs, feedlots and dairies, respectively; $P < 0.05$), wastewater (53 and 117 ARGs; $P < 0.05$) and soil (29 and 20 ARGs, $P > 0.05$). Across all sample types, resistome richness and Shannon's diversity was greater ($P < 0.05$) on feedlots than on dairies at the class and mechanism level. Tetracycline and MLS classes of resistance were more abundant ($P < 0.05$) in the feces of feedlot cattle than in the feces of dairy cows, whereas the beta-lactam

class of resistance was enriched ($P < 0.05$) in feces of dairy cows. The same tendency was observed at the mechanism level (Table 3.1).

Table 3.1**.** Mechanisms of resistance differentially represented ($P < 0.05$) in feedlot and dairy farms separated by type of sample (feces and wastewater) and pairwise comparison (CONV-F vs. CONV-D; NAT-F vs. ORG-D[1]).

	Feces		Wastewater	
Class and mechanism of resistance	CONV-F vs. CONV-D	NAT-F vs. ORG-D	CONV-F vs. CONV-D	NAT-F vs. ORG-D
Tetracylines				
Ribosomal protection protein	+	+	-	+
Tetracycline efflux pumps	+	+	+	+
Inactivation enzymes	+	+	+	+
Macrolide-Lincosamide-Streptogramin				
Macrolide efflux pumps	+	+	-	+
Lincosamide nucleotidyltransferases	+	-	-	+
23S rRNA methyltransferases	+	+	-	+
Streptogramin efflux pumps	-	nd	-	-
Macrolide phosphotransferases	nd	-	+	+
Beta-lactams				
Class A beta-lactamases	-	-	nd	nd
Class C beta-lactamases	-	-	nd	nd
Class D beta-lactamases	-	nd	nd	nd
Penicillin binding proteins	-	-	nd	nd
Mutant porin proteins	nd	nd	nd	-
Aminoglycosides				
O-nucleotidyltransferases	nd	-	-	+
O-phosphotransferases	nd	-	nd	+
Efflux regulators	nd	-	nd	nd
Cationic Antimicrobial Peptides				
Lipid modification	nd	-	nd	nd
Polymyxin B efflux regulator	nd	-	nd	nd
Multi-drug resistance				
MDR regulator	nd	-	+	+
Efflux pumps	nd	-	+	+
Phenicol				
Efflux pumps	nd	nd	+	+
Chloramphenicol acetyltransferases	nd	nd	nd	+
Sulfonamide				
Dihydropteroate synthases	nd	nd	nd	+

[1]CONV-F: conventional feedlot, CONV-D: conventional dairy, NAT-F: natural feedlot, ORG-D: organic dairy.

(+): mechanism significantly enriched in samples collected from feedlots; (-): mechanism significantly enriched in samples collected from dairy farms, nd: no difference in abundance of mechanisms of resistance between feedlot and dairy samples.

When comparing mechanisms of resistance in feedlot and dairy lagoons; overall results varied depending on production practices. Between farms with restricted antibiotic use (NAT-F *vs.* ORG-D), most of the affected mechanisms of resistance were greater in feedlot wastewater compared to dairy wastewater, whereas the differential abundance between conventional lagoons (CONV-F *vs.* CONV-D) varied depending on mechanism of resistance. Fewer differences were observed in the resistome of soil samples between feedlot and dairy farms. Glycopeptide and cationic antimicrobial peptides classes of resistance were higher ($P < 0.05$) in NAT-F soil than in ORG-D soil, while there were no differences ($P > 0.05$) in the soil resistome obtained from conventional feedlot and dairy farms.

Resistance to metals and biocides across farms and types of samples

Sequences aligning to metal and biocide resistance (MBRGs) genes were classified into 12 classes: multiple drugs and biocides (28% relative abundance), multiple metals (20%), copper (13%), peroxide (11%), multiple biocides (9%), mercury (7%), zinc (4%), silver (2%), nickel (2%), multiple metal and biocides (2%), iron (1%) and tellurium (1%). Between production practices, all classes of MBRGs were more abundant ($P < 0.05$) in CONV-F samples than in NAT-F samples, except for peroxide resistance ($P > 0.05$). In dairies, MBRGs classes of resistance had similar ($P > 0.05$) abundance in samples, irrespective of production practices. On CONV farms, MBR gene abundance was greater ($P < 0.05$) in samples from feedlots than from dairies (except for copper which was greater in dairy samples; $P < 0.05$). On farms with restricted use of antibiotics, copper resistance was more abundant ($P < 0.05$) in ORG-D than in NAT-F. Among types of samples (feces vs. soil vs. wastewater), genes conferring resistance to multiple biocides, multiple metals, nickel and zinc were more abundant ($P < 0.05$) in feces than

in environmental samples. On the other hand, peroxide resistance was consistently more prevalent ($P < 0.05$) in environmental samples, especially in soil than in feces samples.

Microbial communities associated with the different resistome.

In feedlots, significant microbiome differences were observed between CONV and NAT samples for feces early, feces late and wastewater (ANOSIM *R*: 0.42, 0.24, and 0.87, respectively). *Proteobacteria* and *Actinobacteria* were more abundant ($P < 0.05$) in "feces early" from CONV-F than from NAT-F, while abundance of *Bacteroidetes* was greater ($P < 0.05$) in "feces late" from steers in NAT-F. In wastewater, *Proteobacteria, Bacteroidetes* and *Firmicutes* were enriched ($P < 0.05$) in CONV-F, while *Actinobacteria* was more abundant ($P < 0.05$) in NAT-F. Microbiome diversity was greater ($P < 0.05$) in feces early and wastewater from NAT-F (Shannon's index: 1.62 and 1.15, respectively) than from samples from CONV-F (1.50 and 0.68, respectively).

Among dairies, microbiome differences ($P < 0.05$) were detected between conventional and organic dairy samples for feces low, soil, and wastewater (ANOSIM *R*: 0.70, 0.36, and 0.98, respectively). *Proteobacteria* were more abundant ($P < 0.05$) in feces from low producing dairy cows in CONV-D compared to ORG-D; while *Firmicutes* and *Bacteroidetes* were more abundant ($P < 0.05$) in dairy cattle feces from ORG-D. Among wastewater samples, abundance of *Bacteroidetes* and *Firmicutes* was greater ($P < 0.05$) in CONV lagoons, while abundance of *Acidobacteria* increased ($P < 0.05$) in lagoons from farms with restricted antibiotic use. Soil from ORG-D harbored a greater ($P < 0.05$) abundance of *Bacteroidetes* than soil from CONV-D. In relation to ecological indexes, Shannon's diversity index for feces low was greater ($P < 0.05$) in ORG-D than in CONV-D (1.64 and 1.16, respectively). Microbiome diversity was greater (*P*

< 0.05) in wastewater CONV-D than in wastewater ORG-D (Shannon index: 1.47 and 0.64, respectively). Richness and diversity did not differ ($P > 0.05$) between soil samples collected from conventional farms and farms with restricted antibiotic use.

Feces, wastewater and soil samples clustered apart in the ordination plot (Figure 3.6). *Proteobacteria* and *Actinobacteria* increased in the soil microbiome relative to the fecal and wastewater microbiome ($P < 0.05$). Conversely, *Bacteroidetes* and *Firmicutes* were enriched ($P < 0.05$) in feces compared to soil and wastewater. Wastewater samples had a greater abundance ($P < 0.05$) of *Proteobacteria* than feces.

Fecal microbiome was more diverse ($P < 0.05$) than soil and wastewater microbiomes (1.49, 0.99 and 0.94 for feces, wastewater, and soil samples, respectively). Weak correlations ($r < 30$) and high dissimilarities ($m^2 > 0.90$) were detected between the resistomes and microbiomes. The strongest relationship between ARGs and the microbial community were found in samples collected from dairies ($P < 0.05$, $r = 0.29$, $m^2 = 0.91$) and conventional farms ($P < 0.05$, $r = 0.30$, $m^2 = 0.91$) (Appendix 6). Among types of samples, feces was the niche with the greatest correlation and goodness of fit ($P > 0.05$, corr. = 0.17, $m^2 = 0.97$).

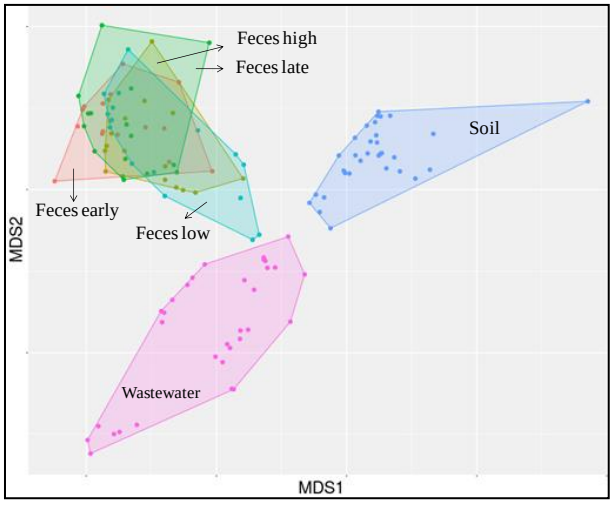


Figure 3.6. Non-metric multidimensional scaling (NMDS) ordination plot showing the microbiome difference at the phylum level between fecal and environmental samples.

Discussion

This study demonstrated that the resistome in beef feedlot and dairy cattle operations is associated with production practices (conventional vs. natural) and type of sample (feces vs. soil vs. wastewater). Although some ARGs were more abundant on conventional farms, feces of animals and the environment harbor a diverse resistome, even when derived from natural farms. This finding was consistent with data from other studies, where ARGs were found from cattle not exposed to antibiotic treatment (Reinstein et al., 2009; Morley et al., 2011; Santamaria et al., 2011; Harvey et al., 2009). It supports the concept that AMR is an ancient phenomenon that does not depend on antimicrobial use to emerge (D'Costa et al., 2011; Stokes and Gillings, 2011). Among fecal samples, animals raised with high levels of antibiotic exposure in conventional pens (late on feeding beef cattle and high producing dairy cows) had the greatest resistome separation compared to their corresponding groups raised without antibiotics. Most mechanisms of resistance were greater in feces from conventionally raised animals compared to those raised without antibiotics. By cattle type, feces from beef cattle generated a larger resistome than those from dairy cattle measured by the amount of AMR hits and the number of ARGs per sample. Antimicrobial drugs are not included in the feed of dairy cows as milk produced from antibiotic-treated cows must be discarded during periods when antibiotic residues can be found in milk (Government Accountability Office, 2011). Abundance of ARGs in feces from feedlots was greater for antibiotics that are commonly used as in-feed ingredients, such as tetracyclines and MLS, while feces from dairies were comprised mainly by resistance to beta-lactams. This finding was consistent with antimicrobial use data reported by the conventional feedlot and dairy. This set of associations between use of antibiotics and ARGs in feces at different levels (type of cattle and production practices) supported the theory that, even though

ARGs can be natural components of bacterial genomes, exposure to antibiotics may at times increase mobilization and dissemination of AMR among microbial communities through horizontal gene transfer and/or clonal expansion of resistant taxa (Looft et al., 2012; Perry and Wright, 2013; Chambers et al., 2015). The fact that some classes of resistance (e.g., aminoglycosides) were found in conventional samples, even though farm managers did not report using those antimicrobial drugs, suggested that ARGs can be selected and enriched without using the respective antimicrobial, either through co-selection of resistance genes or through selection of some portions of the microbiome which also contain these resistance genes. Independent of antibiotics used, diverse ARGs may be ubiquitously distributed in nature and maintained because of their co-localization in complex resistance clusters including antibiotic, metal and biocide resistance (Stoke and Gillings, 2011; Johnson et al., 2015; Pal et al., 2015). For those reasons, it has been hypothesized that ARGs may persist in various types of environments despite discontinued use of antimicrobials (Johnson et al., 2015).

Results suggested that, in feces, the resistome was associated with exposure to antibiotics commonly used in veterinary medicine, whereas the soil resistome was influenced more by diverse and naturally occurring influences (Sengupta et al., 2013). Coincidently, the soil microbiome was dominated by *Proteobacteria* and *Actinobacteria* phyla, which are known for carrying ARGs classified as drug transporters (i.e., efflux pumps and major facilitator superfamily) to cope with the variable exposures found in natural environments (Forsberg et al., 2014). Feces, wastewater and soil microbiomes clustered apart in the microbiome ordination plot, meaning different microbial composition that can be a taxonomic barrier for exchange of ARGs (Hu et al., 2016). In addition, procrustes analysis showed a low correlation between the resistome and microbiome. The lack of congruence between ARGs and microbial communities

suggested that a small fraction of the total bacterial population were antibiotic resistant (MacLean and Vogwill, 2015; Xiao et al., 2016), presence of horizontal gene transfer that can disrupt the link between microbiome and resistome (Forsberg et al., 2014; Johnson et al., 2015), and that exist various drivers associated with changes in the resistome and microbiome in addition to antimicrobial use.

The presence of ARGs in lagoons may reflect be influenced by antibiotics used in animals but also environmental conditions that affect the fate of bacteria harboring AGRs after release into the environment (Peak et al., 2007). For example, *cfx* beta-lactam group of resistance was not detected in any of the wastewater samples (0/32), despite its presence in almost all fecal samples (63/64). It has previously published that manure lagoons of conventional feedlot and swine farms are more likely to contain higher numbers of ARGs when compared to organic farms (Jindal et al., 2006; Peak et al., 2007). However, in our study, this was true only for the comparison of the two dairy farms included in the study. However, we cannot determine whether differences were due to antibiotic use or to manure management. While ORG-D had a dry-lot system where manure was dragged and allowed to dry in pens (minimizing the amount of effluents reaching the lagoon), CONV-D used a water flush system to clean pens, which resulted in large amounts of effluent runoff to the lagoon. If manure management has an impact on composition of the resistome in wastewater lagoons, this opens a new area of research to control ARGs on farm environments. Manure management was similar between CONV and NAT feedlots, in which effluents from pens flowed into respective lagoons. In this case, most mechanisms of resistance were greater in the NAT lagoon, but effluents from some CONV pens also ran off to the natural lagoon.

With regard to soil samples, almost all mechanisms of resistance significantly affected by production practices were enriched in CONV samples. However, we could not establish a causal relationship between use of antibiotics in conventionally raised animals and the soil resistome as soil pH and nutrient availability exerts a strong selection pressure on soil bacteria and ARGs composition (Xiao et al., 2016). There is the concern that land application of wastewater can lead to enrichment of antimicrobial resistant bacteria in soils due to selective pressure exerted by antibiotic molecules, toxic compounds, and ARGs in manure (Ghosh and LaPara, 2007; Cytryn, 2013; Kyselkova et al., 2015; Pornsukarom and Thakur, 2016). Recently, Udikovic-Kolic et al. (2014) and Hu et al. (2015) found that application of manure from cattle that were not exposed to antibiotics increased antibiotic-resistant bacteria in soils, suggesting that manure-derived nutrients are enough to increase relative abundance of ARGs, irrespective of antibiotics used in animals. The present study did not have a reference soil without wastewater application to assess impact on antibiotic-resistant bacteria. However, based on resistome analysis, wastewater shared only 28.0% groups of resistance with the soil resistome, while wastewater and feces shared 82.3%. This suggests that bacteria carrying ARGs present in wastewater were overwhelmed by bacteria in the soil, and diluted in space and time after manure application (Hammesfahr et al., 2008; Heuer et al., 2008; Chee-Sanford et al., 2009; Sengelov et al., 2013).

Metagenomic observational designs offer a cost-effective approach to characterize different resistomes under commercial conditions (Yang et al., 2016, Noyes et al., 2016a Noyes et al., 2016b); however, results must be interpreted in the context of limitations. Environment, diet, cattle source, management practices and location of the farms were cited as confounding factors in attempts to ascertain the relationship between antibiotic use and AMR (Singer et al., 2006; Singer and Williams-Nguyen, 2014; Benedict et al., 2015; Gerzova et al., 2015). In

addition, ARG databases are never fully complete and do not provide context of resistance genes, including taxa (commensal or pathogenic), localization (chromosome or mobile genetic elements) or expression of genes (Fitzpatrick and Walsh, 2016; Crofts et al., 2017). A better understanding of microbial and resistance ecology is relevant to identify production practices and ecological habitats that pose the greater risk for ARGs accumulation and dissemination. Government agencies can use results of this study as an input to make science-based decisions on use of antimicrobials in agricultural production as well to identify research gaps.

CHAPTER IV

USE OF METAGENOMICS TO STUDY THE RESISTOME AND MICROBIOME OF CONVENTIONAL AND RAISED WITHOUT ANTIBIOTIC FEEDLOT CATTLE OVER THE COURSE OF A YEAR

Summary

The objective of this study was to compare the fecal resistome and microbiome of fed cattle from two production systems: conventional (CONV) and raised without antibiotics (RWA) using a shotgun metagenomic approach. A slaughterhouse was visited once per month over a 12-month period for sampling. At each sampling time, 6 cattle lots were selected (3 CONV, 3 RWA) and 10 fecal samples per lot were collected from cattle colons (N = 720). After extracting DNA from individual samples, composite samples were prepared by mixing DNA from each lot into a composite sample (N = 72) and sequenced on an Illumina platform. Metagenomic reads were aligned to a custom database of antimicrobial resistance genes (ARGs) and microbial labels were assigned using a taxonomic classifier. Resistomes of CONV and RWA cattle were significantly different in all seasons. Among the most important mechanisms of resistance, tetracycline inactivation enzymes, macrolide efflux pumps, 23S rRNA methyltransferases, and aminoglycosides O-nucleotidyltransferases were significantly enriched in CONV cattle. The CONV and RWA cattle microbiomes were significantly different at all taxonomic levels across seasons. In general, resistomes and microbiomes of CONV and RWA cattle were not affected by season of the year. In conclusion, ARGs were found in both CONV and RWA cattle, although CONV cattle showed a moderate greater abundance of ARGs.

Introduction

Antimicrobials (AMs) are used in animals to control, prevent and treat infectious diseases. According to the FDA (2015), antimicrobial use in food animals is estimated to account for 80% of all antimicrobials used in the U.S. To accommodate increased consumer demand for reduced antimicrobial use in livestock production, and to profit from the "raised without antibiotics" (RWA) niche, some conventional livestock operations are adopting organic or natural practices which include cessation of the use of all antibiotics (Fox et al., 2008). Consumers perceive meat derived from those RWA systems as safer in terms of the presence of antibiotic resistance genes (ARGs) (Brennan et al., 2003); however, that expectation is controversial from the scientific standpoint, since antimicrobial resistant bacteria (ARB) and ARGs are present in RWA production environments and products (Luangtongkum et al., 2006; Cho et al., 2007; Kazimierczak et al., 2009; Reinstein et al., 2009; Morley et al., 2011; Santamaria et al., 2011).

The majority of studies comparing ARB and/or ARG in conventional and antimicrobial-restricted animal production have historically used traditional approaches to monitor antibiotic resistance bacteria including culture-based and molecular techniques; e.g., antimicrobial susceptibility testing and polymerase chain reaction (PCR). However, the main disadvantage of culture techniques is that only a small fraction of bacteria can be grown in the laboratory (Walsh, 2013; Gerzova et al., 2015). Recently, shotgun metagenomics and next generation sequencing have emerged as a culture-independent approach to study the whole microbial composition ("microbiome") and antibiotic resistance gene reservoir ("resistome") in different environments; i.e., the microbial community in its entirety. Even though there are still limitations in terms of depth of sequencing and coverage to detect rare genes, and questions

about expressing of identified genetics, metagenomics has enabled study of complex microbiomes for presence of known antibiotic resistance genes (ARGs) (Thanner et al., 2016).

The objective of this study was to compare the microbiome and resistome of fed-cattle, over the course of a year, between two differing production systems: raised without antibiotics (RWA) and conventional (CONV), using a shotgun metagenomics approach.

Material and Methods

Study overview

Conventional (CONV) and raised without antibiotics (RWA) cattle were selected for this study to compare the microbiome and resistome present in feces at slaughter. Fecal samples were collected from the colon of individual animals once per month over a 12-month period in a beef processing plant. After DNA extraction, sequencing, and data processing, metagenomic reads were aligned to reference databases for taxonomic classification of bacteria and identification of antibiotic resistance genes. Seasonal comparisons were made for the microbiome and resistome from cattle raised in the two production systems.

Sample collection

Feces were recovered from cattle colons at a commercial beef processing plant over a 12-month period. The establishment was visited once each month from February 2014 to January 2015, collecting a total of 359 and 360 samples from RWA and CONV fed-cattle, respectively. Cattle enrolled in the RWA production group had not received antimicrobials (including ionophores), growth promoters, or animal by-products from birth to slaughter. Data regarding implants, metaphylaxis and parenteral treatments, as well access to in-feed administration of

antibiotics in the CONV group, were not collected. At each visit, 10 fecal samples were obtained from 6 different lots of cattle (3 RWA, 3 CONV) totaling 60 samples per month. The slaughter plant was visited on days processing RWA cattle, and on the same day CONV cattle lots were selected for sampling. Samples were equally distributed by month and production system among 72 different lots of cattle (6 lots x 12 months), defining lot as a group of animals sent to slaughter from the same producer on the days of sampling. Feces were harvested from colons after evisceration by making an incision in the colon with sterilized scissors and then squeezing up to 30 g of fecal material into a sterile sealable plastic bag. Gloves and scissors were changed between samples. After collection, samples were placed in a refrigerated cooler, transported to the laboratory and stored (-20°C) until DNA extraction.

DNA extraction, library preparation and sequencing

Total genomic DNA was extracted from each individual sample using a PowerSoil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) according to manufacturer's instructions and quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA USA). Equal amounts of DNA were added from each of the 10 samples per cattle lot to obtain a 2 ug DNA composite sample. In this step, the number of samples was reduced from 719 individual samples to 72 composite samples (one per lot). Following extraction, DNA from each cattle lot was delivered to the Genomics Core at the U.S. Meat and Animal Research Center (Clay Center, NE) for library preparation using the Illumina TruSeq DNA Sample Preparation Kit (Illumina, Inc., San Diego, CA). Next-generation sequencing was performed on the Illumina NextSeq 500 platform (Illumina, Inc., San Diego, CA) with 8 samples per run. Raw sequencing

data for all 72 samples used in the present study are publicly available at the NCBI BioProject database with accession number PRJNA356291.

Data processing

Raw sequencing reads were trimmed using Trimmomatic version 0.32 (Bolger et al., 2014) to remove Illumina adapters, low quality sequences (Phred score < 15 in a sliding window of 4 nucleotides) and short reads (< 36 nucleotides long). Bovine DNA was removed by mapping trimmed sequence reads to the reference genomes of *Bos indicus* (Canavez et al., 2012) and *Bos taurus* (Zimin et al., 2009) using Burrows-Wheeler Aligner (BWA) version 0.6.2 (Li and Durbin, 2009). For identification of ARGs, trimmed and non-bovine reads were aligned to an antimicrobial resistance custom, non-redundant database using BWA. The resistance database contained ~ 4,000 unique resistance genes combining Resfinder (Zankari et al., 2012), ARG-ANNOT (Gupta et al., 2013), and CARD (McArthur et al., 2013) databases, in addition to the National Center for Biotechnology Information (NCBI) Lahey Clinic beta-lactamase archive. SAMtools (Li et al., 2009) and a custom-developed parsing program were used to generate statistics from the BWA mapping output. Up to 4% (~ 5 bases) of the individual read length was allowed to have mismatches with the mapping location in the reference gene in order to reflect a "hit" between the read and a gene in the database. To decrease the number of false positive results, only ARGs with a read coverage of > 80% over the entire reference gene were considered for downstream descriptive and statistical analysis (Noyes et al., 2016). Genes known to cause resistance as a result of single nucleotide polymorphisms (SNPs) in housekeeping genes were evaluated by visualizing the BWA alignments with Tablet (Milne et al., 2013) and visually confirming that reads aligned with 100% peptide homology. In short, the

ARGV had to have 100% read coverage across the middle 95% of the gene. Then, we looked for places where all of the aligned reads had a SNP and checked if the SNP conferred a synonymous or non-synonymous mutation. If synonymous mutation (no change in amino acid) was detected, we kept the aligned reads for downstream analysis; if non-synonymous mutation (change in amino acid) was observed, we did not count the aligned reads. The genes identified in our samples and included in this post-processing verification step were GYRA, GYRB, PARE, PARC, and RPOB.

Taxonomic labels were assigned to trimmed and non-bovine sequences using Kraken version 0.10.6-beta which utilizes the National Center for Biotechnology Information (NCBI) reference nucleotide database (RefSeq) to classify bacteria at different taxonomic levels (Wood and Salzberg, 2014). In order to increase the sensitivity of taxonomic classification, the optional script kraken-filter was run with a threshold of 0.20 which moves assignments up to higher levels of the taxonomic trees and avoids over-classification of reads at lower taxonomic levels (Peabody et al., 2015). In addition, metagenomic reads were aligned to the Greengenes database (DeSantis et al., 2006) using BWA to identify 16S rRNA sequences in trimmed and filtered reads as a proxy of overall bacterial abundance in each sample.

Statistical analysis of sequencing data

Microbial and resistance count tables were normalized using a scaling-factor approach accounting for varying sequence depth across samples (Paulson et al., 2013). To account for differences in sequence length of ARGs and bacterial load in the samples, we adopted the equation of Li (2015) as a second normalization step; it allowed for ARG abundance to be expressed as copy of resistance gene per copy of 16S-rRNA gene. We used a zero-inflated

Gaussian distribution mixture model included in the metagenomeSeq package (Paulson et al. 2013), that accounts for biases resulting from under-sampling of the microbial community to perform analysis of differential abundance in individual members of the microbiome and resistome. Statistical comparisons were performed following $\log_2$ transformation, which moderated gene-specific variance estimates followed by a multiple-comparison Bonferroni test to report adjusted *P*-values. In general, bacteria taxa and resistance features (class, mechanism, group) with relative abundance > 1% and $\log_2$ fold change over 1 or under -1 (2-fold increase and decrease in abundance, respectively) were considered for biological and statistical interpretation as metagenomeSeq is more accurate for genes with higher abundance and log fold change (Jonsson et al., 2016).

The initial statistical model was to test for the fixed interaction term between production system and season including batch (samples sequenced in the same run in the sequencer) as a covariate and location of the farm (producer) as a random effect. For the purpose of this study, seasons were defined as winter (December-January-February), spring (March-April-May), summer (June-July-August), and fall (September-October-November). Tests of main fixed effects (production system and season) were conducted only if the interaction term was not significant. A critical value $\alpha = 0.05$ was used for all statistical analysis.

Data were analyzed using the multivariate ordination technique, non-metric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM) included in the vegan package version 2.2-2 (Oksanen et al., 2012) to investigate whether the overall microbiome and resistome differed significantly between production system. The NMDS plots with a stress value below 0.2 were considered a good fit (Clarke, 1993). In addition to a *P*-value, ANOSIM calculates an *R*-value, which can range from 0 to 1 and which indicates the extent to which

microbiomes or resistomes differ between groups. For purposes of this study, *R*-values ranging from 0.00-0.25, 0.26-0.50, 0.51-0.75, and 0.76-1.00 were interpreted as weak, mild, strong, and very strong group separation, respectively. Richness (*S*, e.g. number of unique taxa or ARGs counted in a sample) and Shannon's diversity index (*H*, e.g. number and proportion of unique taxa or ARGs counted in a sample) were compared between groups using generalized linear models. Procrustes analysis was performed on NMDS ordinations of the microbiome and resistome for CONV and RWA systems at each season of the year. Briefly, procrustes analysis was applied to evaluate how closely the microbiome and resistome mimicked each other by superimposing, translating and rotating the microbiome ordination shape to fit the resistome ordination shape (Okansen, 2015). The objective was to minimize the sum of the squared term (m^2) and maximize the correlation coefficient between the two ordinations.

Results

Sequencing and bioinformatic processing

After removing low quality bases and bovine sequences, an average of 81,036,886 reads per sample (min. 8,295,016; max. 301,800,317) were available for downstream analysis. Across the 72 samples, a total of 7,948,454 reads (mean of 110,395; min. 12,784; max.503,921) aligned to the antibiotic resistance gene database corresponding to 0.07% of total non-bovine trimmed reads. The amount of normalized reads aligned to the resistance database differed between CONV and RWA samples (44,044 and 32,042 reads per sample, respectively), while no differences were observed ($P > 0.05$) between seasons. In addition, 167,487 reads/sample (min. 19,941; max. 588,285) aligned to16S rRNA sequences as an estimate of the overall bacterial abundance per sample. The number of reads aligned to the 16S rRNA database did not differ

significantly between CONV and RWA cattle or across seasons; however the ratio of normalized ARGs to 16S rRNA genes per sample tended to be greater in CONV cattle than in RWA cattle (0.43 and 0.38, respectively, $P = 0.059$), mainly caused by differences ($P < 0.05$) in summer (0.56 and 0.22, for CONV and RWA, respectively) (Figure 4.1).

Resistome

After removing sparse features (resistance features at any level detected in less than 4 samples), 176 ARGs were detected corresponding to 12 classes, 28 mechanisms, and 95 groups of resistance. On average, colons from CONV cattle harbored more ARGs ($P < 0.05$) than RWA cattle (89 and 75 ARGs per sample, respectively).

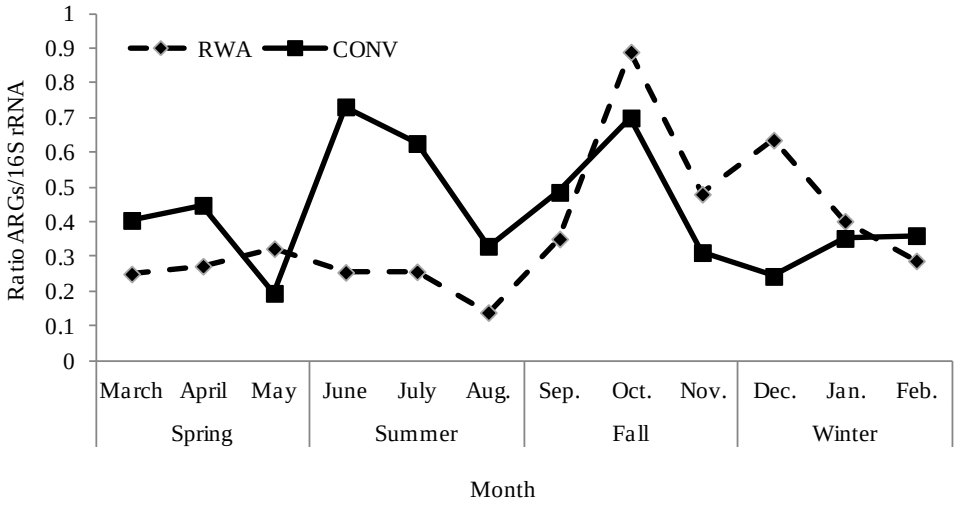


Figure 4.1. Monthly evolution of the ratio of normalized reads aligned to antibiotic resistance genes (ARGs) to reads aligned to 16S rRNA genes for conventional (CONV) and raised without antibiotic (RWA) cattle.

The amount of ARGs per sample was not affected ($P > 0.05$) by season of the year among system of production averaging 80 (min. 43; max. 121), 73 (min. 39; max. 111), 88 (min. 41; max. 150) and 77 (min. 33; max. 117) ARGs per sample during fall, winter, spring and

summer, respectively. The CONV cattle harbored more ARGs than RWA cattle during spring (107 and 63 ARGs per sample, respectively; $P < 0.05$). All groups of resistance were found in at least one sample in CONV and RWA cattle groups, except for ERMA, ERMC, ERMT, ERMX (23S rRNA mechanisms of resistance), and OXA (class D mechanism) found in 4, 5, 6, 8, and 3 samples, respectively; but only in CONV cattle. Interestingly, 11 groups of resistance were found in all samples from either CONV or RWA including ANT6 (aminoglycoside), MEFA and LNUC (macrolide-lincosamide-streptogramin, MLS), and the tetracyclines TET32, TET40, TET44, TETB, TETO, TETQ, TETR, and TETW.

The most abundant genes were assigned to tetracycline resistance (74% in CONV and 77% in RWA cattle, expressed as relative abundance of normalized reads among those aligning to ARGs), MLS (22% CONV; 19% RWA), beta-lactam (0.9 % CONV, 1.3% RWA), aminoglycoside (1.2% CONV, 1.1% RWA) and multi-drug (0.9% CONV, 1.2% RWA) classes of drug resistance. The relative abundance of groups of resistance within the main classes of antibiotics is shown in Figure 4.2. At the mechanism level, tetracycline ribosomal protection proteins (97% CONV, 98% RWA), MLS efflux pumps (86% CONV, 78% RWA), class A beta-lactamases (90% in both CONV and RWA), aminoglycoside O-nucleotidyltransferases (66% CONV, 50% RWA), and multi-drug efflux pumps (62% CONV, 59% RWA) were the most common mechanisms of resistance within each of the main classes of resistance.

The cattle production method was associated with differences ($P < 0.05$) in the resistome at the class, mechanism and group level during all seasons (Figure 4.3 a-d). Seventeen groups of resistance differed between CONV and RWA cattle, most of them (n = 16) during winter. Groups conferring resistance to aminoglycosides (ANT6), beta-lactams (ACI), macrolide-lincosamide-streptogramin (ERMF, ERMQ, MEFA) and tetracyclines (TETM, TETQ, TETX)

were more abundant ($P < 0.05$) in CONV than in RWA cattle. These groups were detected in all CONV samples (n = 36) except TETM (n = 34), and in 36 (ANT6, MEFA, TETQ), 35 (ACI), 34 (ERMQ), 31 (ERMF, TETX) and 27 (TETM) RWA samples. The average $\log_2$ fold change was 1.41 which means the overall abundance of those groups in CONV samples more than double the abundance in RWA samples (min. 0.64, max. 2.24 $\log_2$ fold change, TETQ and ERMF, respectively). We observed higher resistome richness (number of different resistance determinants per sample) during the spring in CONV cattle than RWA cattle at the class (average of 5.2 and 4.2 classes per sample, respectively), mechanism (11.1and 9.8 mechanisms, respectively), and group levels (63 and 38 groups, respectively), while richness was not affected ($P > 0.05$) by production methods during remaining seasons at any resistance level. In general, season of the year had a weak effect on the resistome composition of CONV and RWA cattle.

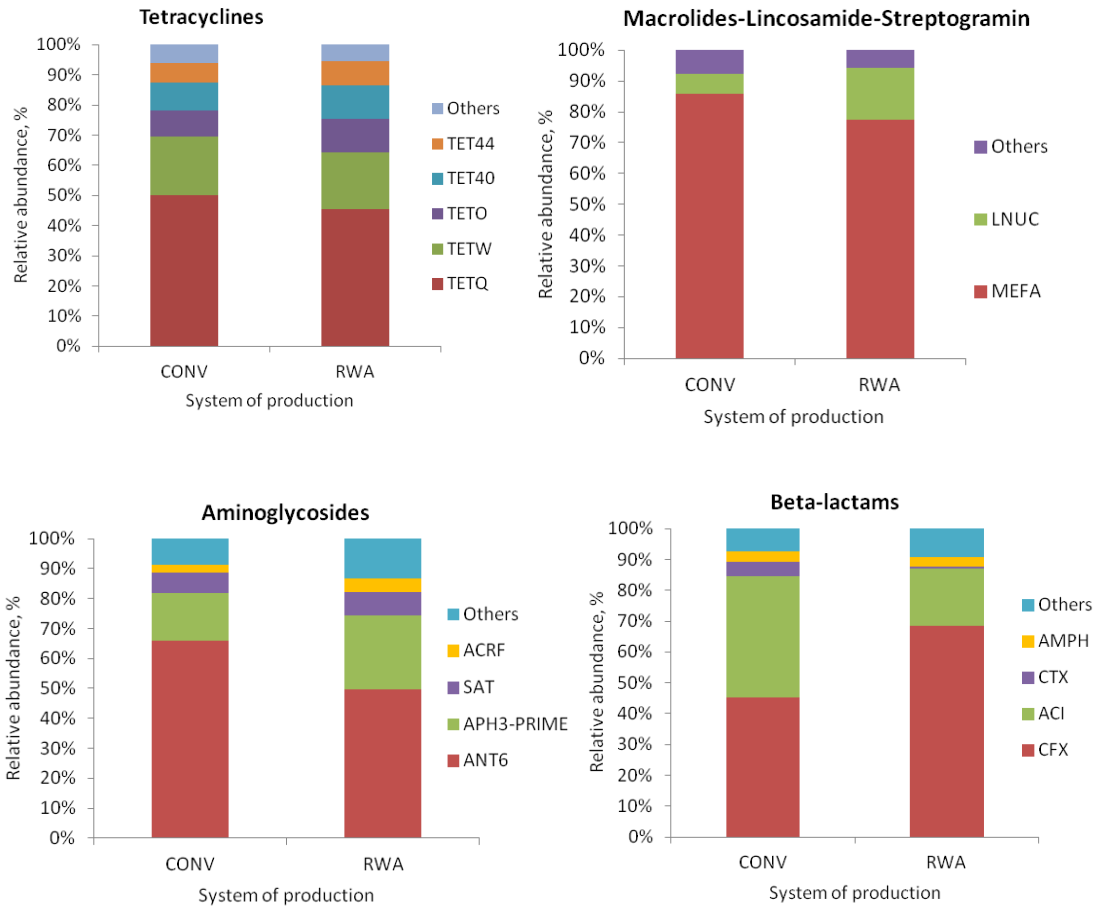


Figure 4.2. Relative abundance of groups of resistance within the main four classes of resistance separated by system of production (CONV = conventional; RWA = raised without antibiotics).

The resistome of CONV and RWA cattle was affected by season of the year at the group level (R: 0.09 and stress: 0.16 for CONV; R: 0.11 and stress: 0.13 for RWA). At the mechanism level, only the resistome of RWA cattle was affected by season (R: 0.08, stress: 0.09), while there was a tendency (P = 0.08) for a season effect in CONV cattle (R: 0.06, stress: 0.13). However, the R coefficient was consistently < 0.25 meaning a weak separation of the resistomes. There was no statistically significant separation of CONV and RWA resistomes at the class level by season. In CONV cattle, resistome richness at the class level was greater in the spring (9 classes per sample) than during the remaining of seasons (7 classes per sample). Shannon

diversity index within CONV and RWA cattle did not differ between seasons ($P > 0.05$) at any resistance level.

Microbiome

Across the 72 samples, we identified 26 phyla, 46 classes, 112 orders, 209 families and 471 genuses of bacteria aggregated at each taxa level. At the higher taxonomic level, the majority of hits to bacteria sequences belonged to *Firmicutes* (36%), *Bacteroidetes* (35%), *Proteobacteria* (17%), *Spirochaetes* (9%), and *Actinobacteria* (3%). Figure 4.4 shows the abundance of the most common taxa found across all samples at the class, order, family, and genus level. The interaction production system by season of the year was affected in 8 phyla, 13 classes, 27 orders, 58 families and 188 genuses ($P < 0.05$). None of the most abundant taxa (described in Figure 4.4) were affected ($P > 0.05$) by the interaction term. System of production (CONV *vs.* RWA) had an effect ($P < 0.05$) on the microbiome at all taxa levels, averaged over seasons ($P < 0.05$; ANOSIM $R < 0.10$), except at phylum level ($P = 0.17$).

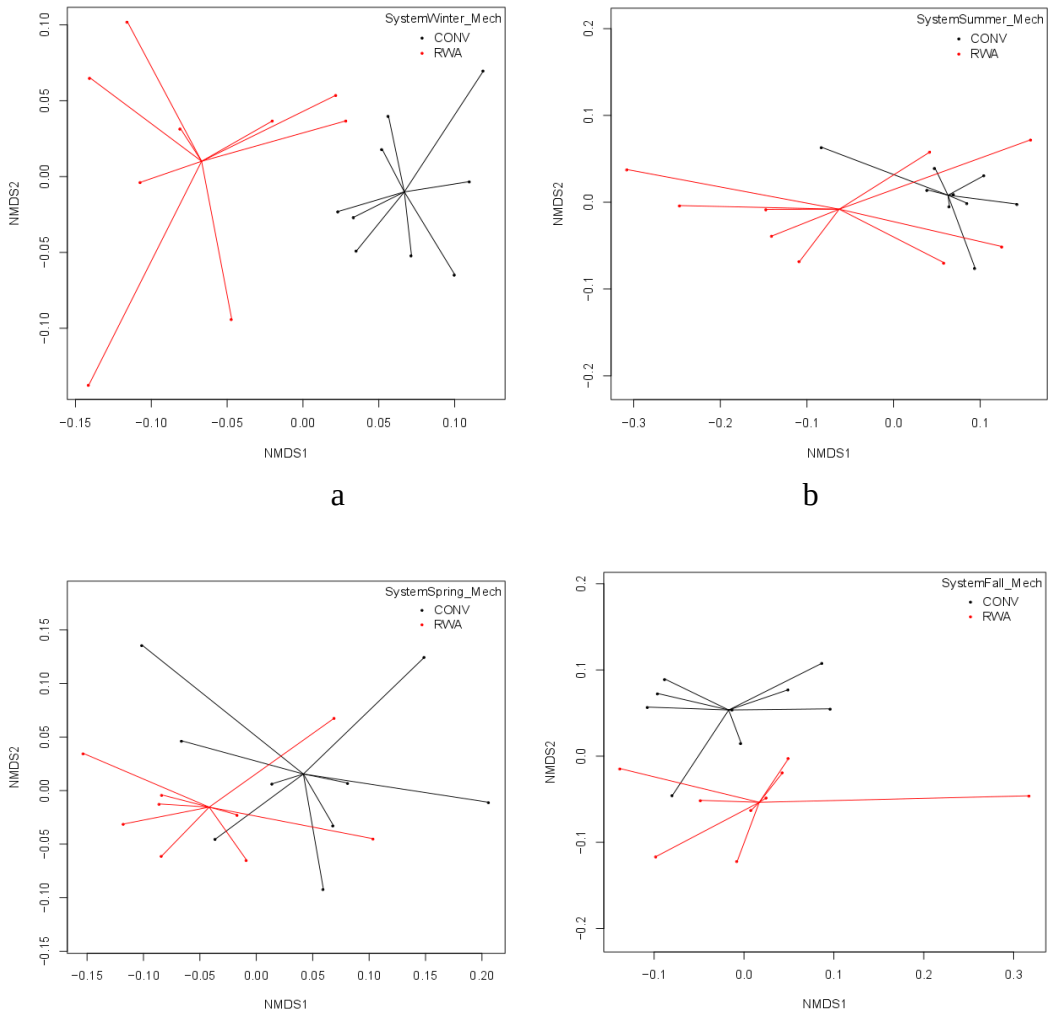


Figure 4.3 a-d. Non-metric multidimensional scaling (NMDS) ordination of conventional (CONV, black) and raised without antibiotics cattle (RWA, red) conducted at the mechanism of resistance level by season of the year: winter (Figure 4.3a, R: 0.45, stress: 0.08), summer (Figure 4.3b, R: 0.27, stress: 0.05), spring (Figure 4.3c, R: 0.21, stress: 0.12), and fall (Figure 4.3d, R: 0.28, stress: 0.07).

Among the most relevant taxa, *Clostridia* (class) and *Clostridiales* (order) were more abundant in RWA cattle. Among minor taxa, *Bifidobacteria* (order), *Bifidobacteriaceae* and *Eubacteriaceae* (family), and *Bifidobacterium* and *Eubacterium* (genus) were more abundant ($P < 0.05$) in the colons of RWA cattle compared to CONV cattle. The overall microbiomes of

CONV and RWA cattle were most different in winter (R = 0.21), spring (R = 0.16), and summer (R = 0.17), but not in the fall (*P* = 0.21).

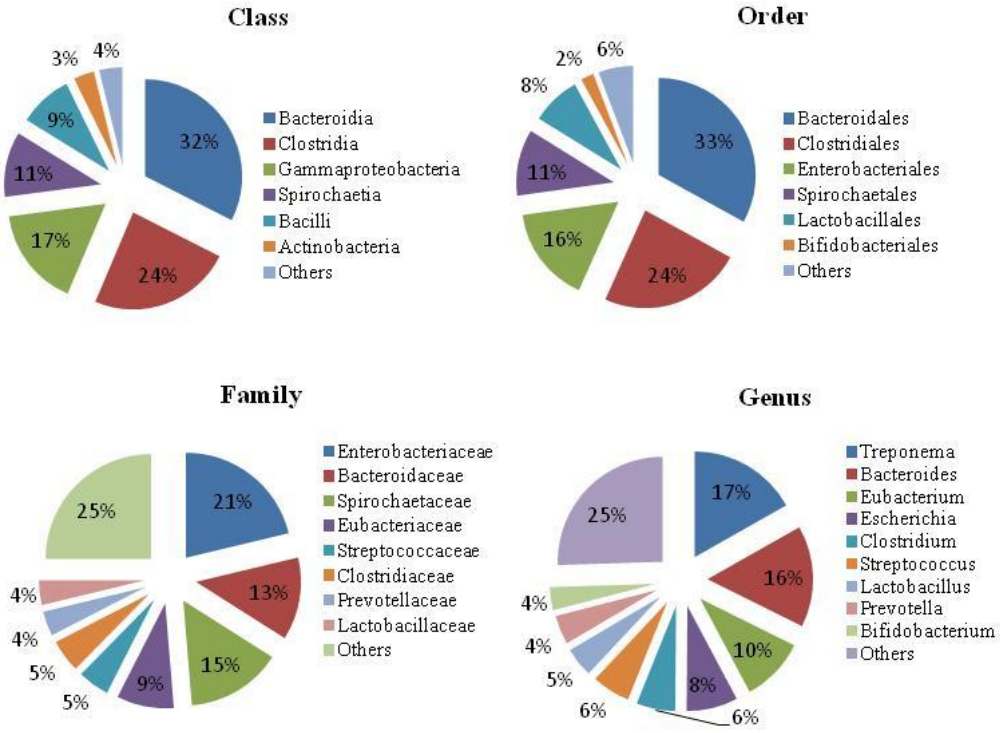


Figure 4.4. Relative abundance of classified bacteria aggregated at class, order, family, and genus level averaging over all samples. "Others" includes taxa with less than 2% (class and order) and 4% (family and genus) relative abundance.

Shannon's diversity index differed between systems of production at the class and order level (lower diversity in RWA cattle than in CONV), caused mainly by differences in summer (*P* < 0.05), while the diversity was not affected (*P* > 0.05) by season of the year. Richness was not affected (*P* > 0.05) by the system of production.

Based on the statistical analysis of the NMDS ordination, we did not observe a seasonal effect on the microbiome at any taxonomic level, either averaged over system of production, or within CONV and RWA groups (*P* > 0.05). Shannon's diversity index and richness were not affected (*P* > 0.05) by season of the year. Procrustes analysis showed that the resistomes at the

class, mechanism, and group level were correlated ($P < 0.05$) to the overall microbiome in each season of the year. The strongest correlation was detected at the group of resistance level based on the goodness of fit and correlation coefficients (Figure 4.5).

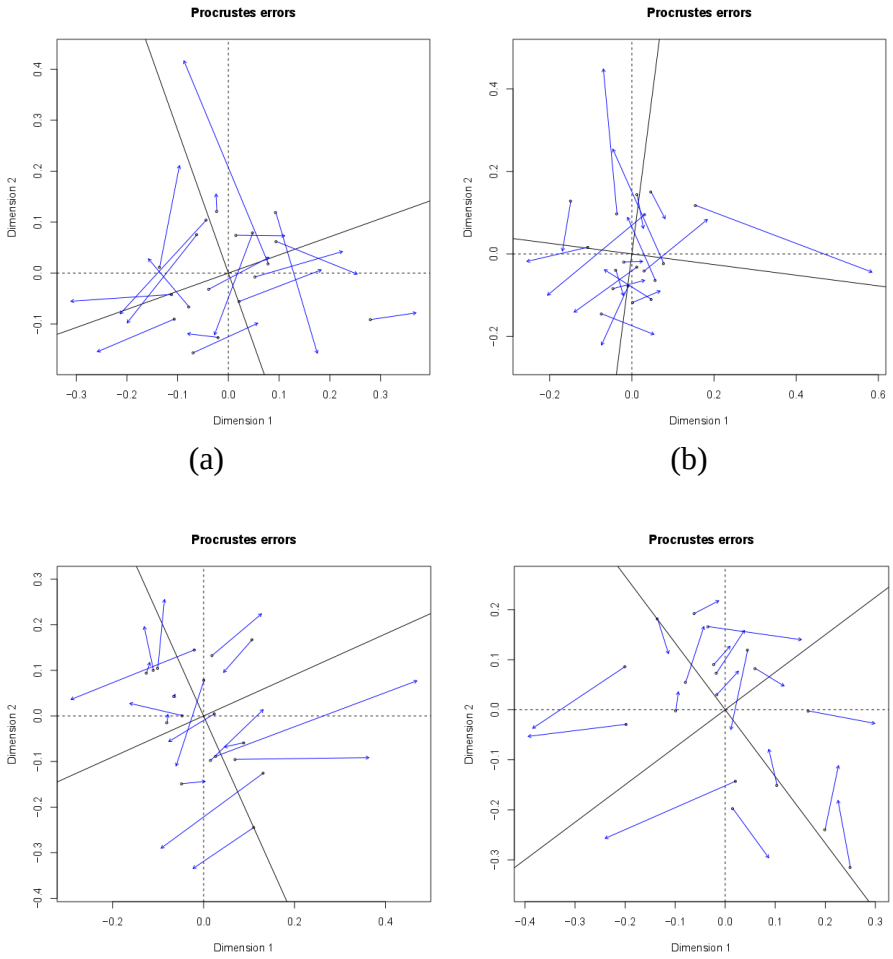


Figure 4.5. a-d. Procrustes analysis showing the correlation ($P < 0.05$) between the resistome across system of production between antibiotic resistance genes (ARGs) and taxa composition in fall ($m^2 = 0.39$, r = 0.78, Figure 4.5a), winter ($m^2 = 0.45$, r = 0.74, Figure 4.5b), spring ($m^2 = 0.31$, r = 0.83, Figure 4.5c), and summer ($m^2 = 0.37$, r = 0.79, Figure 4.5d). Black lines connect the same sample in the microbiome and resistome. Shorter distances between the same samples mean higher correlation between the two ordinations.

Discussion

In this study we evaluated the resistome and microbiome in the colons of feedlot cattle that were raised conventionally or without exposure to antibiotics over a year. Results showed that the cattle resistome harbors diverse resistance genes, even in animals raised without exposure to antibiotics in natural systems of production. This finding agreed with results of previous studies, which reported presence of antibiotic resistant bacteria and resistance genes in cattle, pigs, and chickens raised without antibiotics, in organic and natural operations (Price et al., 2005; Luangtongkum et al., 2006; Kazimierczak et al., 2009; Reinstein et al., 2009; Santamaria et al., 2011; Zwonitzer et al., 2016). In others fields, ARGs were observed in infants (Fohuy et al., 2014; Gosalbes et al., 2015), isolated human populations (Pallecchi et al., 2007; Clemente et al., 2015) and in uninhabited places (D'Costa et al., 2011; Bhullar et al., 2012) that were unlikely to come in contact with antibiotics. Such results suggest that ARGs are a consequence of bacterial evolution and that exposure to antibiotic molecules is not a pre-requisite for emergence of antibiotic resistance (Sengputa et al., 2013). On the other hand, from an environmental perspective, presence of ARGs in RWA cattle suggested that those genes are present in the environment, either as a part of the background pool of naturally occurring ARGs, or due to dispersion of antibiotic resistant bacteria from conventional farms or human environments followed by horizontal gene transfer from such resistant strains to members in the microbiome found in natural farms (Sengputa et al., 2013). Moreover, antibiotic resistance can be further modified by co-selection for heavy metals (Pal et al., 2015). For cattle, heavy metals like copper and zinc are usually included in excess in diets of natural cattle to replace conventional antibiotics (Reinstein et al., 2009). Because ARGs are transmitted in the

environment, Durso et al. (2012) proposed to determine baseline background levels of ARGs before comparing the impact of antibiotic use on the resistome.

Tetracycline, macrolide-lincosamide-streptogramin (MLS), beta-lactam and aminoglycoside resistance genes were present in feces from all cattle, regardless of the production system in which cattle were raised. Resistance to tetracycline is known to be ubiquitous and present even when animals are not fed antibiotics (Alexander et al., 2008; 2011). The most prevalent ribosomal protection protein genes found in this study (*tet*O, *tet*Q and *tet*W) could easily be transferred between bacteria, as they have been associated with mobile genetic elements such as plasmids, transposons, and integrons (Roberts, 2005). With regard to resistance to MLS, efflux pumps encoded by the *mef*A gene were the most common mechanism of resistance, even though bacteria resistant to macrolides usually carry *erm* genes as the most common mechanism of resistance (Portillo et al., 2000; Beukers et al., 2015). Resistance to beta-lactams was dominated by the groups CFX and ACI, both encoding class A beta-lactamases which are the most common mechanism of bacterial resistance to beta-lactams (Yang et al., 1999). Aminoglycosides were the third most abundant class of resistance to antibiotics found in this study, although aminoglycosides are not commonly used in feedlots. Interestingly, Pal et al. (2015) reported a plasmid co-localization of resistance genes to aminoglycosides, macrolides and heavy metals (cadmium and zinc) suggesting co-selection for resistance towards those antibiotics and promotion of horizontal gene transfer.

There was a preponderance of mechanisms conferring resistance to tetracyclines, macrolides, beta-lactams and aminoglycosides in CONV cattle compared to RWA cattle, the greatest differences in resistomes observed during winter. Assuming a relatively constant baseline level of exposure to in-feed antibiotics throughout the year for health optimization and

prevention of disease, we would typically expect an increase in antibiotic metaphylaxis to prevent the spread of infectious diseases during winter, when stocker cattle usually are transported to feedlots at the end of the grazing season. This can create a stronger environmental selective pressure for the cattle in pens ready for slaughter in the present study. Additionally, colder temperatures during winter are less effective for biodegradation of antibiotics molecules and attenuation of ARGs in the environment (Pei et al., 2007; McKinney et al., 2010), thereby maintaining a greater selective pressure.

Normalization of resistance genes to the 16S rRNA gene present in all bacteria taxa provides an estimate of the proportion of the microbial community carrying ARGs and also accounts for variations in the bacterial load between samples (Thames et al., 2012). Cattle raised on a conventional system showed greater amounts of ARGs per copy of 16S rRNA gene than RWA cattle during the summer months (June, July, and August). This could be due to a combination of clonal proliferation of strains harboring ARGs and/or horizontal transfer of antibiotic resistance determinants between different bacterial species.

It has been reported that the microbiome of the large intestine and rectum of cattle is dominated by strict anaerobes and facultative anaerobes (Dowd et al., 2008; Shanks et al., 2011). This supports findings of the present stud where the predominant bacterial groups found at higher taxonomic levels were *Bacteroidetes* and *Firmicutes*. *Eubacterium* and *Bifidobacterium* were the two most important genuses in terms of relative abundance, and they were more abundant ($P < 0.05$) in RWA than in CONV cattle. Similar to our results, Gerzova et al. (2015) found that members of the *Bifidobacteriaceae* family increased in the microbiome of organic pigs compared to conventional pigs. We did not find a separation ($P > 0.05$) of fecal microbiomes across seasons suggesting that colon microbial communities in feedlot cattle are

highly constant across the year and dominated by well adapted taxa. Seasonal variation in microbiome composition has been reported for agricultural and human environments (Smit et al., 2001; Knapp et al., 2012; Davenport et al., 2014; Asakura et al., 2016; Kable et al., 2016). It may be harder to detect bacterial changes when using a metagenomic approach as dominance of sequences belonging to most abundant taxa can mask differences in less abundant taxa. We found a correlation ($P < 0.05$) between the microbial composition and the resistome across systems of production at the class, mechanism and group level using procrustes analysis. This suggested that the different microbiomes found in CONV and RWA cattle may be the main driver shaping the resistome in cattle.

In the context of microbial and antibiotic resistance ecology, shotgun metagenomics allowed identification of multiple taxa and resistance genes compared to traditional culture and polymerase chain reaction approaches (PCR) targeting specific organisms and genes (Schmieder and Edwards, 2015). However, the fact that we identified 176 unique resistance genes in cattle colons did not mean that they were fully expressed and functional, thereby conferring phenotypic resistance. For that reason, Forslund et al. (2013) proposed the term 'resistance potential' instead of 'resistance' to reflect potential differences in gene expression and regulation that can affect phenotypic resistance. In addition, and from a public health perspective, resistance genes harbored in animals' microbiomes do not necessarily imply a significant risk for spread and transmission to clinically relevant pathogens. Other limitations of this study included the lack of information about the type, dose, duration and frequency of exposure to antibiotics in CONV animals. Additionally, we did not have access to the location of the farms from which feeder cattle were derived, which potentially may have been located close to a source with heavy antibiotic use, thereby increasing risk of acquiring resistance organisms or genes by

environmental routes of transmission (runoff, air, wildlife, etc.). To overcome this issue, Singer et al. (2015) proposed to include, not only the location of the farm but also a spatial characterization of the surrounding area (proximity to streams, urban populations and other agricultural premises, soil type, vegetation characteristics, etc.) to account for potential confounding variables. For that reason, based on our results, we can establish an association between the cattle rearing method and the ecology of the resistome, but we could not establish a causal relationship. With regard to the microbiome, a system effect (CONV *vs.* RWA) can be confounded with population differences in known variables able to cause microbial community shifts in cattle, such as diet, feed efficiency, geography, and animal feeding operation (Fernando et al., 2010; Shanks et al., 2011; Petri et al., 2012; Myer et al., 2015).

To our knowledge, this is the first published study evaluating the resistome and microbiome of CONV and RWA cattle over the course of an entire year using a metagenomic approach. Antibiotic resistance genes were found in both CONV and RWA cattle, although CONV cattle showed a greater abundance of ARGs. Season had no significant impact on the resistome or microbiome of cattle. Further research is needed to understand the relationship between antibiotic use and the resistome at the individual animal level, as well as the contribution of environmental background ARGs in conventional versus natural systems of production.

CHAPTER V

ASSOCIATION BETWEEN ANTIMICROBIAL USE AND ANTIMICROBIAL RESISTANCE BACTERIA IN FEEDLOT CATTLE: A SYSTEMATIC REVIEW AND META-ANALYSIS

Summary

Antimicrobials in feedlot cattle are administered to prevent and control diseases. The objective of this study was to assess the research question: Is antimicrobial use (AMU) in feedlot cattle associated with antimicrobial resistance (AMR) in bacteria? The initial screening of peer-reviewed literature identified 344 unique studies, of which, 32 were selected to be part of the present systematic review addressing AMR in *Escherichia coli* (n = 24), *Enterococcus* spp. (n = 6), *Salmonella* spp. (n = 4), *Campylobacter* spp. (n = 3), and *Mannheimia haemolytica* (n = 3). Overall, 60% (95% CI: 26% to 88) of the observational studies and 50% (95% CI: 30% to 70%) of the controlled trials reported a positive association between AMU and AMR in bacteria recovered from feedlot cattle. Meta-analysis provided evidence for an increase in average relative risk (RR) of resistant bacteria associated with antibiotic use. Isolates recovered from treated cattle were 2.5 times (95% confidence interval: 1.7 – 3.5) as likely to show antibiotic resistance as isolates recovered from unexposed animals. Risk of resistance increased with animal-defined daily doses (DDDs). One unit increase in animal DDDs corresponded to a change of 1.3 (95% conf. interval: 1.1, 1.7) units in terms of the average RR. Improving our understanding between AMU and AMR may help to improve decisions and policies about antibiotic use in feedlot cattle.

Introduction

Antibiotic resistant bacteria have a significant public health and economic impact worldwide. In Europe, 25,000 people die per year as a result of multidrug-resistant bacteria costing around € 1.5 billion per year (ECDC/EMEA Joint Technical Report, 2009). In U.S., it is estimated that more than 2 million people become infected annually with bacteria resistant to antibiotics, causing 23,000 deaths each year (CDC, 2015). Use of antimicrobials in food producing animals has been hypothesized to be a contributor to emergence and dissemination of antibiotic resistant bacteria of human importance (Mathew et al., 2007; Oliver et al., 2011); i.e., those causing treatment failure when treatment is required in humans. Although it is generally accepted that antimicrobial resistance (AMR) is a natural and ancient phenomena (D'Costa et al., 2011; Wright, 2012), much debate exists on the occurrences of AMR bacteria in beef cattle and the influence of antimicrobial use (AMU) during production (Schmidt, 2015).

Several individual studies have evaluated relationships between AMU and AMR bacteria in feedlot cattle, but through various types of study design (randomized controlled trials, observational studies, etc.), using different target organisms (*E. coli, Salmonella, Campylobacter*, etc.), measures of antimicrobial exposures (continuous or categorical variable) and resistance outcomes (presence/absence of resistant isolates, proportion of resistant isolates in exposed and unexposed groups, etc.), among other factors. The systematic review and meta-analysis is a method to record, summarize and analyze such variability in the scientific literature based on eight steps: 1) define the review question, 2) conduct an extensive search for studies, 3) select relevant studies from the results of the search, 4) collect data from relevant studies, 5) assess the risk of bias in relevant studies, 6) synthesize the results, 7) interpret and discuss the results, and 8) perform a meta-analysis if possible (O'Connor and Sargeant, 2015). This

methodic and systematic approach is what distinguishes systematic reviews from traditional literature reviews (Khan et al., 2003). Systematic reviews assessing effects of AMU on development of AMR were conducted in animal species such as chickens and pigs (Burow et al., 2014; Simoneit et al., 2015); however, to our knowledge, there is no published systematic review addressing the same question for beef cattle. The main objective of the present systematic review and meta-analysis was to identify, report, and investigate the published knowledge on the effect of AMU on AMR in bacteria recovered from feedlot cattle. Results reported in the present study may provide scientific evidence for future risk assessments that endeavor to model the effects of AMU in feedlot cattle on meat safety and antimicrobial resistance in humans.

Materials and Methods

Formulation of the systematic review question

We followed the P.I.C.O. format (Population, Intervention, Comparison, Outcome) for developing the question addressed by this systematic review, as recommended by the European Food Safety Authority (2010): Is antimicrobial use in feedlot cattle associated with antimicrobial resistance in bacteria? The population of interest was feedlot cattle (calves, steers, and heifers) of any breed and the intervention was the use of any class of antibiotics commonly used in feedlot cattle, including low and therapeutic dose administration by any route (in-feed, oral, parenteral). The outcome of interest was antibiotic resistance in bacteria recovered from samples collected from feedlot cattle. The comparison of interest was to evaluate difference in resistance outcomes between antibiotic treated animals and untreated control animals in controlled studies, and to assess the association between antimicrobial exposures and AMR in observational studies.

Literature search and study selection

This systematic review was conducted between January and April 2017 by two independent reviewers. Any disagreement was resolved by the expert opinion of a third-reviewer. PubMed, EBSCO and Web of Science™ databases were searched using the following Boolean search terms: (feedlot OR finishing OR growing) AND (cattle OR calves OR steers OR heifers) AND (antibiotic OR antimicrobial) AND resistan* AND bacteria. Additionally, the reference sections from selected articles also were screened to identify other relevant articles. Duplicated studies were removed creating a non-redundant database, which was subjected to a three-step screening process. First, each retrieved study was assessed based on the title and abstract. Those articles clearly beyond the scope of the research question were eliminated (e.g., studies assessing the association between AMU and AMR on pigs or chickens). Remaining studies were retained as complete articles and read in full length to confirm relevance and pertinence according to the research question. A study was defined as one publication and trials were defined as the different comparisons within a study. One study could have more than one trial, but not all trials would be selected for inclusion in the systematic review. For example, Lefebvre et al. (2005; 2006) and Diarra et al. (2009) evaluated the impact of monensin, steroid growth promotants and in-feed antimicrobials on AMR in *E. coli*; but, only the trial evaluating an in-feed antimicrobial was selected for this review as growth promotants are not antibiotics and *E. coli* has intrinsic resistance to monensin (Agga et al., 2016). In the final screening, a quality assessment was performed for the selected studies based on the experimental design (i.e., randomize control trial, cohort, observational), randomization (yes or no), bias and confounders (i.e., were they addressed?), and external validity (i.e., can the results be generalized to all cattle populations?). Based on reviewer's opinions, studies were ranked as high, medium and low

quality. For example, high quality studies used randomization techniques, invoked blinding of personnel to treatment groups, controlled for confounding variables (source of cattle, previous antimicrobial exposure, etc.) and addressed relevant classes of antimicrobials in commercial feedlots. Studies considered to be of low quality were removed from further analysis.

Evaluation of the association between AMU and AMR

Using procedures reported by Bell et al. (2014), each study was assigned a dichotomous outcome based on the relationship between AMU and AMR found in each article (1 = positive association between AMU and AMR; 2 = no association between AMU and AMR). A binomial test was then performed to investigate whether a significantly greater number of positive associations between exposure and resistance were found in the comparisons. The proportion under the null hypothesis (no association between resistance and exposure) was 50% (i.e., the probability of a positive association was equal to the probability of a non-positive association). A descriptive analysis and discussion of results was performed for each group of organism included in the present systematic review (*Escherichia coli, Salmonella, Campylobacter, Mannheimia haemolytica*, and *Enterococcus*).

First, a random effect meta-analysis was conducted among studies that reported the proportion of resistant isolates in control and treated animals to calculate pooled relative risk (RR). Then, animal defined daily doses (DDDs), and time between end of antibiotic use and collection of samples, and duration of antibiotic treatment (days) were included in a mixed effects model. Each antimicrobial treatment was converted to DDDs and multiplied by the duration of treatment (days) to standardize antimicrobial exposures across different studies

(Noyes et al., 2016). Data were analyzed using R version 3.1.3 and metafor package (Viechtbauer, 2010).

Results and Discussion

Description of the studies.

The process of study selection for the systematic review is summarized in Figure 5.1. After removing duplicate studies retrieved from different publication databases, we created a non-redundant list containing 344 unique studies. Most of these (87.5%) were excluded during the first screening step as they were not performed in growing or finishing beef cattle, samples were not collected from animals, or there was a lack of information regarding antimicrobial use in the study population. After careful examination of the remaining 43 studies, 12 (25.6%) were removed from downstream analysis. In most of these instances, studies were removed because they did not use culture-based techniques for isolation of resistant bacteria; instead they used culture-independent techniques such as polymerase chain reaction, metagenomics and whole genome sequencing. In the last screening step, no further studies were removed because of low quality. Of the selected studies (n = 32), 22 (68.8%) were controlled experiment trials and 10 (31.2%) were observational studies (Tables 5.1 and 5.2, respectively). In general, randomized controlled trials addressed confounding and bias through proper experimental design, but they generally used small cattle populations in research facilities compromising their external validity. Observational studies usually included information about multiple antibiotic exposures in large populations of cattle under commercial conditions, but were more vulnerable to bias and confounding (source of cattle, previous antibiotic exposure, etc.) due to lack of experimental design and control (Noyes et al., 2015).

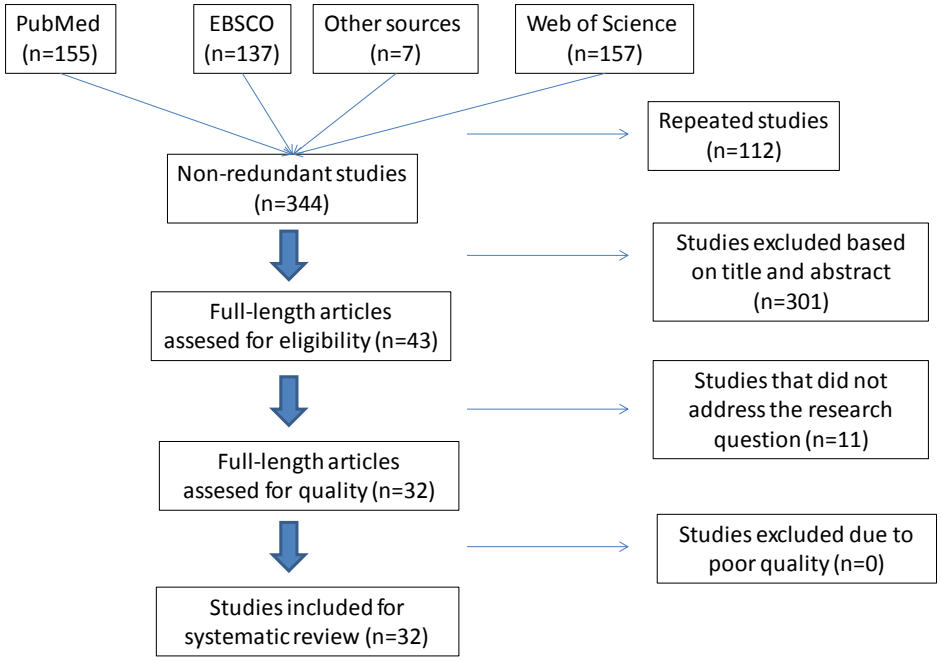


Figure 5.1. PRISMA flow chart showing the flow of articles through the different phases of the systematic review addressing the question: Is the use of antimicrobials in feedlot cattle associated with an increased proportion of antimicrobial resistant bacteria?

Bacterial populations targeted were *Escherichia coli* (*E. coli*, n = 24 studies), *Enterococcus* spp. (n = 6), *Salmonella* spp. (n = 4), *Campylobacter* spp. (n = 3), and *Mannheimia haemolytica* (*M. haemolytica*, n = 3). Antimicrobial resistance in enteric bacteria was assessed by Stabler et al. (1982) and was considered as *E. coli*. Six studies evaluated AMR simultaneously in more than one group of organisms. Most studies examined resistant bacteria in fecal samples collected either from the rectum or pen floor, except for the *M. haemolytica* studies which was recovered from nasopharyngeal samples. Twenty-five percent of the studies were conducted under commercial conditions and all selected studies were carried out in Canada (n = 19) and U.S. (n = 13). As a result, publication bias was not discarded in the present systematic review. In addition, it was possible that some studies that did not find an association between AMU and AMR were not published and included in the present review. This may increase type I error, or the likelihood of incorrectly concluding that associations exist between AMU and AMR

(Dickersin, 1990). In 49% of the studies, resistance was classified using the broth microdilution technique, whereas 25% and 20% of the studies used disk diffusion and agar dilution techniques, respectively. Two studies (6%) assessed antimicrobial resistance performing only selective enrichment and/or plating on selective agar.

Overall, 60% of the observational studies (95% CI: 26% to 88%; $P > 0.05$) reported at least one positive association between AMU and AMR in bacteria recovered from feedlot cattle in their conclusions. Among those which reported a positive association, 66.7% (n = 4) clearly stated concerns about the strength or implications of the association, including small differences in AMR between exposed and unexposed cattle, questionable biological relevance of such differences, and concerns about how well AMU predicts AMR due to confounding factors. Several measured or unmeasured confounding factors could contribute to that association, including environment, diet, cattle source, previous exposure to antibiotics, days on feed, type of susceptibility test, feedlot hygienic practices, timing and route of exposure, and season of the year, among others. Other limitations of observational studies mentioned by the authors were the difficulty of obtaining detailed information about AMU, which may result in recall bias, the limited ability to study rare exposures and/or rare outcomes, and the ability to evaluate associations when in-feed medication and individual antimicrobial treatment occurred at the same time (Checkley et al., 2008; Benedict et al., 2015).

Table 5.1. Information on intervention protocols for controlled trials studying the association between antimicrobial use and the isolation of resistant bacteria from fecal samples in feedlot cattle.

Study	Authors	Organism	Interventions	Dose	Route	Follow up (days)
1	Stabler et al., 1982	Enteric bacteria	Control OTC[1] OTC + CTC[4] CTC low CTC high	 5 mg/.45 kg BW[2] x 3 doses Idem + 70 mg CTC/head 70 mg/head 700 mg/head	 SC[3] SC + F[5] F F	45
2	O'Connor et al. 2002	*E. coli*	CTC CTC+OTC	3-8 mg/lb BW x 16 days Idem + 9 mg/lb BW	F F + SC	76
3	Berge et al. 2005	*E. coli*	Control Florfenicol	- 39.6 mg/kg BW	- SC	42
4	Inglis et al., 2005	*Campylobacter jejuni*, *Campylobacter hyointestinalis*	Control CTC CTC+S[6] Virginiamycin Monensin Tylosin phosphate	- 11 pm 350 + 350 mg/head/d 250 mg/head/d 25 ppm 11 ppm	- F F F F F	246
5	Lefebvre et al. 2005	*E. coli O157*	Control Oxytetracycline	- 200 mg/ml	- IM[7]	165
6	Lefebvre et a. 2006	*E. coli*	Control Oxytetracycline	- 200 mg/ml	- IM	165

Table 5.1. (Cont'd)

Study	Authors	Organism	Interventions	Dose	Route	Follow up (days)
7	Lowrance et al., 2007	*E. coli*	Control CCFA[8] 1-dose CCFA 2/3-dose CCFA 3-doses	- 6.6 mg/kg BW 4.4 mg/kg BW 6.6 mg/kg BW x 3	- SC SC SC	28
8	Alexander et al., 2008	*E. coli*	Control CTC CTC + S Virginiamycin Monensin Tylosin	- 11 ppm 44 ppm 31 ppm 25 ppm 11 ppm	- F F F F F	315
9	Coe et al., 2008	*E. coli*	Control Tilmicosin Florfenicol	- 10 mg/kg BW 40 mg/kg BW	- SC SC	210
10	Jacob et al., 2008	*E. coli, E. coli O157:H7, Enterococcus, Salmonella*	Control Monensin Monensin + Tylosin	- 300 mg/head/d 300 + 90 mg/head/d	F	136
11	Platt et al. 2008	*E. coli, Enterococcus*	Control CTC	- 22 mg/kg BW	- F	33
12	Sharma et al., 2008	*E. coli*	Control CTC CTC + S	- 350 mg/head 350 mg/head each	- F F	225

Table 5.1. (Cont'd)

Study	Authors	Organism	Interventions	Dose	Route	Follow up (days)
13	Diarra et al. 2009	*E. coli* non-O157	Control Oxytetracycline	- 200 mg/ml	- IM	165
14	Alexander et al. 2010	*E. coli*	Control CTC + S	- 44 ppm	- F	179
15	Checkley et al. 2010	*E. coli*	Control OTC Long acting OTC	- 2 g/animal/d x 14 days 20 mg/kg BW	- F SC	248
16	Mirzaagha et al. 2011	*E. coli*	Control CTC CTC+S Virginiamycin	- 11 ppm 44 ppm 31 ppm	- F F F	246
17	Kanwar et al., 2013	*E. coli*	CCFA all animals CCFA all animals + CTC CCFA one animal CCFA one animal + CTC	CCFA: 6.6 mg/kg BW CTC: three separate 5-day regimes 22 mg/kg BW	SC F	26
18	Zaheer et al., 2013	*Mannheimia haemolytica, Enterococcus*	Control Tilmicosin Tulathromycin Tylosin	- 10 mg/kg BW 2.5 mg/kg BW 11 ppm	- SC SC F	28

Table 5.1. (Cont'd)

Study	Authors	Organism	Interventions	Dose	Route	Follow up (days)
19	Edrington et al., 2014	*E. coli, E. coli* O157:H7, *Salmonella*, *Enterococcus*	Control Virginiamycin Virginiamycin Virginiamycin commercial	- 0.7 mg/kg 8.9 mg/kg 0.06 mg/kg	- F F F	49
20	Amachawadi et al., 2015	*Enterococcus*	Low Copper High Copper Low Copper + Tylosin High Copper + Tylosin	10 mg/kg 100 mg/kg 10 + 10 mg/kg 100 + 10 mg/kg	F F F F	28
21	Beukers et al., 2015	*Enterococcus*	Control Tylosin	- 11 ppm	F	225
22	Agga et al., 2016	*E. coli*	Control CTC	- 10 mg/lb BW x 5 days	- F	117

[1] OTC: oxytetracycline, [2] BW: Body weight, [3]SC: subcutaneous, [4] CTC: chlortetracycline, [5]F: feed, [6]S: sulfamethazine, [7]IM: intramuscular, [8]CCFA: ceftiofur crystalline-free acid.

Table 5.2. Description of observational studies investigating the association between exposures to antimicrobials and antimicrobial resistance in feedlot cattle.

Study	Authors	Organism	Feedlots	Pens	Animals	Sample
23	Dargatz et al. 2002	*Salmonella*	100	200	-	Feces
24	Inglis et al. 2006	*Campylobacter*	4	-	2,622	Feces
25	Checkley et al. 2008[1]	*E. coli*	1	12	447	Feces
26	Rao et al. 2010	*E. coli*, *E. coli* O157, *Salmonella* and *Campylobacter*	21	84	8,688[2]	Feces
27	Morley et al. 2011	*E. coli*	3	84	9,470	Feces
28	Schmidt et al. 2013[1]	*E. coli*	1	-	763	Feces
29	Alexander et al. 2013[3]	*Mannheimia haemolytica*	4	-	5,814	Nasopharyngeal
30	Benedict et al. 2015[3]	*E. coli*	4	305	5,849	Feces
31	Noyest et al. 2015[3]	*Mannheimia haemolytica*	4	-	5,498	Nasopharyngeal
32	Noyes et al. 2016[3]	*E. coli*	4	300	-	Feces

[1]Research feedlot, the remaining feedlots corresponded to commercial facilities; [2]Estimated based on average number of animals placed on pens; [3]Studies carried out from the same surveillance project

Among controlled studies, 55% (95% CI: 32% to 76%; $P > 0.05$) reported a positive association between AMU and AMR. The probability of finding a positive association in controlled studies was greater among studies addressing *Enterococcus* spp. resistance (83%; 95% CI: 36% to 100%; $P = 0.22$) and weaker for those addressing *E. coli* resistance (44%; 95% CI: 21% to 69%; $P = 0.63$). *Salmonella* spp., *Campylobacter* spp., and *M. haemolytica,* where studies, included only one controlled trial for each such that there was no strong experimental evidence to determine an association between AMU and AMR.

The relationship between AMU and AMR may vary within a study depending on which bacteria and class of antibiotics are being considered. For example, Jacob et al. (2008) reported that tylosin use was associated with increased macrolide resistance in *Enterococcus,* whereas in the same experiment, cattle exposed to tylosin had a decreased proportion of *E. coli* isolates resistant to tetracyclines. For that reason, is important to conduct not only separate analysis between AMU and AMR for each group of bacteria, but also measure resistance to antibiotics not used in the study. Bacteria may exhibit co-resistance to multiple classes of antibiotics due to the presence of antibiotic resistance genes in mobile genetic elements (Wales and Davies, 2015). Results from studies in the present review suggested that administration of in-feed chlortetracycline can lead to resistance to tetracyclines, as well as to other classes of antibiotics, including sulfamethoxazole, ampicillin and chloramphenicol (Alexander et al., 2008; 2010; Sharma et al. 2008; Mirzaagha et al., 2011). In addition, use of ceftiofur (Lowrance et al., 2007) and florfenicol (Berge et al., 2005) were associated with isolates that showed simultaneous resistance to multiple classes of antibiotics.

Escherichia coli studies

A total of 24 studies attempted to determine the association between AMU and AMR in *E. coli* isolates recovered from feedlot cattle; studies were distributed into 6 observational studies and 18 controlled trials. Among the observational studies (Checkley et al., 2008; Rao et al., 2010; Morley et al., 2011; Schmidt et al., 2013; Benedict et al., 2015; Noyes et al., 2016), resistance among *E. coli* isolates irrespective of the cattle group (exposed or unexposed to antibiotics) was most common to tetracycline, streptomycin, sulfadiazine, sulfisoxazole, and sulfamethoxazole (Figure 5.2).

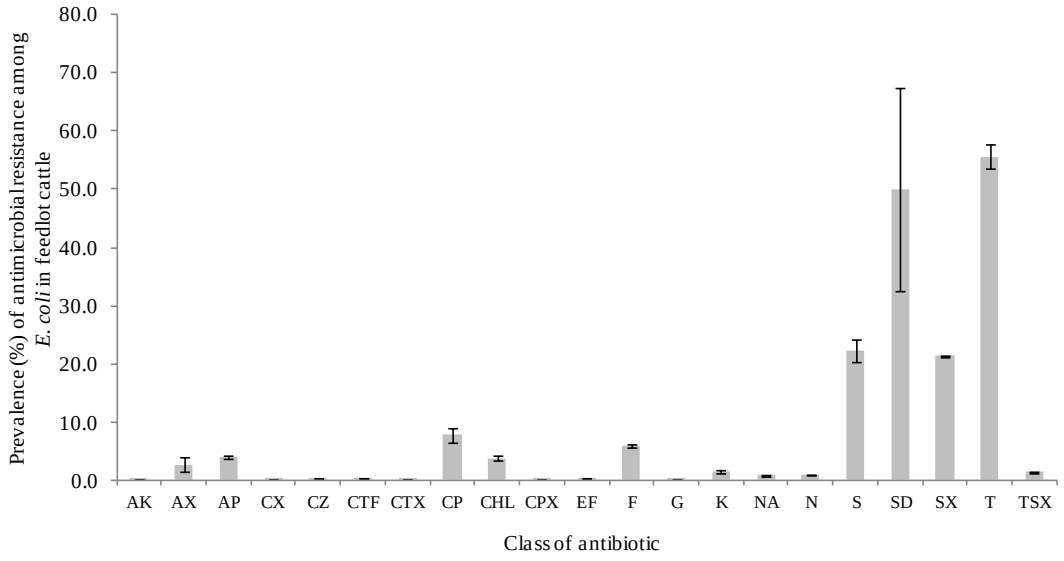


Figure 5.2. Percentage (± standard error of the mean) of resistance among *E. coli* isolates recovered from feedlot cattle in observational studies. Each mean is the average of at least 2 different studies. AK: amikacin, AX, amoxicillin, AP: ampicillin, CX: cefoxitin, CZ: ceftazidime, CTF: ceftiofur, CTX: ceftriaxone, CP: cephalothin, CHL: chloramphenicol, CPX: ciprofloxacin, EF: enrofloxacin, F: florfenicol, G: gentamicin, K: kanamycin, NA: nalidixic acid, N: neomycin, S: streptomycin, SD: sulfadiazine, SX: sulfisoxazole, T: tetracycline, TSX: trimethoprim-sulfamethoxazole.

Despite the high level of resistance, tetracyclines continue to be efficacious for prevention and treatment of diseases in feedlot cattle (Rao et al., 2010; Benedict et al., 2015) and are not considered to be a first-line antibiotic option to treat human infections. For that reason, the impact of the eventual association between tetracycline use and tetracycline-resistant *E. coli* on animal and human health is under debate.

According to conclusions, two of the observational studies did not find an association between AMU and AMR (Checkley et al., 2008; Schmidt et al., 2013), three studies found a small to moderate association (Rao et al., 2010; Morley et al., 2011; Benedict et al., 2015), and one study found detectable associations between AMU and AMR for specific drugs (Noyes et al., 2016) (Table 5.3). The most common positive association was between exposure to tetracycline and tetracycline resistant-*E. coli* (Rao et al., 2010; Morley et al., 2011; Benedict et al., 2015; Noyes et al., 2016). Among observational studies, resistance to critically important antibiotics defined by the WHO (2014), such as 3rd and 4th generation cephalosporin and penicillins occurred at low levels. All observational studies cited in the present systematic review were performed before the Veterinary Feed Directive (2015) went into effect in 2017. This rule promoted judicious use of in-feed antimicrobials in feedlot cattle by requiring enhanced veterinary oversight. It will be interesting to evaluate in further studies the impact of the VFD implementation on development and dissemination of AMR in feedlot cattle. Tetracycline use was by far the most common class of antibiotic used as an antimicrobial intervention in *E. coli* controlled studies (Table 5.4).

Table 5.3. Summary of significant associations found in observational studies between antimicrobial use and antimicrobial resistance in *E. coli* in confined cattle.

Study	Exposure to	Resistance to[1]
Checkley et al., 2008	Tetracycline, tilmicosin	No resistance to AMP, ENR, TET, GEN, SMX, TMP/SSS, TMP[2]
Rao et al., 2010	Tetracycline	Tetracycline, streptomycin, sulfadiazine
Morley et al., 2011	Several antibiotics	Chloramphenicol, streptomycin, sulfamethoxazole, tetracycline
Schmidt et al., 2013	Ceftiofur	No resistance to extended-spectrum cephalosporins
Benedict et al., 2015	Tetracycline Beta-lactam	Tetracycline, trimethoprim sulfa Streptomycin (-)
Noyes et al., 2016	Tetracycline Sulfonamide Macrolide	Tetracycline (OR: 1.1 – 3.2)[3] Sulfisoxazole (OR: 1.4 – 2.5) Ampicillin (-) (OR: 0.03 – 0.2)

[1] (-): exposure to the corresponding class of antibiotic was associated with a decrease probability of recovering the resistance outcome.
[2] AMP: ampicillin, ENR: enrofloxacin, TET: tetracycline, GEN: gentamicin, SMX: sulphamethoxazole, TMP/SSS: trimethoprim/sulfanilamide, TMP: trimethoprim
[3] Odds ratio 95% confidence interval

Studies that evaluated in-feed antimicrobials were more likely to find a positive relationship between AMU and AMR in *E. coli* (64%; 95% CI: 31% to 89%; $P > 0.05$) than studies that used antibiotics injected intramuscularly or subcutaneously (25%; 95% CI: 3% to 65%; $P > 0.05$). This suggested that the route of administration and/or length of the exposure may play a critical role in AMR ecology as bacteria may not be exposed equally when antimicrobials are administered by different routes. Zhang et al. (2013) suggested that oral administration of antibiotics has a stronger effect on AMR than other drug delivery approaches (i.e., injections) by exposing gut microbiota to a greater selective pressure. Under long-term in-feed antimicrobial exposure, sensitive strains are more likely to remain in low concentrations;

whereas resistant strains have more time to overcome the cost of resistance through compensatory adaptation (MacLean and Vogwill, 2015).

Overall, 6 out of the 18 experimental studies (33.3%) for which *E. coli* resistance were evaluated (Stabler et al., 1982; Berge et al., 2005; Lefebvre et al., 2005; Lowrance et al., 2007; Platt et al., 2008; Agga et al., 2016) found a short-term positive association between AMU and the prevalence of *E. coli* resistant isolates, in most cases, between 2 and 20 days post treatment. Sensitive strains may survive and recover after exposure to antibiotics, thereby overwhelming and displacing resistant strains in the long term. For that reason, likelihood of finding an association between antimicrobial use and AMR decreases with time after exposure. Given the short-lived nature of resistant phenotypes and the fact that most antimicrobials are used early in the feeding period, Noyes et al. (2016) questioned the role of antimicrobial use in cattle with respect to dissemination of resistance through the food chain. In addition, the fact that susceptible *E. coli* counts usually decline significantly after administration of antibiotics increases the probability of detecting a resistant isolate which might have been present in the animal before treatment (Singer et al., 2008). For that reason, studies that did not enumerate *E. coli* cannot determine whether the increase of AMR observed immediately after antibiotic administration was due to an increased population of resistant bacteria or due to a decreased population of susceptible bacteria (Platt et al., 2008). On the other hand, 4 studies (23.5%) reported a significantly increased resistant *E. coli* with the feeding period (Lefebvre et al., 2006; Sharma et al., 2008; Checkley et al., 2010; Kanwar et al., 2013).

Table 5.4. Summary of results from controlled studies evaluating the effect of in-feed or subcutaneously injected antimicrobials in *E. coli* resistance in fecal samples from feedlot cattle.

Study	Main result
In-feed	
Stabler et al., 1982[1]	Use of CTC increased the prevalence of TET resistant bacteria
Jacob et al. 2008	Exposure to monensin and tylosin decreased the percentage of isolates resistant to CTC and OTC
Platt et al. 2008	Exposure to CTC did not have a large long-term effect on *E. coli* resistance
Sharma et al. 2008	Use of CTC resulted in higher TET resistant- *E. coli* shedding
Alexander et al. 2008	Use of CTC or CTC + S increased the prevalence of TET and AMP resistant *E. coli* in cattle
Alexander et al. 2010	Use of CTC + S increased the prevalence of AMP and TET resistant *E. coli*
Checkley et al. 2010[2]	OTC increased the proportion of animals carrying TET resistant *E. coli* isolates from arrival to pre-slaughter
Mirzaagha et al. 2010	Use of CTC + S increased resistance to SFX and CHL in AMP and TET resistant *E. coli*
Kanwar et al. 2013	In-feed CTC exacerbated CF *E. coli* resistance levels following CCFA injection
Edrington et al. 2014	No differences were observed in antimicrobial resistance of *E. coli* due to exposure of virginiamycin.
Agga et al. 2016	Short term use of CTC had no long-term impact on antimicrobial resistant *E. coli*
Parenteral	
Stabler et al., 1982[1]	Injectable OTC had no long-term impact on antimicrobial resistant enteric bacteria
O'Connor et al., 2002	The use of injectable OTC, in addition to in-feed CTC, was associated with CHL and SFS *E. coli* resistance
Berge et al., 2005	Florfenicol treatment did not have a large long-term effect on *E. coli* resistance
Lefebvre et al., 2005	Injectable OTC had no long-term impact on antimicrobial resistant *E. coli* O157
Lefebvre et al., 2005	Injectable OTC was not associated with the presence of antibiotic-resistant *E. coli*
Coe et al., 2008	Tilmicosin or florfenicol treatment did not have an effect on *E. coli* resistance
Diarra et al., 2009	Injectable OTC was not associated with antibiotic-resistant non-O157:H7 Shiga toxin-producing *E. coli*
Lowrance et al., 2009	Injectable CCFA had no long-term impact on antimicrobial resistant *E. coli*
Checkley et al., 2010[2]	Injectable OTC increased the proportion of animals carrying TET resistant *E. coli* isolates from arrival to pre-slaughter, but not as strong as the use of in-feed OTC

AMP: ampicillin, CCFA: ceftiofur crystalline-free acid, CF: ceftiofur, CHL: chloramphenicol, CTC: chlortetracycline, OTC: oxytetracycline, S: sulfamethazine, SFX: sulfamethoxazole, SFS: sulfisoxazole TET: tetracycline.

[1,2] Same studies evaluating both in-feed and injectable antimicrobials

Control trials assessing AMR in *E. coli* reported different resistance outcomes, including prevalence of resistant isolates in control and treated groups (50% of studies), mean counts of resistant *E. coli* (20%), prevalence of animals carrying resistant isolates (15%), and distribution of minimum inhibitory concentrations (MIC) in *E. coli* isolates (15%). Tetracycline resistant *E. coli* counts were consistently greater in cattle exposed to in-feed tetracycline than in animals unexposed to tetracycline (mean ± standard error: 4.85 ± 0.75 and 5.82 ± 1.09 log CFU/g feces, respectively) (Figure 5.3).

Salmonella studies

Four studies addressed the question of whether or not AMR in *Salmonella* was associated with AMU in feedlot cattle. Three were observational studies (Dargatz et al, 2002; Rao et al., 2010; Morley et al., 2011), while the remaining study was a controlled trial (Jacob et al., 2008).

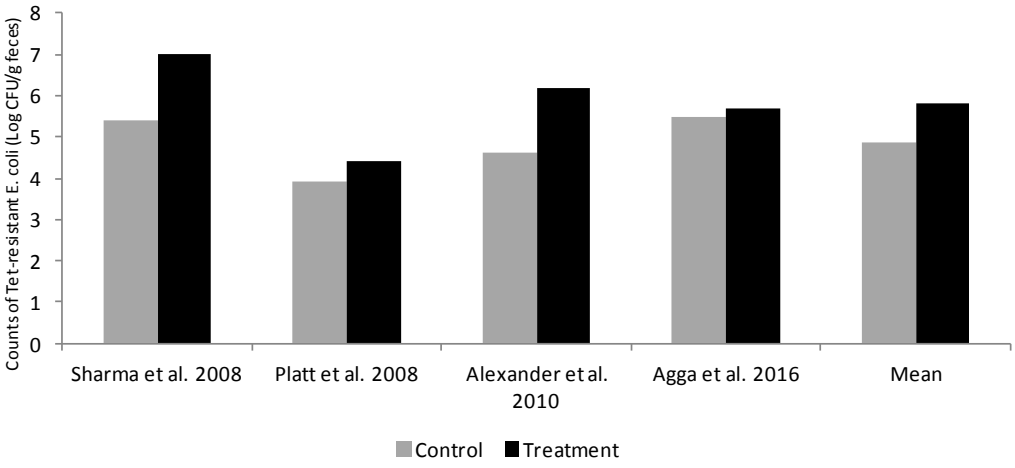


Figure 5.3. Concentration of tetracycline-resistant *E. coli* in the feces of feedlot cattle exposed (treatment) and unexposed (control) to tetracyclines.

All four *Salmonella* studies did not provide enough evidence of an association between AMR and AMU for *Salmonella.* In a large study comprising more than 100 feedlots, Dargatz et al. (2002) reported that AMR in *Salmonella* was not related to the presence of in-feed antimicrobials at the time of sample collection. Rao et al. (2010) and Morley et al. (2011) could not study an association between AMR and AMU because of the low numbers of *Salmonella* isolates recovered from fecal samples (0.95% and 0.73% positive samples, respectively). In the controlled trial, Jacob et al. (2008) found that all isolates (n=21) were resistant to clindamycin and macrolides, and susceptible to gentamicin, neomycin, tetracyclines, and ampicillin, irrespective of treatment (exposed or not to in-feed tylosin). This was not consistent with two additional studies determining AMR characteristics of *Salmonella* spp. isolated from feedlot cattle in U.S. (Dargatz et al., 2013; 2016), but lacking AMU data, and for that reason, not formally included in this systematic review. They found that most isolates recovered were susceptible to several antimicrobials, but more commonly to tetracyclines, sulfisoxazole, streptomycin, ampicillin, or chloramphenicol.

Campylobacter studies

A total of three studies evaluated the association between AMU and AMR in *Campylobacter* isolates recovered from feces of feedlot cattle in Alberta, Canada (Inglis et al., 2005; 2006; Rao et al., 2010). Both studies conducted by Inglis et al. (2005, 2006) found significant development of resistance to tetracyclines in *Campylobacter* isolates after exposure to in-feed chlortetracycline (alone or in combination with sulfamethazine) and long-acting injectable oxytetracyline. The association was more evident when cattle were fed a grain-based diet compared to when cattle were fed a forage-based diet. The majority of resistant isolates were

obtained from animals housed in a few pens, thereby suggesting that transmission of isolates rapidly occurred among penmates (Inglis et al., 2005). In addition, and only for *C. hyointestinalis*, Inglis et al. (2005) observed an increase of erythromycin-resistant isolates when chlortetracycline was included in the diet. In the observational study conducted by Rao et al. (2010), exposure to antimicrobials (mainly in-feed sulfonamides, macrolides and tetracyclines) was not associated with detectable differences in AMR among *C. jejuni* isolates recovered from 21 feedlots. However, resistance to doxycycline was greater in isolates collected from pre-slaughter animals than those collected from newly arrived animals but the authors questioned its practical relevance (26.7% and 44.3% prevalence of AMR in *C. jejuni* in newly arrived and pre-slaughter cattle, respectively). In a similar study, which was not part of the present review due to the lack of AMU data, Englen et al. (2005) reported an overall tetracycline resistance of 51.6% in *Campylobacter* isolates recovered from 73 feedlots in the U.S.

Enterococcus studies

In six studies, researchers investigated AMR in *Enterococcus* spp. isolated from feedlot cattle exposed to in-feed or parenteral macrolides (n = 4), in-feed streptogramin (n = 1), and in-feed chlortetracycline (n = 1). Tylosin phosphate, a macrolide included in the feed to reduce incidence of liver abscesses in feedlot cattle (Nagaraja and Chengapp, 1998; Beukers et al., 2015), was the most common exposure to asses *Enterococcus* resistance. The four studies that evaluated in-feed supplementation of tylosin (Jacob et al., 2008; Zaheer et al., 2013; Amachawadi et al., 2015; Beukers et al., 2015) found greater levels ($P < 0.05$) of *Enterococcus* resistance towards macrolides in isolates obtained from the exposed groups than those from the control groups (average 49% and 24%, respectively). Similarly, Platt et al. (2008) found that the

proportion of tetracycline-resistant *Enterococcus* isolates was greater in cattle exposed to in-feed chlortetracycline than in unexposed cattle. Their study did not evaluate resistance to macrolides, but it is well known that co-occurrence of both macrolide and tetracycline resistance genes in fecal microbiomes of beef cattle may occur (Chan et al., 2008). One study (Edrington et al., 2014) examined the effect of feeding distilled grains containing different levels of virginiamycin residues on AMR in *Enterococcus* spp. isolates. Virginiamycin is a streptogramin antibiotic used to control bacterial growth during the fermentation process in the ethanol industry (Paulus et al., 2012). Edrington et al. (2014) did not find an impact of virginiamycin residues in distiller's grain fed to cattle on AMR of *Enterococcus* spp. isolates.

Mannheimia haemolytica studies

Three studies assessed the influence of antimicrobial use on AMR in *M. haemolytica* isolates recovered from nasopharyngeal swabs in feedlot cattle (Alexander et al., 2013; Zaheer et al., 2103; Noyes et al., 2015). These studies were included in the present review despite the fact that they did not collect fecal samples as the rest of the studies evaluated in this review did, mainly because of the economic importance of *M. haemolytica* and bovine respiratory disease in feedlot cattle (Booker et al., 2008; Fulton et al., 2009). Two studies found no association between macrolide use (parenteral tulathromycin or tilmicosin, in-feed tylosin) and macrolide-resistance in *M. haemolytica* isolated from feedlot cattle in western Canada (Alexander et al., 2013; Zaheer et al., 2013). Similarly, Noyes et al. (2015) found no association between antibiotic exposure (mostly to tetracyclines and macrolides) and *M. haemolytica* resistant to single drugs. However, parenteral administration of antimicrobials in the penmates of sampled animals

increased the odds of recovering multiply-resistant M. haemolytica, probably due to contagious spread (Noyes et al., 2015).

Meta-analysis

Meta-analysis is usually the final step of systematic reviews. It uses statistical techniques to pool results of similar studies for quantitative analyses (Schechner et al., 2013). In the present review, the possibility of performing a meta-analysis between AMU and AMR was limited due to a lack of a consistent measure to report resistance outcome for each group of organisms. Figure 5.4 shows the pooled average relative risk (RR) of selected randomized controlled trials that reported the proportion of isolates resistant to diverse antimicrobials in control and exposed groups, which was the most common outcome reported among studies. For example, the first trial corresponds to Stabler et al. (1982) (study 1 in Table 5.1), the intervention was oxytetracycline (OTC) and the outcome was isolates resistant to tetracycline (Tet). The relative risk (RR) of resistance was greater ($P < 0.05$) in isolates recovered from cattle exposed to antibiotics than in those recovered from unexposed cattle. Isolates recovered from treated cattle were 2.5 times (95% confidence interval: 1.7 – 3.5) as likely to show antibiotic resistance as the isolates recovered from unexposed animals (log RR: 0.9; 95% CI: 0.5 – 1.3). Similar results were obtained for *E. coli* (RR: 1.9; 95% CI: 1.2 – 3.2, $P < 0.05$) and *Enterococcus* (RR: 2.7; 95% CI: 1.5 – 5.2, $P < 0.05$) when separate meta-analysis were performed for each group of bacteria (Appendix 7 and 8). However, heterogeneity was detected among all studies (Cochran's Q = 158; $P < 0.05$), suggesting that other variables not considered in the random model influenced results. We next included three fixed variables as covariates in the model: (i) cumulative days of antimicrobial exposure before sampling (mean: 50 days, min. 2, max. 197), (ii) time between last

antimicrobial exposure and collection of samples (mean: 13 days, min. 0, max. 36), and (iii) animal defined daily doses (DDDs). The first two variables were not useful ($P = 0.25$ and $P = 0.17$, respectively), while DDDs appeared to influence the RR ($P < 0.05$).

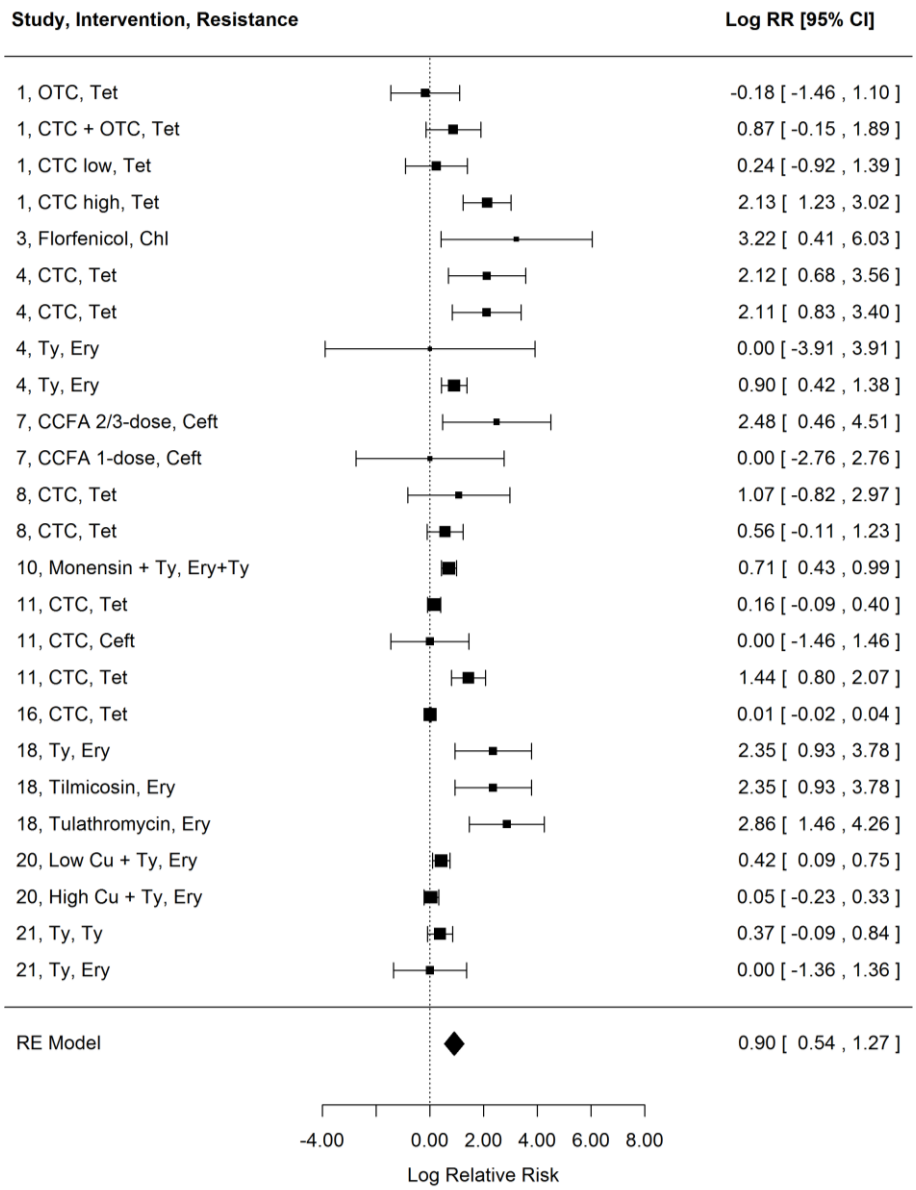


Figure 5.4. Relative risk (log scale) for antibiotic-resistance in bacteria isolates collected from feedlot cattle exposed to antibiotics using a random effect model. See Table 5.1 for a description of studies and interventions. CCFA: ceftiofur crystalline-free acid, CTC: chlortetracycline, Cu: Copper, OTC: oxytetracycline, Ty: Tylosin, Ceft: ceftiofur, Chl: chloramphenicol, Ery: erythromycin, Tet: tetracycline, Ty: tylosin.

Figure 5.5 shows a plot of the RR as a function of DDDs. One unit increase in animal DDD corresponded to a change of 1.3 (95% conf. interval: 1.1, 1.7) units in terms of average RR. Animal DDDs also were important ($P < 0.05$) for *E. coli* and *Enterococcus* individual models. One unit increases in animal DDDs corresponded to a change of 1.3 (95% conf. interval: 1.1, 1.5) and 2.1 (95% conf. interval: 1.1, 3.9) average RR for *E. coli* and *Enterococcus*, respectively.

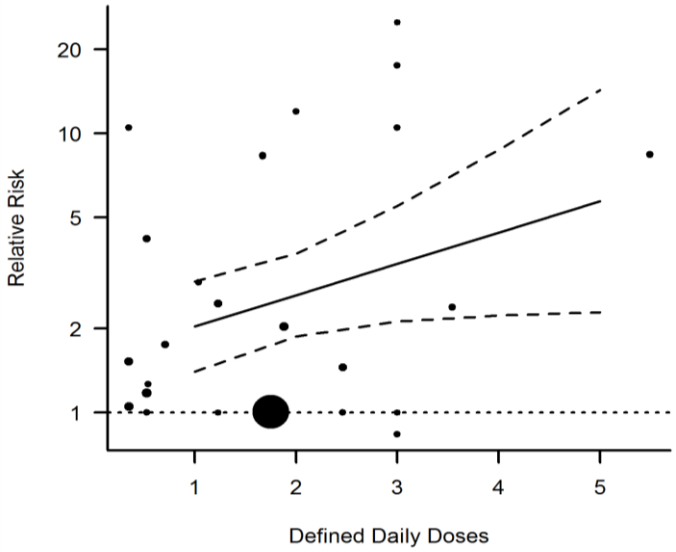


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Figure 5.5. Relative risk of antibiotic resistance versus defined daily doses (dotted lines represent 95% confidence interval).

For *E. coli*, RR associated with antibiotic exposure decreased with increased time between last exposure and collection of samples (Figure 5.6). Studies with larger last exposure-sampling windows tended to register a greater average RR. The effect of categorical variable route of administration (in-feed or parenteral) on the RR w also was assessed. Both in-feed and parenteral use of antibiotics led to an increased average RR ($P < 0.05$). The estimated average RR for studies using in-feed and parenteral treatment was 2.3 (95% conf. interval: 1.5, 3.6) and 3.0 (95% conf. interval: 1.4, 6.4) (Appendix 9). The wide confidence intervals for estimates of

association between AMU and AMR indicated low precision or presence of random (noise) variation from studies included in the present systematic review (Carlson and Morrison, 2009).

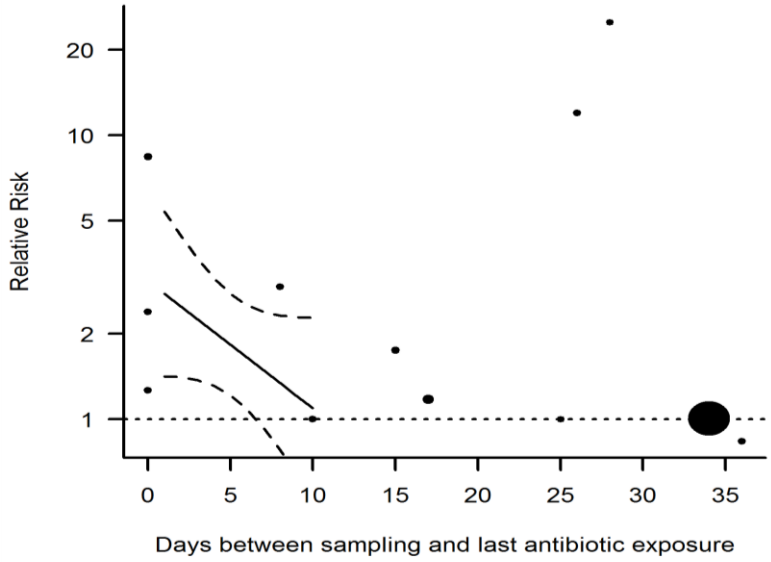

Figure 5.6. Relative risk of antibiotic versus time (days) between last exposure and collection of fecal samples (dotted lines represent 95% confidence interval).

Based on the meta-analyses performed, cattle exposed to antibiotics were more likely to have resistant isolates compared to unexposed cattle. This was based on the observation that resistance isolates were found in control cattle to a lesser extent than in treated cattle. However, antibiotic resistance was measured as the proportion of resistant isolates among all isolates in each group (control and treated). Schechner et al. (2013) reported that an increase in the proportion of isolates that are resistant may not necessarily correspond with an increase in the absolute numbers of resistant bacteria, particularly when the entire community of bacteria (i.e., the microbiome) is considered. It is known that antibiotics significantly reduce the number of susceptible isolates in a microbial community, which can lead to a rise in the proportion of resistant isolates even if the number of resistant isolates remains unchanged (Schwaber et al., 2004).

Final considerations

Results from the present systematic review can be used as an input to better understand relationships between AMU and AMR in feedlot cattle, but correct interpretation and context is required. Some studies (Inglis et al., 2006; Alexander et al., 2008; Sharma et al., 2008; Jacobs et al., 2008; Zaheer et al., 2013; Agga et al., 2016) used selective procedures during enrichment and culture in order to recover rare resistant organisms in the feces of feedlot cattle. Although selective enrichment and plating can provide a better understanding of the resistant population, it overestimates resistant organisms in the overall bacteria population, and therefore cannot be used to estimate the amount of resistant bacteria entering the food supply in more comprehensive risk assessments (Tragesser et al., 2006). To establish the public health consequences of AMU in feedlot animals was beyond the objectives of this study. To accomplish that broader goal, Tragesser et al. (2006) identified missing data, such as the likelihood of ARGs from feedlots entering the food supply, the frequency that those ARGs are ingested by consumers, and the probability that consumers develop undesirable health outcomes due to ARGs derived from feedlot cattle treated with antimicrobials. Finally, while AMR may occur, advantages of using antibiotics in feedlot cattle may outweigh disadvantages in aspects such as animal health, animal welfare, use of critically important antibiotics in food-producing animals, food safety, food costs, length of feeding, and environmental contamination.

In summary, we found evidence of an association between antibiotic use and antimicrobial resistance, especially for *E. coli* and *Enterococcus* isolated from feedlot cattle treated with tetracycline and macrolides (tylosin), respectively. Results can be used as important information to address the complex relationship between antibiotic use and antibiotic resistant bacteria prevalence in cattle, but more comprehensive studies that consider the relationship

between antibiotic use in cattle and antibiotic resistant bacteria in humans are needed as a part of a farm to fork approach to tackle antimicrobial resistance.

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APPENDIX

Appendix 1. Antimicrobial usage in early feeding pens (n = 8) in the conventional feedlot.

	Days on feeding and (animals/pen) at the time of sampling							
	1 (274)	5 (262)	5 (268)	8 (264)	8 (276)	22 (282)	29 (270)	29 (284)
Antimicrobial ingredient (Dose equivalent)	**Percentage of animals treated since the beginning of feeding**							
Monensin sodium (25 mg/kg diet DM[1])	100	100	100	100	100	100	100	100
Chlortetracycline (35 mg/kg diet DM)	100	100	100	100	100	100	100	100
Ceftiofur sodium (1.0 mg/kg BW[2])	0	0.4	0	5.3	0	0.35	1.1	3.5
Florfenicol (40 mg/kg BW)	6.2	0	0	2.3	6.5	0	0	1.8
Oxytetracyline (20 mg/kg BW)	97.8	100	100	100	98.5	100	100	100
Tulathromycin (2.5 mg/kg BW)	0	0	0	0	0	0	0	0.7
Trimethoprin (2.67 mg/kg BW), Sulfadoxine (13.33 mg/kg BW)	0	0	1.1	4.5	2.2	1.1	0	2.1
Sulfanilamide (73.13 mg/kg BW), Sulfathiazole (73.13 mg/kg BW), Sulfamethazine (48.75 mg/kg BW)	0	0	0	0.38	0	0	0	0

[1]DM: Dry Matter

[2]BW: Body Weight

Appendix 2. Antimicrobial usage in late feeding pens (n = 8) in the conventional feedlot.

	Days on feeding and (animals/pen) at the time of sampling							
	181 (93)	215 (206)	233 (272)	240 (284)	244 (45)	246 (270)	286 (83)	301 (261)
Antimicrobial ingredient and dose equivalent	**Percentage of animals treated since the beginning of feeding**							
Monensin sodium, 25mg/kg DM[1]	100	100	100	100	100	100	100	100
Chlortetracycline, 35 mg/kg DM	100	100	100	100	100	100	100	100
Chlortetracycline, 1 g/head/d	100	0	0	100	100	100	100	100
Chlortetracycline, 6 g/head/d	100	0	0	100	100	100	100	100
Ceftiofur sodium,1.0 mg/kg BW[2]	8.6	21.8	25.4	23.6	6.7	20.4	10.8	23.0
Ceftiofur crystalline free, 6.6 mg/kg BW	0	0	1.5	1.8	4.4	3.3	9.6	7.7
Enrofloxacin, 7.7 mg/kg BW	0	0	0	0	0	0	3.6	0.38
Florfenicol, 40 mg/kg BW	9.7	0	0	0.35	0	1.1	6.0	5.4
Oxytetracyline, 20 mg/kg BW	8.6	2.4	9.2	3.2	48.9	35.6	57.8	10.3
Oxytetracyline, 30 mg/kg BW	92.4	0	0	0	0	0	1.2	0
Oxytetracyline ,6.67 mg/kg BW	9.7	0	0	0	0	0	0	0
Tylosin tartrate, 29 mg	100	100	100	100	100	100	100	100
Tulathromycin, 2.5 mg/kg BW	1.1	100	0.37	100	100	100	100	100
Trimethoprin (2.67 mg/kg BW) and Sulfadoxine (13.33 mg/kg BW)	6.5	2.9	8.8	12.7	0	5.5	7.2	10.3

[1]DM: Dry Matter

[2]BW: Body Weight.

Appendix 3. Antimicrobial usage in low and high producing pens in the conventional dairy farm.

			Number of cows treated[1]	
Antimicrobial	**Class**	**Dose equivalent**	**Low producing pen**	**High producing pen**
Ceftiofur crystalline free acid	Beta-lactam	6.6 mg/kg BW[2]	16	23
Ceftiofur hydrochloride	Beta-lactam	2.2 mg/kg BW	21	16
Ceftiofur hydrochloride	Beta-lactam	125 mg per quarter	15	21
Ampicillin	Beta-lactam	6.0 mg/kg BW	90	32
Number of treatments within 60 days of sampling			13	35

[1] Number of cows presents in the pen at the time of sampling and treated with the corresponded drug at least one year before our sampling date.

[2]BW: Body Weight

Appendix 4. Groups of resistance found only in conventional samples.

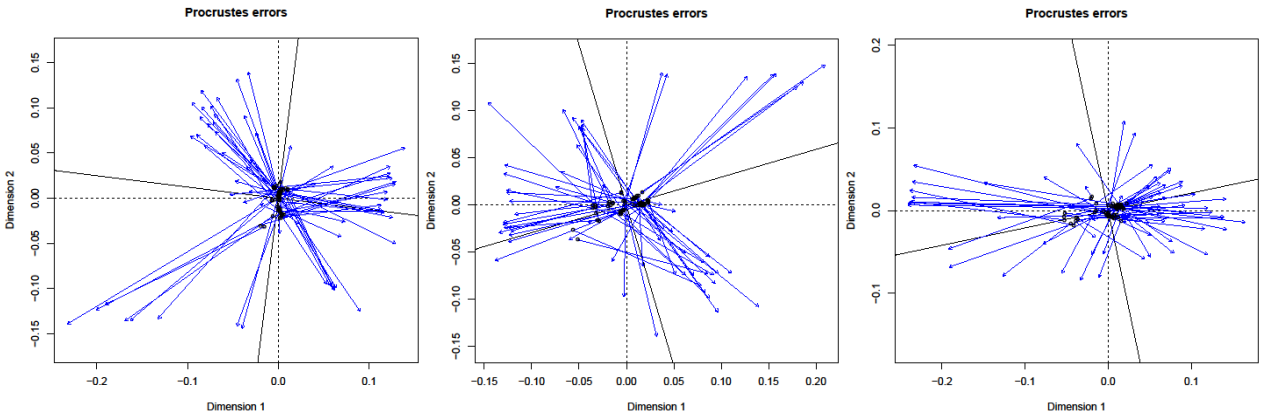
Resistance		Positive samples		
Group	Class	Feces[1]	Wastewater[2]	Soil[3]
ANT4-PRIME	Aminoglycosides	1	0	0
APH6-PRIME	Aminoglycosides	2	0	0
PME	Beta-lactams	2	0	0
RAHN	Beta-lactams	0	1	0
CPS	Beta-lactams	0	4	0
LRA	Beta-lactams	0	0	1
BJP1	Beta-lactams	0	0	1
OXA	Beta-lactams	18	5	0
FOSK	Fosfomycin	4	0	0
BRP	Glycopeptide	1	0	0
VANHA	Glycopeptide	0	0	1
MEXV	Multi-drugs	0	0	1
OQXA	Multi-drugs	0	6	0
OQXB	Multi-drugs	0	8	0
MEXH	Multi-drugs	0	0	1
MEXC	Multi-drugs	0	0	1
ROBA	Multi-drugs	0	1	0
ERM	MLS[4]	8	7	2
ERMS	MLS	0	0	1
ERMT	MLS	9	0	0
OLEI	MLS	0	0	1
OLEB	MLS	0	0	2
CARA	MLS	0	0	3
LNUF	MLS	3	13	0
SULI	Sulfonamides	6	1	0
TET31	Tetracyclines	1	1	0
TETG	Tetracyclines	2	0	0
TETS	Tetracyclines	1	0	0
DFRF	Trimethoprim	2	1	0

[1] Out of 32 fecal samples
[2] Out of 16 wastewater samples
[3] Out of 16 fecal samples
[4] Macrolide-Lincosamide-Streptogramin

Appendix 5. Ten most abundant unique groups of resistance in the different types of samples based on the amount of normalized reads (ordered from more to less abundance).

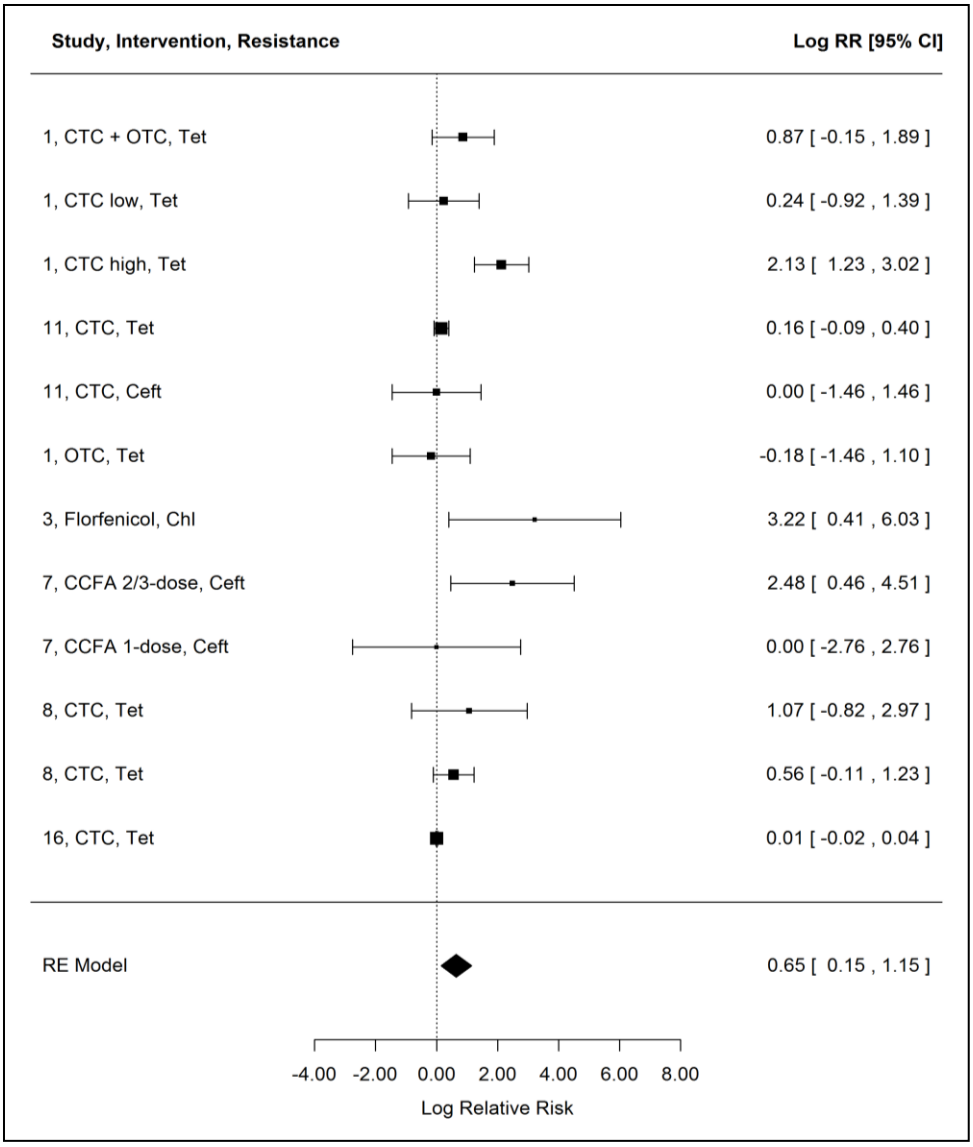
Unique for feces		Unique for wastewater		Unique for soil	
Group[1]	Class	Group[1]	Class	Group[1]	Class
CFX	Beta-lactam	TET39	Tetracycline	CEOB	Multi-drug
MDTC	Multi-drug	OQXB	Multi-drug	RPH	Rifampin
ACRF	Aminoglycoside	MEXT	Multi-drug	VANRO	Glycopeptide
PMRC	CAP[1]	OQXA	Multi-drug	NOVA	Aminocoumarin
EVGS	Multi-drug	CPS	Beta-lactams	TLRC	MLS[2]
MDTB	Multi-drug	EREA	MLS[2]	MEXB	Multi-drug
ACRD	Aminoglycoside	ROBA	Multi-drug	DRRA	Multi-drug
MDTO	Multi-drug	RAHN	Beta-lactam	MEXQ	Multi-drug
MDTP	Multi-drug			CEOA	Multi-drug
PBP2	Beta-lactam			MEXN	Multi-drug

[1] CAP: cationic antimicrobial proteins
[2] MLS: macrolide-lincosamide-streptogramin

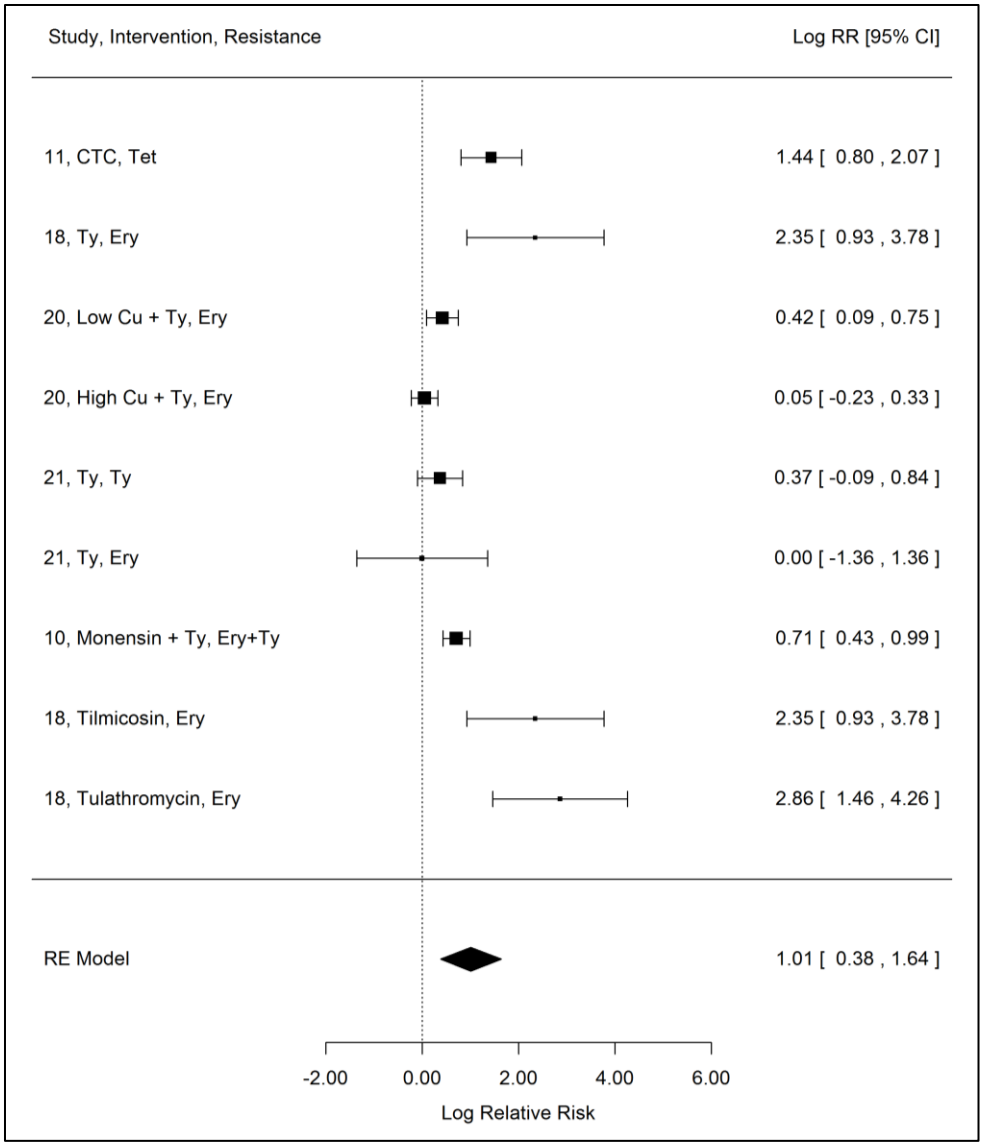


(a) (b) (c)

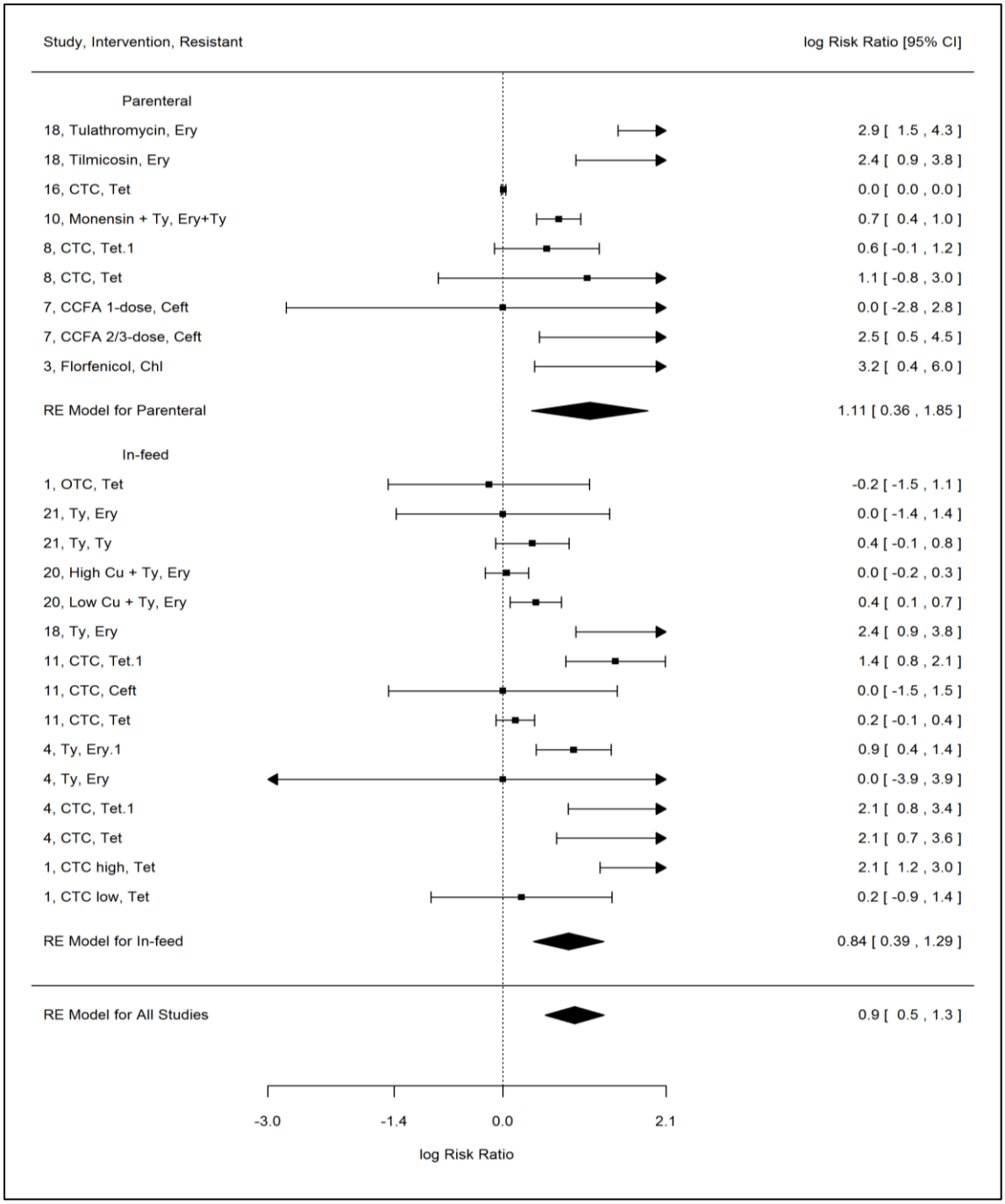
Appendix 6. Procrustes analysis showing the correlation between the resistome (class level) and microbiome (phylum) in samples collected from (a) dairy farms ($P < 0.05$), (b) conventional farms ($P < 0.05$) and (c) feces ($P > 0.05$). Lines connect the same sample in the microbiome and resistome. Shorter distances between the same samples mean higher correlation between the two ordinations.



Appendix 7. Relative risk (log scale) for antibiotic-resistance in *E. coli* isolates collected from feedlot cattle exposed and unexposed to antibiotics using a random effect model.). See Table 5.1 for a description of studies and interventions. CCFA: ceftiofur crystalline-free acid, CTC: chlortetracycline, OTC: oxytetracycline, Ceft: ceftiofur, Chl: chloramphenicol, Tet: tetracycline.



Appendix 8. Relative risk (log scale) for antibiotic-resistance in *Enterococcus* isolates collected from feedlot cattle exposed and unexposed to antibiotics using a random effect model. See Table 5.1 for a description of studies and interventions. CCFA: ceftiofur crystalline-free acid, CTC: chlortetracycline, OTC: oxytetracycline, Ceft: ceftiofur, Chl: chloramphenicol, Tet: tetracycline.



Appendix 9. Relative risk (log scale) for antibiotic-resistance in bacteria isolates collected from feedlot cattle exposed and unexposed to antibiotics using a random effect model discriminated by route of administration of antibiotics (in-feed or parenteral). See Table 5.1 for a description of studies and interventions. CCFA: ceftiofur crystalline-free acid, CTC: chlortetracycline, OTC: oxytetracycline, Ceft: ceftiofur, Chl: chloramphenicol, Tet: tetracycline.