Metagenomic analysis of antibiotic resistance genes in the fecal microbiome following therapeutic and prophylactic antibiotic administration in dairy cows

Lindsey Renee Caudle

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Committee

Katharine F. Knowlton, Chair

Amy J. Pruden

Michael S. Strickland

Robert M. Akers

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Keywords: shotgun metagenomic sequencing, antibiotic resistance, dairy cow

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ABSTRACT

The use of antibiotics in dairy cattle has the potential to stimulate the development and subsequent fecal dissemination of antibiotic resistance genes (ARGs) in bacteria. The objectives were to use metagenomic techniques to evaluate the effect of antibiotic treatment on ARG prevalence in the fecal microbiome of the dairy cow and to determine the temporal excretion pattern of ARGs. Twelve Holstein cows were assigned to one of four antibiotic treatments: control, pirlimycin, ceftiofur, or cephapirin. Fecal samples were collected on d -1, 1, 3, 5, 7, 14, 21, and 28. Samples were freeze-dried and subjected to DNA extraction followed by Illumina paired-end HiSeq sequencing and quantitative polymerase chain reaction (qPCR). Illumina sequences were analyzed using MG-RAST and the Antibiotic Resistance Gene Database (ARDB) via BLAST. Abundance of *ampC*, *ermB*, *tetO*, *tetW*, and 16S rRNA genes were determined using qPCR. All data were statistically analyzed with PROC GLIMMIX in SAS. Antibiotic treatment resulted in a shift in bacterial cell functions. Sequences associated with "resistance to antibiotics and toxic compounds" were higher in ceftiofur-treated cows than control cows. Ceftiofur-treated cows had a higher abundance of β-lactam and multidrug resistance sequences than control cows. There was no effect of treatment or day on fecal *tetO* and *ermB* excretion. The relative abundances of *tetW* and *ampC* were higher on d 3 post-treatment than d 5 and d 28. In conclusion, antibiotic use in dairy

cattle shifted bacterial cell functions and temporarily increased antibiotic resistance in the fecal microbiome.

Keywords: shotgun metagenomic sequencing, antibiotic resistance, dairy cow

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TABLE OF CONTENTS

ABSTRACT ..... **ii**

ACKNOWLEDGEMENTS ..... **iv**

TABLE OF CONTENTS ..... **vii**

LIST OF TABLES ..... **x**

LIST OF FIGURES ..... **xi**

Chapter 1: INTRODUCTION **1**

Chapter 2: REVIEW OF LITERATURE **4**

ANTIOBIOTIC USE IN THE DAIRY INDUSTRY AND ASSOCIATED PROBLEMS **4**

MECHANISMS OF ANTIBIOTIC RESISTANCE **5**

Cephalosporin Resistance 7

Lincosamide Resistance 9

Ionophore Resistance 10

BOVINE ANTIBIOTIC METABOLISM AND RESIDUE EXCRETION **11**

Metabolism and Excretion of Cephalosporins 11

Metabolism and Excretion of Lincosamides 13

METHODS FOR MEASURING ANTIBIOTIC RESISTANCE **13**

Traditional Culture-Based Methods 13

Quantitative Real-Time Polymerase Chain Reaction 15

Metagenomic Sequencing 17

Functional Analysis 21

ANTIBIOTIC RESISTANCE IN BOVINE BACTERIA **24**

Gastrointestinal (GI) Tract 24

Feces 24

ENVIRONMENTAL EFFECTS AND POTENTIAL IMPACT 30

CONCLUSION AND RESEARCH OBJECTIVES 31

REFERENCES 33

Chapter 3: METAGENOMIC ANALYSIS OF ANTIBIOTIC RESISTANCE GENES IN THE FECAL MICROBIOME FOLLOWING THERAPEUTIC AND PROPHYLACTIC ANTIBIOTIC ADMINISTRATION IN DAIRY COWS 45

ABSTRACT 45

INTRODUCTION 46

MATERIALS AND METHODS 48

Animals and Experimental Treatments 48

Antibiotic Administration 49

Sample Collection 50

Metagenomic Analysis 51

Real-Time Quantitative Polymerase Chain Reaction (qPCR) Analysis 54

RESULTS 55

Metagenomic Results: MG-RAST Analysis 55

Metagenomic Results: BLAST Analysis using the ARDB 58

Q-PCR Results 58

DISCUSSION 60

MG-RAST Analysis 60

BLAST/ARDB Analysis 64

qPCR Analysis 66

CONCLUSIONS 70

REFERENCES 105

APPENDIX 112

LIST OF TABLES

Table 1 Summary of responses to antibiotic treatment for each analysis 72

Table 2 MG-RAST analysis: effect of ceftiofur crystalline-free acid antibiotic treatment on bacterial species diversity in dairy cow feces 73

Table 3 MG-RAST analysis: effect of ceftiofur crystalline-free acid antibiotic treatment on the abundance of bacterial cell functions in dairy cow feces 74

Table 4 MG-RAST analysis: effect of ceftiofur crystalline-free acid antibiotic treatment on the abundance of "phages, prophages, transposable elements, plasmids" sequences in dairy cow feces 83

Table 5 MG-RAST analysis: effect of ceftiofur crystalline-free acid antibiotic treatment on the abundance of "virulence, disease, and defense" sequences in dairy cow feces 85

Table 6 BLAST/ARDB analysis: effect of ceftiofur crystalline free acid antibiotic treatment on the abundance of resistance gene sequences in dairy cow feces 88

Table 7 BLAST/ARDB analysis: effect of ceftiofur crystalline free acid antibiotic treatment on the proportion of resistance gene sequences in dairy cow feces.....91

Table 8 qPCR analysis: effect of antibiotic treatment on antibiotic resistance gene absolute abundance in dairy cow feces.....93

Table 9 qPCR analysis: effect of antibiotic treatment on antibiotic resistance gene relative abundance in dairy cow feces.....96

LIST OF FIGURES

Figure 1. MG-RAST analysis: abundance of "cell division and cell cycle" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. 78

Figure 2. MG-RAST analysis: abundance of "clustering-based subsystems" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment.. 79

Figure 3. MG-RAST analysis: abundance of "metabolism of aromatic compounds" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. 80

Figure 4. MG-RAST analysis: abundance of "secondary metabolism" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment..... 81

Figure 5. MG-RAST analysis: abundance of "stress response" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. 82

Figure 6. MG-RAST analysis: abundance of "pathogenicity islands" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment..... 84

Figure 7. MG-RAST analysis: abundance of "resistance to antibiotics and toxic compounds (RATC)" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. 86

Figure 8. BLAST/ARDB analysis: abundance of antibiotic resistance gene (ARG)-like sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. 87

Figure 9. BLAST/ARDB analysis: abundance of beta-lactam resistance genes in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment..... 89

Figure 10. BLAST/ARDB analysis: abundance of multidrug resistance genes in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment..... 90

Figure 11. BLAST/ARDB analysis: abundance of beta-lactam resistance genes in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment..... 92

Figure 12. qPCR analysis: abundance of 16S rRNA (log10 gene copies per 1.0 g freeze-dried feces) in fecal samples. 95

Figure 13. qPCR analysis: (A) Abundance of *tetO* (log10 gene copies per 1.0 g freeze-dried feces) in fecal samples. (B) Relative abundance of *tetO* (gene copies/ 16S rRNA) in fecal samples collected..... 98

Figure 14. qPCR analysis: (A) Abundance of *tetW* (log10 gene copies per 1.0 g freeze-dried feces) in fecal samples. (B) Relative abundance of *tetW* (gene copies/ 16S rRNA) in fecal samples collected..... 100

Figure 15. qPCR analysis: relative abundance of *ampC* (gene copies/ 16S rRNA) in collected fecal samples.. 102

Figure 16. qPCR analysis: (A) Abundance of *ermB* (log10 gene copies per 1.0 g freeze-dried feces) in fecal samples. (B) Relative abundance of *ermB* (gene copies/ 16S rRNA) in collected fecal samples 103

Chapter 1: INTRODUCTION

Even at an early stage, the development of antibiotic resistance was recognized as a potential problem of antibiotic use. Alexander Fleming, upon his discovery of penicillin, said "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them" (Fleming, 1945). Today, the resistance problem continues to grow. Gram-negative bacterial isolates were recovered from ICU patients all over the United States from 1994 – 2000 and, in that time span, susceptibility to several antibiotics were reduced including ciprofloxacin by 10% and gentamicin by 6% (Neuhauser et al., 2003). Antibiotic resistance is clearly on the rise and must be addressed.

In addressing this global problem, attention has fallen on the animal industry and its use of antibiotics. In 2011, the FDA reported that 3.29 million kg of antibiotics were sold for human pharmaceutical purposes while 13.5 million kg of antibiotics were sold for use in domestic livestock (FDA, 2011;2012). Therefore, it is estimated that of the total antimicrobials sold annually, roughly 80% is used by the livestock industry. This usage of antibiotics has implications for soil and water microbiomes through effects of excreted antibiotics (Sarmah et al., 2006) as well as the dissemination of antibiotic resistance from livestock fecal bacteria.

The three main mechanisms of antibiotic resistance transfer include (1) conjugation, which occurs via plasmids, (2) transformation, which is the uptake of naked DNA, and (3) transduction, which is the transfer of resistance genes via a bacteriophage (Barbosa and Levy, 2000). Resistance genes coding for resistance mechanisms to β-lactams [i.e. altering penicillin binding proteins or producing β-lactamases (McDermott

et al., 2003)] or to macrolide-lincosamide-streptogramins [i.e. obtaining an rRNA methylase which inhibits drug binding to the 50S ribosomal subunit (Roberts, 2004)] can be spread rapidly between bacteria.

Previously, cultured-based methods like agar disc diffusion tests and dilution-based tests (Schmieder and Edwards, 2012) were used to identify antibiotic resistant bacterial isolates. However, newer quantitative methods like qPCR (Steffan and Atlas, 1991) can analyze for specific resistance genes present in the environmental sample, not just in culturable bacteria. Today, metagenomic methods of analysis like 454 pyrosequencing (Sirohi et al., 2012) and Illumina sequencing (Fox et al., 2009) allow for the study of entire bacterial metagenomes. Metagenomic methods are enhanced by functional analysis of the sequencing output via databases like BLAST, ARDB, the SEED, and MG-RAST.

The effect of antibiotic treatment on cattle fecal microbiomes has been a topic of study for quite some time yielding results both consistent with and contradictory to any directly linked hypotheses. For instance, fecal bacteria from conventionally-raised cows tend to be more resistant to antibiotics than that from cows raised organically (Sato et al., 2005; Halbert et al., 2006) but the increase in resistance in fecal bacteria of cows treated with antibiotics is only transient (Tragesser et al., 2006; Singer et al., 2008). Even cattle never exposed to antibiotics shed antibiotic resistant bacteria (Durso et al., 2011; Thames et al., 2012). Finally the link between feeding antibiotics to cattle and fecal antibiotic resistance is weak, with conflicting results (Inglis et al., 2005; Edrington et al., 2006; Jacob et al., 2008; Mirzaagha et al., 2011). What is known is that runoff from farms contain higher levels of antibiotic resistance genes than other water sources (Santamaría

et al., 2011) and that land applying contaminated cattle feces to vegetable plots results in resistance genes making their way into and on produce (Marti et al., 2013). Further research is necessary to complete the picture of antibiotic use on antibiotic resistance to determine how to appropriately prevent the dissemination of those resistance genes.

Chapter 2: REVIEW OF LITERATURE

ANTIOBIOTIC USE IN THE DAIRY INDUSTRY AND ASSOCIATED PROBLEMS

Antibiotics are compounds with antimicrobial properties and can be synthetic, semi-synthetic, or found naturally in the environment (Phillips et al., 2004). Resistance to these compounds is a natural occurrence, developed by bacteria to aid in their survival against other antibiotic-producing microorganisms in the environment (Phillips et al., 2004). However, it wasn't until Alexander Fleming accidentally discovered penicillin in 1928 that this natural relationship between bacteria (and other organisms) began to play a role in human and animal health.

Antibiotics are commonly used for therapeutic and prophylactic purposes in livestock, as well as for growth promotion. In 2000, a survey by the Animal Health Institute revealed that in 1998, roughly 8 million kg of antimicrobials were used in the U.S. livestock industry, with 6.6 million kg of that being for prophylactic and therapeutic use (McEwen and Fedorka-Cray, 2002). In 2011, the FDA reported that 3.29 million kg of antibiotics were sold for human pharmaceutical purposes while 13.5 million kg of antibiotics were sold for use in domestic livestock (FDA, 2011;2012). Therefore, it is estimated that of the total antimicrobials sold annually, roughly 80% is used by the livestock industry. The FDA further reported that 5.6 million kg of the antibiotics distributed to livestock were tetracyclines, making it the most widely-used drug class (FDA, 2011).

In the dairy industry, common antibiotic uses involve the treatment or prevention of diarrhea and pneumonia in dairy calves, as well as the prevention (dry-cow therapy) or

treatment of mastitis in dairy cows (McEwen and Fedorka-Cray, 2002). Unlike in the beef industry, lactating dairy cows receive few antimicrobials through their feed (McEwen and Fedorka-Cray, 2002). The two most common types of antibiotics used on conventional dairy farms are penicillins and cephalosporins, as these are the primary choices for treatment of respiratory disease, mastitis, metritis, and foot problems (Zwald et al., 2004).

While antibiotics used as growth promoters in livestock are not used in human medicine, those used for disease treatment and prevention often are from the same drug classes as antibiotics used in human medicine (Phillips et al., 2004). The broad use of these classes of antibiotics has resulted in disease treatment problems in the human health sector. Antibiotic resistance first became a problem in the 1950s with the emergence of penicillin resistance in hospitals across the world, followed by gentamicin resistance in the 1970s (O'Brien, 2002). Soon after, resistance to amikacin, sulfonamides, and the "last-resort" drug, vancomycin, followed (O'Brien, 2002). However, the contribution of animal antibiotic use to this growing resistance problem in human medicine is still in question.

MECHANISMS OF ANTIBIOTIC RESISTANCE

An organism is resistant to an antibiotic when it is capable of surviving and reproducing upon exposure. There are three main categories of biochemical mechanisms by which resistance occurs: inactivating the antibiotic compound, preventing high cellular levels of the antibiotic via drug efflux pumps or reduced permeability, and altering the antibiotic cell target (Pehrsson et al., 2013). Resistance to a specific antibiotic

can be due to a single resistance mechanism or a combination of these mechanisms utilized by the cell (Barbosa and Levy, 2000).

There are two general types of mechanisms by which bacteria can obtain antibiotic resistance: intrinsic and acquired (Alekshun and Levy, 2007). Intrinsic resistance comes from antibiotic resistance genes (ARGs) naturally found on the bacterium's chromosome while acquired resistance comes from the transfer or mutation of genes targeted by an antibiotic (Alekshun and Levy, 2007). Intrinsic resistance is spread vertically between mother and daughter cells through proliferation, while acquired resistance is spread horizontally between different bacteria through a variety of transfer mechanisms (Licht and Wilcks, 2006).

Conjugation is the most common transfer mechanism for acquired resistance and involves the exchange of either chromosomal or extra-chromosomal DNA via cell-to-cell contact (Barbosa and Levy, 2000). Plasmids are extra-chromosomal, double-stranded, circular DNA that often carry multiple resistance genes (O'Brien, 2002). Transposons are chromosomal DNA segments that are cut from the chromosome and transferred to new bacterial chromosome where it is integrated (Licht and Wilcks, 2006). While plasmids are capable of independent replication, transposons instead depend on replication of the host chromosome (Licht and Wilcks, 2006). Both plasmids and transposons allow for the rapid and relatively easy spread of antibiotic resistance among all types of bacteria.

Other mechanisms of acquired resistance transfer include transformation and transduction. Transformation occurs when a bacterium takes up "naked" DNA from its environment (Barbosa and Levy, 2000). This DNA most often comes from the death and lysis of another bacterium, which releases DNA into the environment (McAllister et al.,

2001). Transduction occurs when a bacterium obtains new DNA via infection by a bacteriophage (Barbosa and Levy, 2000). Although these transfer mechanisms are not as common as conjugation, they still play a role in the movement of resistance genes.

Whether intrinsic or acquired, the genes that produce antibiotic resistance within a bacterium through direct or indirect means make up the antibiotic "resistome" (Wright, 2012). This "resistome" includes the "housekeeping" genes that, through mutation or overexpression, produce resistance (Wright, 2012). Therefore, these "housekeeping" genes are considered "proto-resistance" elements (Wright, 2012). This antibiotic "resistome" serves as a resistance gene database to which all bacteria have access.

Cephalosporin Resistance

First- (e.g. cephapirin, cefazolin) and second-generation (e.g. cefuroxime, cefotetan) cephalosporins are referred to as narrow-spectrum cephalospsorins (Jacoby and Munoz-Price, 2005; Seiffert et al., 2013). Third- (e.g. ceftriaxone, ceftiofur) and fourth-generation (e.g. cefepime, cefpirome) cephalosporins are called extended-spectrum cephalosporins (Seiffert et al., 2013). However, all cephalosporin generations fall into the β-lactam drug class (Seiffert et al., 2013).

Cephalosporins, like other β-lactams, function by attacking synthesis of peptidoglycan (or murein), a cell wall component that all bacteria possess with higher levels present in Gram-positive than Gram-negative bacteria (McDermott et al., 2003; Seiffert et al., 2013). There are four major events that occur during peptidoglycan synthesis: (1) cell wall precursors are synthesized in the cytoplasm; (2) precursors are bound to a lipid and then taken across the cytoplasmic membrane; (3) pre-formed glycan components are incorporated into the cell wall; and finally (4) transpeptidation bonding

and subsequent maturation (McDermott et al., 2003). Cephalosporins inhibit peptidoglycan synthesis at stage 4 by blocking the transpeptidation reaction through binding with penicillin-binding proteins (PBPs) (Nikolaidis et al., 2014). PBPs are enzymes that catalyze the process of cross-linking peptides, or transpeptidation (Nikolaidis et al., 2014).

As is true of resistance to other β-lactams, there are four different mechanisms through which bacterial resistance to cephalosporins occurs: (1) altering of the PBP's active site to which the drug binds or acquiring new PBPs that are less susceptible; (2) producing β-lactamases that hydrolyze the β-lactam ring, inactivating the drug; (3) preventing the drug from reaching its target site by changing cell wall porins; and (4) actively pumping the drug out of the cell (McDermott et al., 2003). The mechanisms of altering cell wall porins and developing a drug efflux system both may lead to multiple drug resistance since other drugs may also enter the cell via the same porins or be expelled from the cell via the same efflux system (McDermott et al., 2003).

The major gene class that confers resistance to extended-spectrum cephalosporins (and other β-lactams) is the *bla* class of genes, which encodes for β-lactamases (Seiffert et al., 2013). Another gene that encodes for β-lactamases is *ampC,* but this gene only confers resistance to third-generation cephalosporins (Seiffert et al., 2013). The *bla* genes are often found on mobile genetic elements such as plasmids, transposons, or integrons, while *ampC* tends to be found on either plasmids (*pampC*) or the bacterial chromosome (*campC*) (Seiffert et al., 2013). In the animal industry, the bla_{ESBL} genes encoding CTX-M-types are the most common agents of extended-spectrum cephalosporin resistance with CTX-M-1, -14, and -15 enzyme types being predominant in cattle in the U.S.

(Seiffert et al., 2013). However, the *bla*$_{CMY-2}$ gene, a plasmid-encoded β-lactamase that functions similar to *AmpC*, accounts for roughly 95% of detected *bla* genes in American cattle giving it more interest than the CTX-M-types (Alcaine et al., 2005; Seiffert et al., 2013).

Lincosamide Resistance

Lincosamides are a class of antibiotics that operate by inhibiting bacterial protein synthesis (Roberts, 2004). Protein synthesis is halted when lincosamides bind to tRNA at the 50S subunit preventing transpeptidation, causing tRNA to break away from the ribosome (McDermott et al., 2003). Affinity for the 50S subunit binding site is shared by other antimicrobial classes like macrolides and streptogramin B even though they all possess a different structure (Roberts, 2004). Since they function similarly, these antimicrobials are often linked together and called the Macrolide-Lincosamide-Streptogramin (MLS) group (Roberts, 2004).

Resistance to MLS antibiotics most commonly results when a bacterium obtains an rRNA methylase that inhibits the ability of the MLS to bind to the 50S subunit on the bacterial ribosome (Roberts, 2004). The group of genes responsible for 30 different rRNA methylases is the *erm* genes (Roberts, 2004). The *ermF* gene can actually be found in 20 different genera and both *ermF* and *ermB* are known to be linked to *tet* genes, encoding for tetracycline resistance (Roberts, 2004).

Another mechanism of MLS resistance involves protein transporters that act like an efflux system and pump the incoming antibiotic back out of the cell allowing ribosome function to remain unaltered (Roberts, 2004). There are several types of transporters responsible for this phenomenon including ATP transporters, facilitator transporters, and

eight different transferases (Roberts, 2004). A wide variety of genes code for ATP transporters, with each bacterial genus having its own set of genes, while the genes encoding for facilitator transporters (*lmrA*, *mefA*) and transferases (*lnu*, *vat*) are more specific (Roberts, 2004).

Ionophore Resistance

Ionophores are antimicrobials administered through cattle feed, originally used to treat or prevent intestinal parasites but now with the intention of growth promotion (Callaway et al., 2003). The ionophores fed to cattle belong to the carboxylic group, also known as polyether antibiotics, and are further split into monovalent or divalent polyether categories based on how they transport monovalent (e.g. K^+, Na^+) or divalent (e.g. Ca^{2+}, Mg^{2+}) cations (Butaye et al., 2003). Monensin (Rumensin™), a monovalent polyether antibiotic produced by *Streptomyces cinnamonensis*, exchanges H^+ for either extracellular Na^+ or intracellular K^+, leading to interference with ion gradients and ATP hydrolysis and eventually causing bacterial cell death (Butaye et al., 2003; Callaway et al., 2003). Lasalocid, produced by *Streptomyces lasaliensis*, is another polyether antibiotic that is instead divalent, preferring to transport Ca^{2+} and K^+ (Westley, 1977). Although different in structure, monensin and lasalocid function similarly in their effect on weight gain and feed efficiency in cattle (Berger et al., 1981).

Bacteria become resistant to ionophores via different mechanisms such as preventing the binding of the ionophore to the bacterial cell wall or by reducing cell membrane porin size to prevent ionophore entry (Callaway et al., 2003). Development of ionophore resistance can occur naturally without ionophore exposure, but mainly develops in the presence of ionophore selection pressure (Dawson and Boling, 1983;

Houlihan and Russell, 2003). Ionophore resistance has not been linked to any genetic element so transfer of resistance between bacteria is unlikely (Lana and Russell, 1996; Houlihan and Russell, 2003). Lastly, bacterial resistance to ionophores does not appear to result in additional resistance to therapeutic antibiotics as they have different mechanisms of action for inhibiting bacterial growth or causing bacterial death (Houlihan and Russell, 2003).

BOVINE ANTIBIOTIC METABOLISM AND RESIDUE EXCRETION

When an antibiotic is administered to an animal it is not fully absorbed, resulting in residues being excreted in either feces or urine. In fact, from a single antibiotic dose up to 90% may be excreted through urine; other antibiotics are excreted via feces (up to 75%) (Sarmah et al., 2006). Antibiotics may be excreted as the parent compound or as metabolites, but the distinction makes little difference as metabolites may be converted back into the parent compound upon excretion (Sarmah et al., 2006). These excreted antibiotics can persist in the environment and may contribute to the maintenance and development of antibiotic resistance as environmental bacteria are exposed to these compounds (Sarmah et al., 2006). Therefore, antibiotic metabolism, excretion, and environmental degradation are important.

Metabolism and Excretion of Cephalosporins

Cephalosporin antibiotics are semisynthetic and are adapted from cephalosporin C, a compound naturally produced by *Cephalosporium acremonium* (Alcaine et al., 2005). There are a variety of cephalosporin forms that can be administered either intramammary (e.g. cephapirin), or intramuscularly and subcutaneously (e.g. ceftiofur crystalline free acid sterile suspension or CCFA-SS).

In cattle, CCFA-SS is routinely given behind the ear and is quickly metabolized upon injection (Washburn et al., 2005; Volkova et al., 2012). When metabolized by the body, ceftiofur becomes desfuroylceftiofur, which remains active because the β-lactam ring is maintained (Washburn et al., 2005; Volkova et al., 2012). It has been shown that subcutaneous administration of ceftiofur in beef calves results in blood concentrations that remain at or above 0.2 μg/mL for up to 8 days post-administration (Washburn et al., 2005). When excreted in cattle, roughly 65% of ceftiofur and desfuroylceftiofur exit through the urine, while roughly 35% does so through the feces (Volkova et al., 2012). About 29% of intramuscularly injected ceftiofur was detected in cattle feces 8 hours post-injection, with 37% detected after 12 hours (Volkova et al., 2012). The majority of ceftiofur recovered in feces does not show any kind of antimicrobial activity even though the ceftiofur is believed to cross over the wall of the large intestine with its β-lactam ring still intact (Volkova et al., 2012).

The first-generation cephalosporin, cephapirin, is routinely given intramammary for the purpose of treating or preventing mastitis (dry cow therapy) (Pol and Ruegg, 2007). When two doses of 275 mg cephapirin sodium were given per quarter at 12-hour intervals, both cephapirin and its metabolite, desacetyl-cephapirin, were detected in the milk of healthy and mastitic cows for up to 48 hours post-administration of the second dose (Cagnardi et al., 2014). Since cephapirin is highly ionized and has low lipid solubility, it is not very capable of reaching deeper udder tissues and blood (Gehring and Smith, 2006). Therefore, most cephapirin excretion is via milk.

Metabolism and Excretion of Lincosamides

Modern lincosamide antibiotics are semisynthetic derivatives of the naturally produced lincomycin, including clindamycin and pirlimycin (Morar et al., 2009). These antibiotics are effective against Gram-positive bacteria but can be used against some Gram-negative bacteria and protozoa (Morar et al., 2009). Both clindamycin hydrochloride and pirlimycin hydrochloride are approved for animal use in the United States (FDA, 2014).

When pirlimycin is infused into the cow's udder it is slowly absorbed across the udder membrane/blood barrier with blood concentrations being highest 6 to 12 hours post-treatment (Hornish et al., 1992). What remains unabsorbed in the udder is quickly expelled in the first few milkings following treatment, resulting in roughly 50% of the dose being excreted in the milk as intact pirlimycin (Hornish et al., 1992). Once the absorbed pirlimycin is circulated throughout the vascular system, about 10% of the total dose is excreted through the urine and about 24% through the feces (Hornish et al., 1992). Of the pirlimycin excreted renally, 80% remains intact while 8% is metabolized into pirlimycin sulfoxide (Hornish et al., 1992). Of that excreted via feces, 45% remains intact while only 1.5% is metabolized into pirlimycin sulfoxide (Hornish et al., 1992). Overall, about 89% of the total administered pirlimycin dose is recovered in the milk, urine, and feces (Hornish et al., 1992).

METHODS FOR MEASURING ANTIBIOTIC RESISTANCE

Traditional Culture-Based Methods

The two main traditional methods for studying the antibiotic resistance phenotype in bacteria are the agar disc diffusion test and the dilution-based test (Schmieder and

Edwards, 2012). Both tests involve first isolating the bacteria of interest from an infected human or animal and then evaluating their ability to grow following antibiotic exposure (Cockerill, 1999). In order to accurately perform these tests, the Clinical Laboratory Standards Institute (CLSI), formerly known as the National Committee for Clinical Laboratory Standards (NCCLS), provides standards for media preparation, incubation requirements, and explains how to properly interpret the results (Cockerill, 1999). Standards are provided for aerobic and anaerobic bacteria and for yeasts, and all are updated regularly (Cockerill, 1999).

The agar disk diffusion test involves soaking a paper disc with an antibiotic and placing the disc onto agar that has been seeded with the bacteria of interest (Gavin, 1957). The agar can be prepared a variety of ways, including seeding the entire agar with bacteria or pouring inoculated agar (seed layer) over nutrient agar (base layer) (Gavin, 1957). The antibiotic will diffuse through the agar, producing a zone of inhibition around the paper disc because of its effect on the bacteria (Gavin, 1957; Papich, 2013). The larger the zone of inhibition (diameter that is measured in millimeters) the more susceptible the bacteria are to the antibiotic (Papich, 2013). An ideal zone is one that is well defined and easy to identify and large plates with multiple discs are often used in order to obtain accurate zone measurements (Gavin, 1957). Although an agar disc diffusion test is simple, it is limited to antibiotics that will readily diffuse through agar and the results of one antibiotic cannot be compared to another (Gavin, 1957). And, although the minimum inhibitory concentration (MIC) of a bacterium can be estimated based on the size of the zone of inhibition it should not be used to identify the actual MIC value (Papich, 2013).

A dilution-based test, or microdilution test, is the way to directly obtain the MIC value of a bacterium (Papich, 2013). The MIC is defined as the lowest concentration of an antibiotic that prevents visible bacterial growth (Papich, 2013). The test requires inoculating a multiple wells of a well plate with the bacteria of interest and then adding different dilutions of an antibiotic to the wells as a serial dilution (Papich, 2013). This MIC is determined based on a standard and is reported as μg/mL for the first antibiotic concentration in which the bacteria is unable to grow (Papich, 2013). The level of resistance to an antibiotic can then be determined based on CLSI classifications for MIC values: S, susceptible; I, intermediate; or R, resistant (Papich, 2013).

The advantages of using culture-based methods are that they enable the phenotype to be studied, requiring no extrapolations on the expression of a genotype, and they allow for a comprehensive view of the resistance expressed (Cockerill, 1999). However, the disadvantages are that most bacteria cannot be cultured, the tests are slow as they are based on organism growth, and results may be misleading as these bacteria are tested under artificial conditions (Cockerill, 1999). To overcome these disadvantages, newer genetic-based methods like quantitative real-time polymerase chain reaction and metagenomic sequencing were developed.

Quantitative Real-Time Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method that can detect resistance genes within an environmental sample based on the exponential amplification of a targeted DNA sequence (Steffan and Atlas, 1991). The three stages of PCR include: (1) melting the double-stranded DNA down to single-stranded DNA; (2) annealing primers to the target DNA; and (3) using DNA polymerase to extend the DNA through nucleotide

addition (Steffan and Atlas, 1991). Oligonucleotide primers are used to hybridize to the DNA surrounding a target sequence, enabling the duplication of that specific sequence through PCR (Steffan and Atlas, 1991). It is possible to amplify several target sequences simultaneously through the use of multiple primer sets, a process called multiplex PCR (Steffan and Atlas, 1991).

Quantitative real-time polymerase chain reaction (qPCR) is a form of PCR that allows for quantification during sequence amplification by using fluorescence (Wilhelm and Pingoud, 2003). This reaction relies on a reporter molecule (TaqMan or SYBR Green) that increases its fluorescence level based on the accumulation of PCR product within an amplification cycle (Sirohi et al., 2012). An amplification curve involves three phases: (1) an initial lag phase where there is no product accumulation; (2) an exponential phase; and (3) a plateau phase (Wilhelm and Pingoud, 2003). It is in the exponential phase that the threshold and signal curve must intersect in order for quantification to occur (Wilhelm and Pingoud, 2003). Where the two intersect is called the threshold value (C_T) and this value is used to quantify the number of genes present in the sample based on the number of cycles needed for the signal to reach the threshold (Wilhelm and Pingoud, 2003).

qRT-PCR has become an important tool in the detection and quantification of resistance genes within a bacterial community (Sirohi et al., 2012). However, due to limited knowledge of resistance genes and the subsequent lack of available primers, it can only be implemented to detect the few well-studied genes within a sample, leaving other genes unidentified (Yang et al., 2013).

Metagenomic Sequencing

Metagenomics involves the study of all metagenomes whether bacterial, viral, or fungal, present in an environmental sample (Penders et al., 2013). There are three different approaches for analyzing a metagenome: targeted (PCR-based), functional, and sequenced-based (shotgun) metagenomics (Penders et al., 2013). Targeted metagenomics involves using real-time PCR to analyze samples for known resistance genes (Penders et al., 2013). Functional metagenomics requires placing DNA fragments of interest into a host (ex. *E. coli*) via a vector (e.g. a plasmid), and then testing the transformant for antibiotic resistance expression by plating it on an antibiotic-containing media (Penders et al., 2013). If the transformant grows then its DNA is sequenced (Penders et al., 2013). Lastly, sequenced-based metagenomics involves extracting DNA from a given sample, fragmenting it, randomly sequencing the extracted DNA, and then comparing the sequence output to a database to identify resistance genes (Penders et al., 2013). The two most common techniques used for sequence-based metagenomic sequencing are Roche 454 Pyrosequencing and Illumina GAIIX Sequencing.

Roche 454 Pyrosequencing

Roche 454 pyrosequencing is a technique based on the "sequencing-by-synthesis" principle, meaning a sample is sequenced by monitoring DNA synthesis via bioluminescence (Sirohi et al., 2012). This was the first "next-generation" system to be commercially successful (Liu et al., 2012). Pyrosequencing is, at its core, a revolutionized version of Sanger DNA sequencing, which was developed in the late 1970s (Ahmadian et al., 2006). The Sanger technique was the first to use DNA synthesis as a means of sequencing samples and it involved the incorporation of both

deoxyribonucleotide triphosphates (dNTPs) and dideoxyribonucleotide triphosphates (ddNTPs) used to create DNA fragments (Ahmadian et al., 2006). These fragments were then separated by size and analyzed using gel electrophoresis (Ahmadian et al., 2006). In contrast, pyrosequencing is performed by sequentially adding nucleotides to a primed template, thus determining the sequence of the template based on the order of the synthesized complementary DNA strand (Ahmadian et al., 2006).

The first step in the enzymatic pyrosequencing reaction is emulsion PCR, resulting in the production of a DNA template which is then used for sequencing (Novais and Thorstenson, 2011). This involves isolating and fragmenting genomic DNA, followed by ligating "A" and "B" adaptors to either end of the single-stranded DNA fragments (Novais and Thorstenson, 2011). A magnetization process is then used to bind these fragments to specialized beads, with one fragment per bead, before isolating each fragment-bead complex in a droplet of a "PCR-reaction-mixture-in-oil emulsion" (Novais and Thorstenson, 2011). Within the droplet, amplification of the fragment occurs, producing ten million copies on the surface of the bead: the DNA template (Novais and Thorstenson, 2011). The emulsion is then broken and the DNA strands contained on the bead are denatured into single-stranded DNA (Margulies et al., 2005). The DNA-containing bead is then loaded into a well on a fiber optic slide (PicoTiterPlate^{TM}) along with other beads containing the enzymes needed for the pyrophosphate sequencing step (Margulies et al., 2005).

The pyrophosphate-sequencing phase begins by adding deoxynucleotides (dATP, dCTP, dGTP, or dTTP) stepwise to the well of the fiber optic plate (França et al., 2002). If the deoxynucleotide is complementary to the base in the template strand then it is

incorporated via DNA polymerase and a pyrophosphate (PPi) is released, directly proportionate in amount to the number of deoxynucleotides incorporated (ex. 1 PPi for each 2 dATPs incorporated) (França et al., 2002). Released PPi are converted to ATP via ATP sulfurylase, providing the energy for the oxidation of luciferin by luciferase, thus generating light (Ronaghi and Elahi, 2002). The light generated is detected by a photon detector device, producing a pyrogram that is analyzed to determine the sequence of the DNA template strand (Ronaghi and Elahi, 2002).

There are two types of pyrosequencing approaches: solid-phase sequencing and liquid-phase sequencing (França et al., 2002). Solid-phase sequencing is a three-enzyme reaction and involves a washing step between each deoxynucleotide addition to remove the deoxynucleotides that were not incorporated in order to prevent interference with the reaction (França et al., 2002). Liquid-phase sequencing is similar to solid-phase except that it has a nucleotide-degrading enzyme, apyrase, making it a four-enzyme reaction (França et al., 2002). The use of apyrase makes the washing steps between deoxynucleotide addition unnecessary (França et al., 2002).

Multiplexing can be used in pyrosequencing to sequence several samples simultaneously by barcoding the DNA templates prior to sequencing (Novais and Thorstenson, 2011). The pyrogram produced by multiplexed pyrosequencing is then deconvoluted to obtain separate data for each DNA template (Ronaghi and Elahi, 2002). Using this approach reduces the cost of analyzing samples.

Illumina Genome Analyzer (GAIIx) Sequencing

Like Roche 454 pyrosequencing, Illumina GAIIX Sequencing is also based on the "sequencing-by-synthesis" principle. However, Illumina produces ten times more

sequence information than 454 pyrosequencing at roughly the same cost, but with shorter read lengths and a longer run time (Fox et al., 2009). Therefore, the newer Illumina technology has become the preferred approach for applications that require deep sequencing and where shorter read lengths are sufficient (Fox et al., 2009).

Illumina implements solid-phase amplification and begins by fixing adaptors to the fragmented sample DNA strands, or DNA library (Fox et al., 2009; Liu et al., 2012). The double-stranded DNA is then denatured into single strands and attached to a solid surface, called a single-molecule array or flow cell, followed by bridge amplification (Morozova and Marra, 2008; Liu et al., 2012). The process of bridge amplification involves attaching one end of the single-stranded DNA to the flow cell via an adaptor; the strand then bends over and hybridizes to complementary adaptors also located on the flow cell (Morozova and Marra, 2008). This "bridge" becomes the template for the sequencing step, but is first PCR-amplified to produce about 1000 copies of the template, forming a "cluster" (Morozova and Marra, 2008). The flow cell will ultimately hold more than 40 million clusters, all created from the original DNA library (Morozova and Marra, 2008).

The sequencing phase of Illumina involves flooding the flow cell with dNTPs (dATP, dGTP, dCTP, and dTTP), each containing its own fluorescently labeled 3'-reversible terminator (Fox et al., 2009). This "reversible terminator" allows for each cycle of the reaction to occur simultaneously with all four deoxynucleotides present (Imelfort and Edwards, 2009). These fluorescently-labeled terminators each have a different color, so each dNTP will flash its specific color upon incorporation into the complementary strand by DNA polymerase (Morozova and Marra, 2008). The color

signal released is captured by a charge-coupled device (CCD) and thus finishes a single sequencing cycle (Liu et al., 2012). The 3'-terminator is removed prior to the start of the next cycle, a process that is repeated for multiple cycles (Fox et al., 2009).

Functional Analysis

Functional metagenomics is a means of identifying the functions of the genes possessed in a bacterial genome, including functions like transferrable antibiotic resistance (Pehrsson et al., 2013). This process involves shotgun-cloning community DNA and then inserting the library created into an indicator host; the host is selected for the desired function and its DNA is sequenced (Pehrsson et al., 2013).

While this process allows for gene function to be identified, functional analysis (or "profiling") of a sequence instead gives the predicted function of a gene (its gene "profile") (Durso et al., 2011). This is a much simpler process where the community DNA is isolated, sequenced, and the sequences are analyzed against different databases to identify the genes' potential roles in the cell; examples of these databases are the SEED, MG-RAST, BLAST, and ARBD. The genetic information in these metagenomic databases is derived from a variety of resources including previously developed databases, published literature where bacterial genes were identified, and data submissions by users of the databases (Peri et al., 2003; Pruitt et al., 2005; Liu and Pop, 2009).

The SEED

The SEED is a family of databases that stores all publicly accessible genomic sequences and provides the framework for several annotation pipelines including MG-RAST (Disz et al., 2010). The SEED was initially developed by the Fellowship for the

Interpretation of Genomes and was intended to serve as a way for researchers to assemble and manage gene annotations (Overbeek et al., 2004). This family of databases is organized via subsystems, where each subsystem is a group of related biological functions that together form a specific process (Overbeek et al., 2004). It is through these subsystems that an organism's genes can be annotated (Overbeek et al., 2004).

There are four methods by which SEED data can be accessed and utilized, called the SEED servers (Aziz et al., 2012). These four servers are: (1) the Sapling Server; (2) the Annotation Support Server; (3) the RAST Server; and (4) the Metabolic modeling and Flux Balance Analysis (FBA) Server (Aziz et al., 2012). The Sapling Server gives access to the SEED through an entity-relationship data model (ERDB), allowing for direct comparisons against the database (Aziz et al., 2012). The Annotation Support Server enables new protein or DNA sequences to be annotated and assigned subsystems (Aziz et al., 2012). The Rapid Annotation using Subsystems Technology (RAST) server allows new genomes to be submitted and annotated using a web interface (Aziz et al., 2012). Finally, the FBA server allows access to the Model SEED which houses a biochemistry and metabolic model database at the genome level (Aziz et al., 2012). Together these servers provide a wide array of means for annotating and studying genomes and metagenomes.

MG-RAST

"Meta Genome Rapid Annotation using Subsystems Technology " (MG-RAST) is an analysis pipeline used for gene prediction and functional annotation all using the SEED system (Port et al., 2012; Thomas et al., 2012; Overbeek et al., 2014). Once a new

genome is annotated using MG-RAST, it can be made public and added to the SEED for other researchers to reference (Overbeek et al., 2014).

BLAST

"Basic Local Alignment Search Tool" (BLAST) serves as a method of identifying sequence similarity between sequence pairs in order to provide identification (Altschul et al., 1990). This comparison is done by calculating a maximal segment pair (MSP) score, defined as the pair of identical segments between two sequences that has the highest score (Altschul et al., 1990). The best MSP score, between a user-submitted protein or nucleotide sequence and a known database sequence, provides sequence identification (Altschul et al., 1990). BLAST can be used as an individual program or through a web interface maintained by the National Center for Biotechnology Information (NCBI) (McGinnis and Madden, 2004).

There are currently five types of BLAST programs: (1) BLASTP; (2) BLASTN; (3) BLASTX; (4) TBLASTN; and (5) TBLASTX (Pertsemlidis et al., 2001). BLASTP involves the submission of an amino acid sequence and then identifying the sequence using a protein sequence database (Pertsemlidis et al., 2001). BLASTN takes a nucleotide sequence and compares it against a nucleotide sequence database (Pertsemlidis et al., 2001). BLASTX takes a nucleotide sequence and translates it in order to identify the resulting protein using a protein sequence database (Pertsemlidis et al., 2001). TBLASTN translates a protein sequence and identifies its nucleotide sequence by comparing it against a nucleotide sequence database (Pertsemlidis et al., 2001). Finally, TBLASTX compares nucleotide translations against the translations found in a nucleotide sequence database (Pertsemlidis et al., 2001).

ARDB

The "Antibiotic Resistance Gene Database" (ARDB) enables the identification and classification of antibiotic resistance genes (Liu and Pop, 2009). The antibiotic resistance gene sequences were pooled from the NCBI nucleotide and protein databases, as well as from the Swiss-Prot database, and categorized based on their protein sequence similarity (Liu and Pop, 2009). The ARDB operates using the BLAST program, taking BLAST "hits" and grouping them according to resistance type based on their similarity to sequences within the ARDB (Liu and Pop, 2009). The ARDB currently offers information on resistance to 257 antibiotics and 13,293 genes associated with resistance (Liu and Pop, 2009).

ANTIBIOTIC RESISTANCE IN BOVINE BACTERIA

Gastrointestinal (GI) Tract

It is important to separate analysis of the published literature on antibiotic resistance in bovine bacteria into those bacteria housed in the gastrointestinal (GI) tract and those housed in the feces because microbial communities change throughout the digestive tract, potentially impacting the antibiotic resistance gene profile (Frey et al., 2010). However, no published research is available analyzing changes in resistance in different segments of the mature bovine GI tract.

Feces

While the resistance profile of bacteria in the bovine GI tract is important as it directly affects the success of antibiotic treatment, the resistance profile of excreted bovine feces is of even greater importance as it directly impacts the environment and has the potential to impact human health. However, on the question of whether fecal

antibiotic resistance is produced upon antibiotic administration is addressed, the available data are conflicting.

Resistance in Cattle with no Antibiotic Exposure

The first question of interest is whether antibiotic resistance exists in healthy cows with no history of antibiotic exposure. When fecal grab samples were collected from healthy beef heifers and analyzed using shotgun metagenomics about 8% of the sequences were associated with virulence genes (Durso et al., 2011). Of the 8%, roughly 40% of the virulence genes coded for multidrug resistance efflux pumps and 20% coded for fluoroquinolone resistance (Durso et al., 2011). Although the presence of these genes does not necessarily indicate a resistant phenotype, the authors concluded that there is a baseline level of potential resistance in bovine fecal bacteria without any selection pressure from antibiotics (Durso et al., 2011).

Resistance in Organic vs. Conventionally Raised Cattle

Differences have been observed in the resistance profiles of fecal bacteria from cows housed on conventional farms, where antibiotics are routinely administered, and cows housed on organic farms where antibiotics are not allowed. A significantly higher rate of resistance in *E. coli* isolates from conventional dairy cows to ampicillin, streptomycin, kanamycin, gentamicin, chloramphenicol, tetracycline, and sulfamethoxazole was found when compared with organic dairy cows (Sato et al., 2005). While there was an increase in single-drug resistance, there was no effect of farm type on multidrug resistance in *E. coli* isolated from adult dairy cows (Sato et al., 2005). When tested specifically for cephalosporin resistance, fecal samples had significantly more ESBL-producing *E. coli* in cows from conventional dairy farms where the use of third-

and fourth-generation cephalosporins was common (Dolejska et al., 2011). A significantly higher proportion of *Campylobacter* spp isolates from conventional dairy cows were resistant to tetracycline, requiring a tetracycline concentration four times higher than organic dairy cow *Campylobacter* spp to inhibit the growth of 50% of the isolates (Halbert et al., 2006). However, resistance to erythromycin, clindamycin, ampicillin, ceftriaxone, streptomycin, and many other antimicrobials tested were all statistically similar between conventional and organic dairy cow *Campylobacter* spp isolates (Halbert et al., 2006). These studies show that bacteria isolated from the feces of cows raised on conventional farms tend to be more resistant to some (but not all) antibiotics than those isolated from cows raised under organic conditions.

When considering the issue of antibiotic resistance changes in conventional versus organic or natural systems, the tendency for higher resistance levels in the feces of conventionally raised feedlot cattle is offset by other environmental impacts of the management practice (Morley et al., 2011). When compared with conventionally raised cows, the amount of rearing time required for naturally raised cows (no exposure to antimicrobial drugs, hormone implants, or anthelmintic drugs) is greater by an average of 50 days (Morley et al., 2011). This extended feeding time results in 1,500 extra kg of feces and 750 extra L of urine per feedlot animal (Morley et al., 2011). With daily excretion of antibiotic resistance genes (or antibiotic resistant bacteria) being fairly similar between systems, the increase in fecal and urine output in natural/organic systems may result in a greater overall resistance problem.

Resistance when Treating Cattle with Antibiotics

When cattle are therapeutically treated with antibiotics, impacts on their fecal bacteria are likely. When fecal samples were taken from dairy cows with previous ceftiofur treatment there was no correlation at the cow level between treatment and *E. coli* isolates resistant to ceftriaxone (Tragesser et al., 2006). However, there was a correlation at the herd level where herds with ceftiofur use were 25 times more likely to have cows with reduced-susceptibility *E. coli* (Tragesser et al., 2006). This implies that treating an individual cow with ceftiofur can lead to the spread of extended-spectrum cephalosporin resistance within a herd (Tragesser et al., 2006).

Contradicting the study performed by Tragesser and colleagues, other research showed no impact of previous ceftiofur treatment on the presence of the $bla_{\text{CMY-2}}$ gene in *E. coli* isolates and there was no correlation between use of the drug and susceptibility to ceftazidime at the herd level (Daniels et al., 2009). Another study showed that neither the previous rate of ceftiofur treatment nor treatment itself was associated with the presence of $bla_{\text{CTX-M}}$ or $bla_{\text{CMY-2}}$ genes in *E. coli* isolates from dairy cattle manure (Mollenkopf et al., 2012).

When dairy cows diagnosed with either metritis or interdigital necrobacillosis were treated with either ceftiofur or penicillin there was no significant change in resistance from the pre-treatment baseline in isolated *E. coli* (Mann et al., 2011). When sick dairy cows were treated with ceftiofur, ceftiofur-resistant *E. coli* appeared shortly after antibiotic treatment but remained low in number and were replaced with a susceptible *E. coli* population when the effects of the antibiotic disappeared (Singer et al., 2008).

When beef steers where administered a single subcutaneous injection of florfenicol there was no long-term impact on *E. coli* resistance to a wide variety of antimicrobials, with resistance levels significantly reduced by four weeks post-treatment (Berge et al., 2005). Finally, healthy beef steers were given ceftiofur crystalline-free acid (CCFA) subcutaneous injections, which resulted in *E. coli* populations with reduced-susceptibility to ceftiofur shortly after treatment (Lowrance et al., 2007). However, the *E. coli* returned to pre-treatment susceptibility levels within 15 days of CCFA administration (Lowrance et al., 2007). While the effect of antibiotic treatment on fecal bacteria is not consistent, these studies suggest a short-term increase in resistance after treatment followed by a return to the pre-treatment resistance level.

Resistance when Feeding Cattle Subtherapeutic Antibiotics

The feeding of antibiotics has perhaps the greatest potential impact on the contribution of animal agriculture to the global problem of antibiotic resistance. In 2011, over 4.1 million kg of ionophores and 5.6 million kg of tetracyclines, the most commonly fed antibiotics, were given to domestic food producing animals (FDA, 2011). Together these antibiotic classes made up over 70% of the total antibiotics given to food producing animals in 2011, so clearly the oral route of administration requires attention.

When feedlot steers were fed the ionophore lasalocid there was no effect on fecal coliform resistance to tetracyclines, florfenicol, or sulfonamides (Edrington et al., 2006). Feeding monensin only, or in combination with tylosin, to beef heifers increased the level of macrolide resistance in commensal *Enterococcus* but had no effect on the fecal shedding of *erm*B, a gene coding for macrolide resistance, or *tet*M (Jacob et al., 2008). Feeding monensin, tylosin, or virginiamycin to feedlot steers had no effect on *E. coli*

resistance to ampicillin, tetracycline, or gentamicin (Alexander et al., 2008). When feedlot steers were fed chlortetracycline, chlortetracycline and sulfamethazine, or tylosin phosphate there was no significant effect on the fecal excretion of *tet*, *erm*, or *sul* genes (Alexander et al., 2011). *E. coli* isolated from feedlot steers fed chlortetracycline, chlortetracycline and sulfamethazine, or virginiamycin showed similar resistance levels as control steers to tetracycline, streptomycin, sulfamethoxazole, and ampicillin, indicating no effect of oral antibiotic administration on the expression of resistance in fecal *E. coli* (Mirzaagha et al., 2011). Finally, feeding steer calves chlortetracycline and sulfamethazine or chlortetracycline alone increased the percentage of fecal *C. hyointestinalis* isolates resistant to erythromycin and tetracycline, and feeding chlortetracycline alone increased the percentage of fecal *C. jejuni* resistant to tetracycline when compared to control calves (Inglis et al., 2005). However, in the same study, feeding calves virginiamycin, monensin, or tylosin phosphate resulted in no change in fecal *C. hyointestinalis* resistance to ampicillin, erythromycin, or tetracycline and even decreased the percentage of *C. jejuni* isolates resistant to ampicillin when compared with isolates from control calves (Inglis et al., 2005). Therefore, the published research shows a weak link, if any, between feeding antibiotics and antimicrobial resistance development. In fact, feeding antibiotics may not be the only factor influencing the development of antibiotic resistance in bovine fecal bacteria. Other factors, such as diet, may be major contributors and further research needs to be conducted to determine the clear effect of feeding antibiotics (Mirzaagha et al., 2011).

ENVIRONMENTAL EFFECTS AND POTENTIAL IMPACT

When studying the impact of antibiotic administration in animals on the excretion of ARGs, the major concern is the fate of those ARGs upon excretion. One of the biggest problems is water contamination, as water in close proximity to agricultural activity tends to contain higher levels of ARGs than more distant waters (Pruden et al., 2006). This contamination is likely due to runoff from farms, as resistance genes *tetW* and *tetQ* were found in both the cow feces and runoff from cattle farms (Santamaría et al., 2011). Human contact with the ARGs found in water from an agricultural source is a direct point of entry of these resistance genes to the human microbiome.

Another problem stemming from ARG excretion is the use of contaminated manure as fertilizer for crops. When fresh dairy manure was used to fertilize soil plots growing a wide variety of vegetables, manuring was associated with the presence of *sul2*, *ermF*, *qnrB*, bla_{PSE}, and $bla_{\text{OXA-20}}$ resistance genes on the harvested vegetables (Marti et al., 2013). The consumption of these contaminated vegetables without properly washing or peeling the vegetables provides a means of transfers of these resistance genes from bacteria in the manure to human gut bacteria (Marti et al., 2013).

Finally, the soil that comes in contact with animal manure containing ARGs is yet another major area of concern. It is estimated that the majority of animal manure produced by concentrated animal feeding operations (CAFO) is land applied within a 10-mile radius of the CAFO (Sarmah et al., 2006). This results in high potential soil exposure to manure ARGs. High resistance levels to tetracycline, erythromycin, and streptomycin have all been detected in farmland soil (Popowska et al., 2012). When compared with unmanured soils such as forest and orchard soils, manured vegetable

garden soil possessed bacterial strains with the highest MICs for tetracycline and erythromycin (Popowska et al., 2012). Tetracycline resistance genes were present in the soil of grassland farms where dairy cattle were housed (Santamaría et al., 2011). Manuring vegetable soil plots with fresh dairy or swine manure increased the abundance of bacteria exhibiting resistance to drugs such as amikacin, cefoxitin, and ampicillin (Marti et al., 2013). However, shifts in the soil resistome may have more to do with the bacterial species present in the soil or soil composition than the horizontal gene transfer processes occurring between manure containing antibiotic resistant bacteria and soil bacteria (Forsberg et al., 2014). Furthermore, soil bacteria may not be as capable of transferring resistance to other members of their community like bacteria recognized as human pathogens (Forsberg et al., 2014). Regardless, exposing soils to animal manure tends to result in the production of antibiotic resistance within soil bacteria, making soil a significant reservoir of antibiotic resistance.

CONCLUSION AND RESEARCH OBJECTIVES

In the dairy cow, the role antibiotic administration plays in subsequent fecal excretion of antibiotic resistant bacteria is of major concern. However, the contribution of antibiotic administration alone is unclear due to baseline bacterial resistance in cows without exposure to antibiotics, exposure to environmental resistance genes (e.g. soil or water), and the (until recent) lack of sensitive methods for identifying resistance genes. Fortunately, identification methods and knowledge continue to integrate, improving the understanding of the relationship between antibiotics and resistance.

The objective of this research was to use new metagenomic techniques to evaluate the effect of pirlimycin hydrochloride (mastitis treatment), ceftiofur crystalline free acid

(metritis treatment), and cephapirin benzathine (dry cow therapy) on antibiotic resistance gene prevalence in the fecal microbiome of the dairy cow. A second goal was to determine the temporal pattern of excretion of antibiotic resistance genes to identify key time points for producers to focus their manure management strategies, helping to prevent the spread of antibiotic resistance.

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Chapter 3: METAGENOMIC ANALYSIS OF ANTIBIOTIC RESISTANCE GENES IN THE FECAL MICROBIOME FOLLOWING THERAPEUTIC AND PROPHYLACTIC ANTIBIOTIC ADMINISTRATION IN DAIRY COWS

ABSTRACT

The use of antibiotics in dairy cattle has the potential to stimulate the development and subsequent fecal dissemination of antibiotic resistance genes (ARGs) in bacteria. The objectives were to use metagenomic techniques to evaluate the effect of antibiotic treatment on ARG prevalence in the fecal microbiome of the dairy cow and to determine the temporal excretion pattern of ARGs. Twelve Holstein cows were assigned to one of four antibiotic treatments: control, pirlimycin hydrochloride (PIRL), ceftiofur crystalline free acid (CCFA), or cephapirin benzathine (CEPH). Fecal samples were collected on d -1, 1, 3, 5, 7, 14, 21, and 28. Samples were freeze-dried and subjected to DNA extraction followed by Illumina paired-end HiSeq sequencing and quantitative polymerase chain reaction (qPCR). Illumina sequences were analyzed using MG-RAST and the Antibiotic Resistance Gene Database (ARDB) via BLAST. Abundance of *ampC*, *ermB*, *tetO*, *tetW*, and 16S rRNA genes were determined using qPCR. All data were statistically analyzed with PROC GLIMMIX in SAS. Antibiotic treatment resulted in a shift in bacterial cell functions. Sequences associated with "stress response" were higher and sequences associated with "phages, prophages, transposable elements, plasmids" and "motility and chemotaxis" tended to be higher in ceftiofur-treated cows than control cows. Sequences associated with "resistance to antibiotics and toxic compounds" were higher in ceftiofur-treated cows than control cows. Ceftiofur-treated cows had a higher abundance of β-lactam and multidrug resistance sequences than control cows. There was

no effect of treatment or day on fecal *tetO* and *ermB* excretion. The relative abundances of *tetW* and *ampC* were higher on d 3 post-treatment than d 5 and d 28. In conclusion, antibiotic use in dairy cattle shifted bacterial cell functions and temporarily increased antibiotic resistance in the fecal microbiome.

Keywords: shotgun metagenomic sequencing, antibiotic resistance, dairy cow

INTRODUCTION

Even at an early stage, the development of antibiotic resistance was recognized as a potential problem of antibiotic use. Alexander Fleming, upon his discovery of penicillin, said "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them" (Fleming, 1945). Today, the resistance problem continues to grow. Gram-negative bacterial isolates were recovered from ICU patients all over the United States from 1994 – 2000 and, in that time span, susceptibility to several antibiotics were reduced including ciprofloxacin by 10% and gentamicin by 6% (Neuhauser et al., 2003). Antibiotic resistance is clearly on the rise and must be addressed.

In addressing this global problem, attention has fallen on the animal industry and its use of antibiotics. In 2011, the FDA reported that 3.29 million kg of antibiotics were sold for human pharmaceutical purposes while 13.5 million kg of antibiotics were sold for use in domestic livestock (FDA, 2011;2012). Therefore, it is estimated that of the total antimicrobials sold annually, roughly 80% is used by the livestock industry. This usage of antibiotics has implications for soil and water microbiomes through effects of excreted antibiotics (Sarmah et al., 2006) as well as the dissemination of antibiotic resistance from livestock fecal bacteria.

The three main mechanisms of antibiotic resistance transfer include (1) conjugation, which occurs via plasmids, (2) transformation, which is the uptake of naked DNA, and (3) transduction, which is the transfer of resistance genes via a bacteriophage (Barbosa and Levy, 2000). Resistance genes coding for resistance mechanisms to β-lactams [i.e. altering penicillin binding proteins or producing β-lactamases (McDermott et al., 2003)] or to macrolide-lincosamide-streptogramins [i.e. obtaining an rRNA methylase which inhibits drug binding to the 50S ribosomal subunit (Roberts, 2004)] can be spread rapidly between bacteria.

Previously, cultured-based methods like agar disc diffusion tests and dilution-based tests (Schmieder and Edwards, 2012) were used to identify antibiotic resistant bacterial isolates. However, newer quantitative methods like qPCR (Steffan and Atlas, 1991) can analyze for specific resistance genes present in the environmental sample, not just in culturable bacteria. Today, metagenomic methods of analysis like 454 pyrosequencing (Sirohi et al., 2012) and Illumina sequencing (Fox et al., 2009) allow for the study of entire bacterial metagenomes. Metagenomic methods are enhanced by functional analysis of the sequencing output via databases like BLAST, ARDB, the SEED, and MG-RAST.

The effect of antibiotic treatment on cattle fecal microbiomes has been a topic of study for quite some time yielding results both consistent with and contradictory to any directly linked hypotheses. For instance, fecal bacteria from conventionally-raised cows tend to be more resistant to antibiotics than that from cows raised organically (Sato et al., 2005; Halbert et al., 2006) but the increase in resistance in fecal bacteria of cows treated with antibiotics is only transient (Tragesser et al., 2006; Singer et al., 2008). Even cattle

never exposed to antibiotics shed antibiotic resistant bacteria (Durso et al., 2011; Thames et al., 2012). Finally the link between feeding antibiotics to cattle and fecal antibiotic resistance is weak, with conflicting results (Inglis et al., 2005; Edrington et al., 2006; Jacob et al., 2008; Mirzaagha et al., 2011). What is known is that runoff from farms contain higher levels of antibiotic resistance genes than other water sources (Santamaría et al., 2011) and that land applying contaminated cattle feces to vegetable plots results in resistance genes making their way into and on produce (Marti et al., 2013). Further research is necessary to complete the picture of antibiotic use on antibiotic resistance to determine how to appropriately prevent the dissemination of those resistance genes.

The objective of this research was to use new metagenomic techniques to evaluate the effect of pirlimycin hydrochloride (mastitis treatment), ceftiofur crystalline free acid (metritis treatment), and cephapirin benzathine (dry cow therapy) on antibiotic resistance gene prevalence in the fecal microbiome of the dairy cow. A second objective was to determine the temporal pattern of excretion of antibiotic resistance genes to identify key time points for producers to focus their manure management strategies, helping to prevent the spread of antibiotic resistance.

MATERIALS AND METHODS

Animals and Experimental Treatments

This experiment was conducted under the review and approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (protocol 12-184-DASC). Holstein (n = 12) cows in their first lactation, 110 to 200 days in milk and yielding 30.45 to 40.45 kg of milk daily, were selected from the Virginia

Tech Dairy Center (Blacksburg, VA). Farm records showed that these cows had not received any antibiotic treatment from at least the time of their insemination as heifers.

The study was conducted over 2, 28-day periods. Cows were housed individually in tie-stalls with rubber mats for the first 7 days, were fed a total mixed ration twice daily, and provided *ad libitum* access to water. For the remaining 21 days, cows were housed in a free-stall barn with rubber mats and sawdust, fed a total mixed ration once daily, and given *ad libitum* access to water.

Treatments (n = 4) were the administration of cephapirin benzathine (ToMorrow®, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO), pirlimycin hydrochloride (Pirsue®, Zoetis, Madison, NJ), or ceftiofur crystalline free acid sterile suspension (Excede®, Zoetis, Madison, NJ), as well as a control group where no antibiotic was given. All cows were randomly assigned to the control or one of the antibiotic treatments except for those cows (n = 3) receiving cephapirin benzathine for dry cow therapy. Cows for this treatment were selected based on the previously mentioned criteria as well as a scheduled date for dry-off that overlapped with the time of the experiment. A random number generator was used to assign cows within each treatment to either period 1 or 2, ensuring each treatment was represented in each period.

Antibiotic Administration

Cows (n = 3) on the cephapirin benzathine (CEPH) treatment were dried off on day 0. Virginia Tech Dairy Center (Blacksburg, VA) standard dry-off protocol was followed. The cows were milked and each teat was cleaned with a 4 x 4 gauze pad soaked in 70% isopropyl alcohol. Each teat was injected with 10 mL cephapirin benzathine (300

mg cephapirin) and then sealed with Orbeseal® (bismuth subnitrate, colloidal silicon dioxide, and liquid paraffin; Zoetis, Madison, NJ).

Cows (n = 3) assigned to the pirlimycin hydrochloride (PIRL) treatment were infused with 10 mL of pirlimycin hydrochloride (50 mg pirlimycin) in their front left quarter on days 0 and 1, with 24 hours separating infusions. Prior to each infusion, cows were milked and the teat receiving treatment was cleaned with a 4 x 4 gauze pad soaked in 70% isopropyl alcohol.

Cows (n = 3) assigned to the ceftiofur crystalline free acid (CCFA) treatment were injected subcutaneously with 1.5 mL ceftiofur crystalline free acid sterile suspension (150 mg ceftiofur) per 45.4 kg body weight. Per manufacturer's protocol, cows were injected at the base of the right ear on day 0 and at the base of the left ear on day 3, with injections spaced 72 hours apart. Prior to injection, the area was cleaned with a 4 x 4 gauze pad soaked in 70% isopropyl alcohol.

Sample Collection

Fecal samples were collected from all 12 cows 30 min. prior to antibiotic administration on day 0, and then once daily on days 1, 3, 5, 7, 14, 21, and 28 following treatment. Samples were collected at 1800 h when cows were housed in individual stalls (days 1, 3, 5, and 7) and at 1300 h on days 14, 21, and 28 when cows were housed in a free stall barn (or, for dry cows, when on pasture). Fecal samples were collected rectally, using a clean palpation sleeve and sterile lubricant for each collection. Roughly 500 g of feces was discarded to prevent contamination before 100 g of wet sample was collected in a sterile plastic snap cap vial. Fecal samples were placed in a -20° C freezer within one hour of collection and stored until analysis.

Metagenomic Analysis

Sample Preparation and DNA Extraction

Fecal samples were thawed and 15 g of wet feces/sample was placed into a sterile 50 mL conical and covered with aluminum foil. Samples were frozen for a minimum of 30 min. in a -80°C freezer prior to freeze-drying. Fecal samples were freeze dried (LABCONCO, Kansas City, MO) for 72 hours or until the expected dry matter content (13 – 19% dry matter) was achieved. Freeze-dried samples were mixed with an autoclaved disposable spatula prior to being weighed for DNA extraction.

For DNA extraction, 0.102x g of freeze-dried feces was weighed into a sterile 2 mL centrifuge tube. Samples were extracted in replicates of 6 using a QIAamp DNA Mini Stool Kit (QIAGEN, Germantown, MD), following the Stool Pathogen Detection Protocol with some modifications. First, the ASL Buffer (lysis buffer) was heated prior to use until the precipitate dissolved (at 45 - 50°C). In the lysis step (step 3), samples were heated at 95°C for 6 minutes to improve lysis of Gram-negative bacteria. In step 8, prior to centrifugation, 4 μL of RNase A (Epicentre®, Madison, WI) (conc. of 5 μg/μL) was added, the tube was inverted to mix, and the supernatant was allowed to sit for 3 min. at room temperature. The purpose of adding the RNase A was to rid the sample of any RNA contamination. The recommended step 17 of centrifugation for 1 min. with a new catch tube was included. In step 18 (elution step), 50 μL of Buffer EB (10 mM Tris-Cl) was used because it does not contain EDTA, which inhibits Illumina library preparation. Also, the Buffer EB was heated at 60°C for 2 min. prior to pipetting it onto the column and allowed to sit on the column for 6 min. prior to centrifugation; this aided in the binding of

the buffer to the DNA on the column. Finally, all centrifugation steps were carried out at 16,100 x *g*. The eluted DNA was immediately stored at -20°C until further processing.

Sample Purification and Quality Testing

The DNA concentration of each sample replicate (n = 6) was quantified using a Qubit® 2.0 Fluorometer (Life Technologies™, Grand Island, NY) with the Qubit® dsDNA HS Assay Kit. Samples were submitted to the Virginia Bioinformatics Institute Genomics Research Laboratory (Blacksburg, VA) for Solid Phase Reversible Immobilization (SPRI) Purification, using Agencourt AMPure XP. This is a purification process that uses magnetic beads to bind amplicons larger than 100 bp, ridding the sample of contaminants like salts or primer dimmers (Beckman Coulter, Inc., 2013). After purification, the DNA concentrations of each sample replicate were again quantified using a Qubit® 2.0 Fluorometer with the Qubit® dsDNA HS Assay Kit. Sample replicates were run on an electrophoresis gel with 2.66 μL loading dye (sybergreen, monensin blue, and glycerol) to 4 μL sample. Lastly, sample replicates were analyzed on a NanoPhotometer® (Implen, Inc., Westlake Villiage, CA) to confirm that the 260/280 value of each replicate was approximately 1.8, indicating pure DNA. The best two replicates of each sample, determined by 260/280 ratio resembling pure DNA, highest DNA concentration, and adequate gel images, were pooled and submitted for library preparation and sequencing.

Library Preparation and Illumina GAIIx HiSeq Sequencing

DNA Seq library preparation and Illumina paired-end HiSeq, 100 cycle, multiplexed sequencing was performed by the Virginia Bioinformatics Institute Genomics Research Laboratory (Blacksburg, VA). HiSeq sequencing was performed on

day -1 (pre-treatment) and day 3 samples for all control and ceftiofur cows (a total of 12 samples sequenced).

BLAST/ARDB Data Analysis

Sequence data analysis for the 12 samples was performed using BLAST to compare sequences to the ARDB by our collaborator Dr. Tong Zhang's lab (Hong Kong, China). An e-value of $1e^{-5}$ was used as the cutoff value for sequence similarity.

MG-RAST Data Analysis

Sequence analysis on the 12 samples was performed using MG-RAST. This involved uploading the paired-end sample sequences, in fastq format, to MG-RAST and removing any bovine host sequences (those associated with the *Bos taurus* genome). The remaining sequences were quality checked and dereplication was performed to remove false sequences (those the result of sequencing error). Of the sequences that passed these quality tests, predicted protein coding regions were determined, assigned annotation based on SEED sequence identification, and assigned to a functional category using the SEED subsystems. Functional data for the paired-end sequences was downloaded from MG-RAST and merged to create one dataset per sample prior to statistical analysis.

For bacterial species diversity, α-diversity values were calculated using MG-RAST and are defined as the antilog of the Shannon diversity index. Therefore, α-diversity values do not represent the actual number of bacterial species.

Statistical Analysis

Statistical analysis of BLAST/ARDB and MG-RAST data was with PROC GLIMMIX in SAS 9.2 (SAS Institute Inc. Cary, NC). A minimum of 40 or more sequences per sample was required for an antibiotic resistance gene class to be analyzed

in the BLAST/ARDB dataset. Samples from day -1 were used as a covariate. Tukey pair-wise comparisons were used to determine treatment differences on day 3. Significance was declared at $P \leq 0.10$. Trends were declared at $P \leq 0.15$.

Real-Time Quantitative Polymerase Chain Reaction (qPCR) Analysis

Sample Preparation and DNA Extraction

All samples were analyzed for 16S rRNA, *tetO*, *tetW*, *ampC*, and *ermB* using qPCR. Fecal samples were thawed and 15 g of wet feces/sample was placed into a sterile 50 mL conical and covered with aluminum foil. Samples were frozen for a minimum of 30 min. in a -80°C freezer prior to freeze-drying. Fecal samples were freeze dried (LABCONCO, Kansas City, MO) for 72 hours or until the expected dry matter content (13 – 19% dry matter) was achieved. Freeze-dried samples were mixed with an autoclaved disposable spatula prior to being weighed for DNA extraction.

DNA extraction was performed using 0.05xx g freeze-dried feces. The FastDNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) was used following manufacturer instructions, including the use of the FastPrep® instrument (MP Biomedicals, Santa Ana, CA). In order to optimize DNA yield, all DNA extracts were incubated for 5 minutes in a 55°C water bath following the addition of DNase/Pyrogen-Free Water to the column. Upon removal from the water bath, the DNA was eluted via centrifugation and immediately stored at -80°C until further processing.

Q-PCR Quantification

Previously reported qPCR protocols were used to quantify the genes 16S rRNA (Suzuki et al., 2000), *ampC* (Shi et al., 2013), *ermB* (Jacob et al., 2008), and *tetO* and *tetW* (Aminov et al., 2001). Primer sequences are provided in the appendix.

Q-PCR quantifications were performed in triplicate using a CFX96™ Real-Time System (Bio-Rad, Hercules, CA). The 10 μl reaction mixture for each well consisted of 5 μl SsoFast EvaGreen (Bio-Rad, Hercules, CA), 0.8 μl (5M) of each forward and reverse primer, 2.4 μl of molecular grade water, and 1 μl of 1:80 diluted sample DNA. Reaction temperatures were 98°C for 2 min. for temperature stabilization, 39 cycles of 98°C to denature the DNA followed by annealing temperatures (60.0°C for 16S rRNA and *tet*W, 50.3°C for *tet*O, 57.8°C for *amp*C), and finally 45 cycles of 98°C for DNA extension.

For resistance gene abundance representation, the absolute abundance value represents the number of ARG copies per gram of freeze-dried feces. The relative abundance value represents the number of ARG copies per copy of 16S rRNA, giving the proportion of ARG abundance relative to total bacterial load.

Statistical Analysis

Statistical analysis was with PROC GLIMMIX in SAS 9.2 (SAS Institute Inc. Cary, NC). Prior to statistical analyses, non-normal data were logarithmically transformed to achieve normality. Samples for day -1 were used as a covariate. If the *P*-value of the treatment by day interaction was < 0.50, Tukey pair-wise comparisons were used to determine treatment differences within each day and day differences within each treatment. Significance was declared at $P \leq 0.10$. Trends were declared at $P \leq 0.15$.

RESULTS

Metagenomic Results: MG-RAST Analysis

Species Diversity: Alpha-Diversity Values

There was no significant effect of CCFA treatment on sample species diversity, as indicated by α-diversity values (Table 2 MG-RAST analysis: effect of ceftiofur crystalline-free acid antibiotic treatment on bacterial species diversity in dairy cow feces).

Functional Sequence Abundance as a % of Total Sample Sequences Assigned Function

There was no effect of CCFA treatment on the fecal abundance of sequences associated with "virulence, disease, and defense" as a percentage of total sample sequences (Table 3).

Control cows had a significantly greater fecal abundance of sequences associated with "cell division and cell cycle" ($P < 0.06$, Figure 1), "clustering-based subsystems" ($P < 0.01$, Figure 2), "metabolism of aromatic compounds" ($P < 0.06$, Figure 3), and "secondary metabolism" ($P < 0.07$, Figure 4) than CCFA-treated cows (Table 3). Control cows also tended to have a greater fecal abundance of sequences associated with "DNA metabolism" ($P < 0.12$) than CCFA-treated cows (Table 3).

CCFA-treated cows had a significantly greater fecal abundance of sequences associated with "stress response" ($P < 0.08$, Figure 5) than control cows (Table 3). CCFA-treated cows also tended to have a greater fecal abundance of sequences associated with "motility and chemotaxis" ($P < 0.13$), (synthesis of) "nucleosides and nucleotides" ($P < 0.12$), and "phages, prophages, transposable elements, plasmids" ($P < 0.15$) than control cows (Table 3).

There was no effect of CCFA treatment on the fecal abundance of sequences associated with (metabolism of) "amino acids and derivatives", (metabolism of) "carbohydrates", "cell wall and capsule", (metabolism of) "cofactors, vitamins, prosthetic

groups, pigments", "dormancy and sporulation", (metabolism of) "fatty acids, lipids, and isoprenoids", "iron acquisition and metabolism", "membrane transport", "miscellaneous", "nitrogen metabolism", "phosphorus metabolism", "photosynthesis", "potassium metabolism", "protein metabolism", "RNA metabolism", "regulation and cell signaling", "respiration", and "sulfur metabolism" (Table 3).

Abundance of Phages, Prophages, Transposable Elements, and Plasmids Subcategories

There was an effect of CCFA treatment on the abundance of sequences associated with "pathogenicity islands" and "phages, prophages" as a % of total "phages, prophages, transposable elements, plasmids" sequences (Table 4). Control cows had a significantly greater ($P < 0.06$, Figure 6) fecal abundance of sequences associated with "pathogenicity islands" than CCFA-treated cows. However, CCFA-treated cows tended to have a greater ($P < 0.11$) fecal abundance of sequences associated with "phages, prophages" than control cows. There was no effect of CCFA treatment on the abundance of sequences associated with "gene transfer agent", "plasmid related functions", "transposable elements", and "other" as a % of total "phages, prophages, transposable elements, and plasmids" sequences (Table 4).

Abundance of Total Virulence, Disease, and Defense Subcategories

There was an effect of CCFA treatment on the abundance of sequences associated with "resistance to antibiotics and toxic compounds" as a % of total "virulence, disease, and defense" sequences (Table 5). Most relevant to our research question, CCFA-treated cows had a significantly greater ($P < 0.01$, Figure 7) fecal abundance of sequences associated with "resistance to antibiotics and toxic compounds" than control cows.

However, there was no effect of CCFA treatment on the abundance of sequences associated with “adhesion”, “bacteriocins, ribosomally synthesized antibacterial peptides”, “detection”, “invasion and intracellular resistance”, “toxins and superantigens”, and “other” as a % of total “virulence, disease, and defense” sequences (Table 5).

Metagenomic Results: BLAST Analysis using the ARDB

There was no significant effect of CCFA treatment on the total abundance of fecal ARG-like sequences (Figure 8). However, there was a significant effect of treatment on β-lactam and multidrug resistance gene sequences as a proportion of total sample sequences (Table 6). CCFA-treated cows had a greater proportion of β-lactam ($P < 0.01$, **Error! Reference source not found.**) and multidrug ($P < 0.02$, Figure 10) resistance genes when compared with control cows. There was also a significant effect of treatment on β-lactam resistance genes as a percentage of total ARG-like sequences (Table 7), with CCFA-treated cows having a significantly greater ($P < 0.01$, Figure 11) percentage of β-lactam resistance genes when compared with control cows.

Q-PCR Results

16S rRNA

There was a significant effect of treatment ($P < 0.03$, Table 8) and day ($P < 0.08$, Table 8) on 16S rRNA abundance in feces. Control cows ($P < 0.06$) and cows treated with CEPH ($P < 0.04$) had a greater abundance of 16S rRNA when compared to cows treated with PIRL (Table 8). On d 3 post-treatment, cows treated with PIRL had a greater abundance of 16S rRNA when compared with cows treated with CEPH ($P < 0.09$, Figure

12). On d 5 post-treatment, control cows had a greater abundance of 16S rRNA when compared with cows treated with PIRL ($P < 0.08$, Figure 12).

Tetracycline Resistance Genes

The absolute abundance of fecal *tetO* was not significantly affected by treatment, day, or treatment by day interaction (Table 8). However, on d 5 post-treatment control cows had a greater absolute abundance of *tetO* when compared with cows treated with PIRL ($P < 0.07$, Figure 13). The relative abundance of *tetO* was also not affected by treatment, day, or treatment by day interaction (Table 9). However, on d 1 post-treatment control cows had a greater relative abundance of *tetO* when compared with cows treated with CCFA ($P < 0.10$, Figure 13).

There was no effect of treatment on the absolute or relative abundance of fecal *tetW*. However, the absolute abundance of fecal *tetW* was significantly affected by day ($P < 0.01$, Table 8) with *tetW* being greater on d 28 post-treatment when compared with d 1 ($P < 0.01$) and d 7 ($P < 0.05$) post-treatment (Figure 14). The relative abundance of *tetW* was also significantly affected by day ($P < 0.04$, Table 9) with *tetW* being greater on d 3 post-treatment when compared with d 5 ($P < 0.03$) and d 28 ($P < 0.10$) post-treatment (Figure 14).

β-lactam Resistance Genes

Absolute abundance of fecal *ampC* was not significantly affected by treatment, day, or treatment by day interaction (Table 8). The relative abundance of *ampC* was significantly affected by day ($P < 0.04$, Table 9) with d 3 post-treatment being greater when compared with d 5 ($P < 0.04$) and d 28 ($P < 0.05$) post-treatment (Figure 15). Although effect of treatment was not significant, cows treated with PIRL had a greater

relative abundance of *ampC* when compared with cows treated with CCFA on d 3 post-treatment ($P < 0.09$, Figure 15).

Macrolide-lincosamide-streptogramin (MLS) Resistance Genes

Absolute abundance of fecal *ermB* tended to be affected by treatment ($P < 0.11$, Table 8) with CEPH-treated cows tending to have greater *ermB* absolute abundance than control ($P < 0.12$) and PIRL-treated ($P < 0.15$) cows (Figure 16). There was no significant effect of day or treatment by day interaction on absolute abundance of *ermB*. On day 1 following treatment, CEPH-treated cows had a significantly greater ($P < 0.08$, Figure 16) absolute abundance of *ermB* than control cows. On day 28 post-treatment, CCFA-treated cows tended to have a greater ($P < 0.11$) absolute abundance of *ermB* than cows treated with PIRL. When looking within treatment, CCFA-treated cows shed significantly greater ($P < 0.10$) amounts of *ermB* on day 14 than day 28 post-treatment.

The relative abundance of *ermB* was not significantly affected by treatment, day, or treatment by day interaction (Table 9). However, on day 28 following treatment CCFA-treated cows had a significantly greater relative abundance of fecal *ermB* than cows treated with PIRL ($P < 0.01$, Figure 16) and CEPH ($P < 0.01$, Figure 16). When looking within treatment, CCFA-treated cows shed a significantly greater ($P < 0.02$) amount of *ermB*, relative to 16S, on day 28 than all other days post-treatment.

DISCUSSION

MG-RAST Analysis

MG-RAST analysis to compare the bacterial cell functions in the fecal microbiomes of cows on the 3rd day following antibiotic (CCFA) treatment showed no effect of treatment on the bacterial species diversity of the fecal microbiome. This

indicates that antibiotic treatment did not result in the death of bacterial populations immediately following treatment. The lack of treatment effect may be a result of the time point analyzed. In a human trial, when ampicillin/sulbactam and cefazolin were administered, the earliest change in fecal diversity was a reduction in Gram-negative bacteria 6 days post-treatment (Pérez-Cobas et al., 2013). In fact, it was 14 days post-treatment before a large shift was observed in the active bacterial populations (Pérez-Cobas et al., 2013). Therefore, in the current study fecal samples taken later after antibiotic treatment must be analyzed to assess the effect of CCFA treatment on bacterial species diversity.

MG-RAST analysis showed there were significant effects of CCFA treatment on several bacterial functional categories. Control cows had a significantly greater proportion of sequences associated with "cell division and cell cycle" than CCFA-treated cows. Although counter-intuitive, this reduction in cell division and cell cycle sequences may be advantageous for the bacteria under antibiotic stress. The tricarboxylic acid (TCA) cycle plays an important role in producing energy for aerobic and facultative anaerobic bacteria, but TCA mutant bacteria are actually better able to survive when exposed to a β-lactam like oxacillin (Thomas et al., 2013). Perhaps the bacteria themselves are responsible for this disruption of the cell cycle, in order to ensure their own survival under antibiotic selection pressure. However, the more accepted explanation is that since CCFA is a cephalosporin, which inhibits peptidoglycan synthesis causing cell death (Lagacé-Wiens et al., 2014), the disruption of cell wall synthesis results in the lack of cell division. Therefore, the reduced incidence of cell division and cell cycle

sequences in the bacteria housed in CCFA-treated cows is due to bacterial cell death upon antibiotic exposure.

Control cows had a significantly greater proportion of sequences associated with "metabolism of aromatic compounds" than CCFA-treated cows. Aromatic compounds make up roughly 20% of the earth's biomass and are mainly produced by plants (Fuchs et al., 2011; Díaz et al., 2013). One major aromatic compound is lignin (Fuchs et al., 2011), a common component of a cow's plant-based diet. It is the responsibility of bacteria to metabolize these aromatic compounds in order to recycle the carbon (Fuchs et al., 2011). The difference between the abundance of aromatic compound metabolism sequences could be due to the disruption of normal bacterial cell functions by antibiotic presence in CCFA-treated cows.

CCFA-treated cows tended to have a greater proportion of sequences associated with bacterial "motility and chemotaxis" than control cows. The increased abundance of sequences associated with motility and chemotaxis in CCFA-treated cows could indicate that the bacteria possessed an increased ability for formation of biofilms to protect them from antibiotic exposure. Motility is a key step in the initial stages of biofilm development (Sauer et al., 2002) and chemotaxis factors are also important in biofilm formation, as it is a method bacteria use to attract other bacteria (Bigot et al., 2013).

CCFA-treated cows tended to have a greater proportion of sequences associated with "phages, prophages, transposable elements, and plasmids" than control cows. This suggests that the bacteria in antibiotic-treated cows possessed a greater ability to acquire and spread antibiotic resistance genes through the use of phages/prophages (transduction) or plasmids (conjugation) (Barbosa and Levy, 2000). When swine were fed antibiotics

(chlortetracycline, sulfamethazine, penicillin) an increase in prophage abundance was detected in the fecal bacteria (Allen et al., 2011). Therefore, this trend for an increase in these types of sequences may reflect an important impact of CCFA treatment on the bacterial resistome.

Control cows had a significantly greater proportion of sequences associated with "secondary metabolism" than CCFA-treated cows. Secondary metabolism involves the natural production of secondary metabolites that often contain antibacterial properties, often as a response to living in an environment with limited nutrient availability (Inaoka et al., 2003). Therefore, it is interesting that bacteria from CCFA-treated cows contained a lower proportion of sequences associated with this function. Perhaps this is due to less competition for nutrients as bacteria susceptible to the antibiotic died off.

CCFA-treated cows had a significantly greater proportion of sequences associated with "stress response" than control cows, suggesting that the bacteria in the gut of treated cows were under stress from the antibiotic selection pressure. Similarly, when *C. trachomatis* were exposed to penicillin, the bacteria became stressed and remained viable but became non-infectious (Kintner et al., 2014). When stressed, *C. trachomatis* were incapable of dividing and were less active metabolically (Kintner et al., 2014). By extrapolation, we might conclude that the stressed bacteria in CCFA-treated cows were relatively static in terms of metabolic function and cell growth. On another note, this increase in stress response could also lead to a delayed change in bacterial species diversity, as mentioned earlier.

Control cows had a significantly greater proportion of sequences associated with "pathogenicity islands" than CCFA-treated cows. Pathogenicity islands are large

segments of DNA (≥10 – 200 kb), often a component of plasmids or bacteriophages, which can contain genes coding for virulence factors such as adhesion, invasion, and Type III and Type IV secretion systems (Hacker and Kaper, 2000). This is surprising because β-lactam exposure was shown to increase the development and transfer of pathogenicity islands between *S. aureus* isolates (Maiques et al., 2006).

CCFA-treated cows had a significantly greater proportion of sequences associated with resistance to antibiotics and toxic compounds than control cows. This was expected because it has been shown that cows receiving antibiotic treatment had increased resistant bacterial isolates or increased fecal shedding of antibiotic resistance genes, even if for only a short interval following treatment (Lowrance et al., 2007; Singer et al., 2008; Alali et al., 2009).

Control cows had a significantly greater proportion of sequences associated with "clustering-based subsystems" than CCFA-treated cows. "Clustering-based subsystems" is a category MG-RAST uses for sequences that share a function, but one that is unknown (Uroz et al., 2013). Therefore, the importance of this difference between the bacteria of control and CCFA-treated cows is unknown.

CCFA-treated cows tended to have a greater proportion of sequences associated with (synthesis of) "nucleosides and nucleotides" than control cows. The reason behind this effect and its subsequent impact is unknown.

Control cows tended to have a greater proportion of sequences associated with "DNA metabolism" than CCFA-treated cows. The reason behind this effect and its subsequent impact is unknown.

BLAST/ARDB Analysis

BLAST/ARDB analysis to compare the fecal microbiomes of cows on the 3rd day following treatment showed no effect of CCFA treatment on overall ARG-like sequence abundance. This lack of effect is partly explained by the dominance (>85% of the sequences associated with antibiotic resistance) in both CCFA-treated and control cows of sequences coding for resistance to the tetracycline class of antibiotics. The presence of antibiotic resistance in the fecal bacteria of cows with no previous exposure to antibiotics is well documented, and the largest resistance category is against antibiotics like tetracyclines that attack drug efflux pumps as a major mode of action (Durso et al., 2011). For instance, genes coding for tetracycline resistance (*tetW* and *tetQ*) were detected in more than 80% of fecal samples collected from cattle raised in grassland-production systems (Santamaría et al., 2011). Therefore, given the lack of impact of CCFA (a cephalosporin) on tetracycline resistance sequences, their overwhelming presence in the current fecal samples may have masked any effect of CCFA treatment on ARG sequence abundance.

Although CCFA treatment had no effect on overall ARG sequence abundance, there was an effect on the proportion of β-lactam and multidrug resistance gene sequences, both of which were higher in ceftiofur-treated cows three days post-treatment than in control cows. Similarly, when feedlot steers were given one dose of CCFA at either 4.4 mg/kg or 6.6 mg/kg, fecal shedding of ceftiofur-resistant *E. coli* isolates was significantly higher on 2, 6, 9, and 16 days following treatment (Lowrance et al., 2007). In a corresponding study beef steers treated with one dose of 4.4 mg/kg CCFA had significantly higher fecal amounts of bla_{CMY-2} on days 3, 7, and 10 following treatment

(Alali et al., 2009). Cows in the current study received two doses of roughly 3.3 mg/kg, somewhat similar to the single 4.4 mg/kg dose in the Lowrance et al. (2007) study, and this resulted in a significant increase in sequences associated with β-lactam resistance on day 3 post-treatment. Another study with more prolonged dosing of CCFA showed similar results. When dairy cows were treated with five doses of 2.2 mg/kg CCFA, significantly more antibiotic resistant *E. coli* were detected in the feces 4, 5, and 6 days following treatment than in control cows (Singer et al., 2008). This was directly correlated with the detection of bla_{CMY-2}. On day 4 post-treatment isolates from two of the treated cows possessed bla_{CMY-2}, while on day 5 four cows did, declining to just one cow by 6 days after treatment (Singer et al., 2008). Combined data from the current and published research provide evidence that when cows are treated with CCFA, fecal shedding of β-lactam-resistant bacteria is a fairly immediate response.

qPCR Analysis

The bacterial genes analyzed using qPCR were (1) 16S rRNA used as a proxy for bacterial abundance and to normalize abundance of measured ARGs; (2) *tetW* and *tetO*, coding for tetracycline resistance; (3) *ampC*, coding for β-lactam resistance; and (4) *ermB*, coding for macrolide-lincosamide-streptogramin resistance, specifically focusing on lincosamide resistance. It is important to note that *ampC*, *tetW*, and *tetO* were detected in the feces of every cow, even the control cows. Resistance gene excretion in control cows may have occurred because of the natural baseline level of resistance genes present in the cow's digestive tract. Even cattle never exposed to antibiotics excrete resistance genes coding for resistance to fluoroquinolones, tetracyclines, β-lactams, vancomycin, and other classes of antibiotics (Durso et al., 2011). The detection of antibiotic resistance

starts even at an early age. Genes encoding for tetracycline (*tetG*, *tetO*, *tetW*, *tetX*), sulfonamide (*sul1*, *sul2*) and macrolide-lincosamide-streptogramin (*ermB*, *ermF*) resistance were detected in the feces of newborn dairy calves (Thames et al., 2012). In fact, even *Enterococcus* spp. from the exotic Russian tortoise displayed resistance to kanamycin, rifampicin, and vancomycin with 59% of the isolates being multi-drug resistant (Nowakiewicz et al., 2013). Therefore, it is not surprising that even fecal bacteria from control cows possessed resistance genes in the current study.

Antibiotic treatment had a significant effect on 16S rRNA abundance with elevated abundance in CEPH-treated and control cows as compared to cows treated with PIRL, suggesting reduced total bacterial counts in PIRL-treated cows. This effect was especially apparent for CEPH vs. PIRL on d 3 post-treatment and control vs. PIRL on d 5 post-treatment. Approximately 24% of the administered intramammary pirlimycin dose is excreted through the feces (Hornish et al., 1992) and exposure of gut bacteria to such a large percentage of the dose may be the cause for reduced bacterial counts in the feces. In contrast, cephapirin does not easily cross from the udder into the blood (Gehring and Smith, 2006) so the bacterial populations in the lower GI tract of the cow remain undisturbed.

Although day post-treatment did affect 16S rRNA abundance, few effects were seen in pairwise comparison of specific days. 16S rRNA gene counts tended to be greater on d 28 post-treatment than on both d 1 and d 7, which could suggest that bacterial populations are affected following antibiotic treatment but eventually return to normal. However, it is important to note that five days after treatment the cows were moved from individual stalls back into the main barn where they are group housed. This change in

housing environment also has the potential to impact the fecal microbiome as cows were again allowed contact with other members of the herd. A herd-level association of ceftiofur treatment and expanded-spectrum cephalosporin resistance was observed in dairy cows, indicating that the use of antibiotics in an individual cow can result in the production of bacterial resistance in the entire herd (Tragesser et al., 2006). The same effect could be true of bacterial diversity where, like antibiotic resistance, bacterial populations could be transferred between members of a herd.

Relative fecal abundance of *ampC* was greater on d 3 than d 5 and d 28 following treatment. Fecal excretion of bla_{CMY-2}, which codes for an ampC-like enzyme and is very closely related to *ampC* (Winokur et al., 2000), has been shown to increase shortly after antibiotic treatment (Alali et al., 2009). Since the two genes are so closely related, it makes sense that they would follow a similar fecal excretion pattern upon exposure to antibiotic treatment. Thus, it can be concluded that there is an almost immediate response of *ampC* excretion to antibiotic administration followed by a return to a baseline-level of resistance gene excretion, similar to the pattern observed in published research focused on other β-lactam resistance genes.

There tended to be an effect of antibiotic treatment on the absolute abundance of *ermB*, with CEPH having the largest effect. Since *ermB* codes for resistance to lincosamides, it was expected that the PIRL treatment would show the largest impact. However, when normalized to 16S the overall treatment effect of CEPH disappeared. Instead, CCFA treatment resulted in the highest relative *ermB* abundance of all treatments. For effect of day, CCFA treatment yielded the highest relative abundance of *ermB* on d 28 post-treatment when compared with all days. The reason for this significant

jump on day 28 is unknown and it cannot be determined if *ermB* levels remained elevated after 28 days following treatment because sampling was not continued.

The effect of antibiotic treatment on the two tetracycline resistance genes, *tetO* and *tetW*, that were analyzed differed. There was no effect of antibiotic treatment or day on the absolute or relative fecal abundance of *tetO*. However, there was a significant day effect for both absolute and relative fecal abundance of *tetW*. Both *tetO* and *tetW* genes code for ribosomal protection proteins (Chopra and Roberts, 2001). Although these genes code for the same resistance mechanism, it is interesting that they responded differently in this study.

The absolute abundance of *tetW* was significantly greater on d 28 when compared with d 1 and d 7 post-treatment. However, when normalized to 16S rRNA, *tetW* was significantly higher on d 3 than d 5 and d 28 post-treatment. It is possible that the higher absolute abundance of *tetW* on d 28 was probably due to the numerically higher abundance of 16S rRNA on d 28 (that is, increased bacteria numbers rather than bacteria with increased copies of tetracycline resistance genes). If the *tetW* increase was due to increased bacterial numbers, this indicates that increased bacterial resistance was not a result of antibiotic treatment. Therefore, it can be concluded that *tetW* fecal abundance was highest three days after antibiotic treatment before declining and remaining at a lower level for the remainder of the sampling period.

Although a tetracycline antibiotic was not administered, the increase in *tetW* relative abundance three days post-treatment could be related to the increase in *ampC* excretion on that same day. Multiple resistance genes may be carried and spread together mainly through extrachromosomal genetic elements such as plasmids (O'Brien, 2002). In

fact, tetracycline resistance genes are known to be associated with *bla* genes, including the plasmid *ampC* gene (Seiffert et al., 2013). Therefore, the *tetW* fecal excretion pattern may be influenced by the excretion of other resistance genes, resulting in increased fecal excretion shortly after antibiotic treatment followed by a low, persistent level of excretion.

CONCLUSIONS

Metagenomic techniques implemented in the current study gave a broad perspective on the effects of antibiotic treatment on the bovine fecal microbiome in terms of bacterial diversity, cell function, and antibiotic resistance profiles. Antibiotic treatment resulted in fecal bacteria that were stressed under antibiotic selection pressure, inhibiting their ability to perform normal cell functions like cell proliferation and degradation of plant products consumed by the cow. However, treatment also induced a wide variety of functions capable of aiding bacterial survival in the presence of antibiotic selection pressure, including functions that aid in biofilm formation and the ability to acquire and transfer resistance genes. In fact, treatment directly increased bacterial sequences associated with resistance to β-lactams and multidrug resistance, indicating that antibiotic treatment increases antibiotic resistance, at least for a short period of time following antibiotic treatment.

A definitive pattern of antibiotic resistance gene excretion following antibiotic treatment is hard to determine. However, the results of this study support the hypothesis that resistance gene excretion increases shortly after antibiotic treatment followed by a gradual return to the baseline level of resistance in the cow's fecal microbiome. Therefore, when determining key time points for producers to focus their efforts when

treating their herd with antibiotics, it seems that the first 5 – 7 days following treatment should be given attention. With the identification of manure treatments to degrade the excreted resistance genes, feces from those days could be isolated and further processed before land application to minimize the transfer of antibiotic resistance to other environments and hosts.

Table 1 Summary of responses to antibiotic treatment for each analysis

Analysis	Specific Measure	Effect of Treatment[1]
MG-RAST	"Virulence, disease, & defense" (VDD), as % of total	NS
	"Resistance to antibiotics & toxic compounds" (RATC), as % of VDD	CCFA > Control
	"Phages, prophages, transposable elements, plasmids" (PPTP), as % of total	CCFA > Control (trend)
	"Pathogenicity islands", as % PPTP	Control > CCFA
	"Phages, prophages", as % PPTP	CCFA > Control
BLAST/ARDB	Total ARG-like sequence abundance	NS
	β-lactam resistance, as prop. total sample sequences	CCFA > Control
	Multidrug resistance, as prop. total sample sequences	CCFA > Control
	β-lactam resistance, as % total ARG-like sequences	CCFA > Control
qPCR	Absolute & relative abundance *tetW*	NS
	Absolute & relative abundance *tetO*	NS
	Absolute & relative abundance *ampC*	NS
	Absolute abundance *ermB*	CEPH > Ctrl & PIRL (trend)
	Relative abundance *ermB*	NS

[1] Significance. $P \leq 0.10$ indicates significance. $P \leq 0.15$ indicates trend. NS indicates $P > 0.15$

Table 2 MG-RAST analysis: effect of ceftiofur crystalline-free acid antibiotic treatment on bacterial species diversity in dairy cow feces

α-Diversity Values				
Antibiotic[1]		Control[2]		P = [3]
LSM[4]	SEM[5]	LSM	SEM	Treatment
356.38	11.93	354.28	11.93	NS

[1] n = 3 subcutaneous antibiotic injection, 1.5 mL ceftiofur crystalline free acid sterile suspension (150 mg ceftiofur activity) per 45.4 kg body weight

[2] n = 3 no antibiotic treatment

[3] Significance. NS indicates $P > 0.10$

[4] Least squares means for treatment

[5] Standard error for LSM

Table 3 MG-RAST analysis: effect of ceftiofur crystalline-free acid antibiotic treatment on the abundance of bacterial cell functions in dairy cow feces

Functional sequences as a % of total sample sequences assigned function					
	Antibiotic[1]		Control[2]		P = [3]
Functional Category	LSM[4]	SEM[5]	LSM	SEM	Treatment
Amino Acids and Derivatives	9.72	0.11	9.65	0.11	NS
Carbohydrates	16.87	0.20	16.78	0.20	NS
Cell Division and Cell Cycle	1.56	0.008	1.60	0.008	< 0.06
Cell Wall and Capsule	3.74	0.11	3.71	0.11	NS
Clustering-based Subsystems	14.50	0.03	14.80	0.03	< 0.01
Cofactors, Vitamins, Prosthetic Groups, Pigments	4.45	0.09	4.48	0.09	NS
DNA Metabolism	5.16	0.03	5.25	0.03	< 0.12
Dormancy and Sporulation	0.43	0.01	0.46	0.01	NS
Fatty Acids, Lipids, and Isoprenoids	2.01	0.07	2.03	0.07	NS
Iron Acquisition and Metabolism	0.60	0.03	0.56	0.03	NS

Table 3 (Cont.) MG-RAST analysis: effect of ceftiofur crystalline-free acid antibiotic treatment on the abundance of bacterial cell functions in dairy cow feces

Functional sequences as a % of total sample sequences assigned function					
	Antibiotic[1]		Control[2]		*P* = [3]
Functional Category	LSM[4]	SEM[5]	LSM	SEM	Treatment
Membrane Transport	1.78	0.03	1.78	0.03	NS
Metabolism of Aromatic Compounds	0.44	0.006	0.47	0.006	< 0.06
Miscellaneous	6.25	0.06	6.40	0.06	NS
Motility and Chemotaxis	0.25	0.003	0.24	0.003	< 0.13
Nitrogen Metabolism	0.49	0.007	0.50	0.007	NS
Nucleosides and Nucleotides	3.72	0.07	3.50	0.07	< 0.12
Phages, Prophages, Transposable Elements, Plasmids	3.07	0.24	2.34	0.24	< 0.15
Phosphorus Metabolism	0.51	0.02	0.50	0.02	NS
Photosynthesis	0.02	0.0006	0.02	0.0006	NS
Potassium Metabolism	0.11	0.007	0.11	0.007	NS

Table 3 (Cont.) MG-RAST analysis: effect of ceftiofur crystalline-free acid antibiotic treatment on the abundance of bacterial cell functions in dairy cow feces

Functional sequences as a % of total sample sequences assigned function					
	Antibiotic[1]		Control[2]		*P* = [3]
Functional Category	LSM[4]	SEM[5]	LSM	SEM	Treatment
Protein Metabolism	11.16	0.13	11.32	0.13	NS
RNA Metabolism	6.29	0.07	6.45	0.07	NS
Regulation and Cell Signaling	0.97	0.01	0.96	0.01	NS
Respiration	1.83	0.06	1.95	0.06	NS
Secondary Metabolism	0.20	0.007	0.23	0.007	< 0.07
Stress Response	1.60	0.02	1.53	0.02	< 0.08
Sulfur Metabolism	0.55	0.02	0.54	0.02	NS
Virulence, Disease, and Defense	1.82	0.03	1.74	0.03	NS

[1] n = 3 subcutaneous antibiotic injection, 1.5 mL ceftiofur crystalline free acid sterile suspension (150 mg ceftiofur activity) per 45.4 kg body weight

[2] n = 3 no antibiotic treatment

[3] Significance. $P \leq 0.10$ indicates significance. $P \leq 0.15$ indicates trend. NS indicates $P > 0.15$

[4] Least squares means for treatment

[5] Standard error for LSM

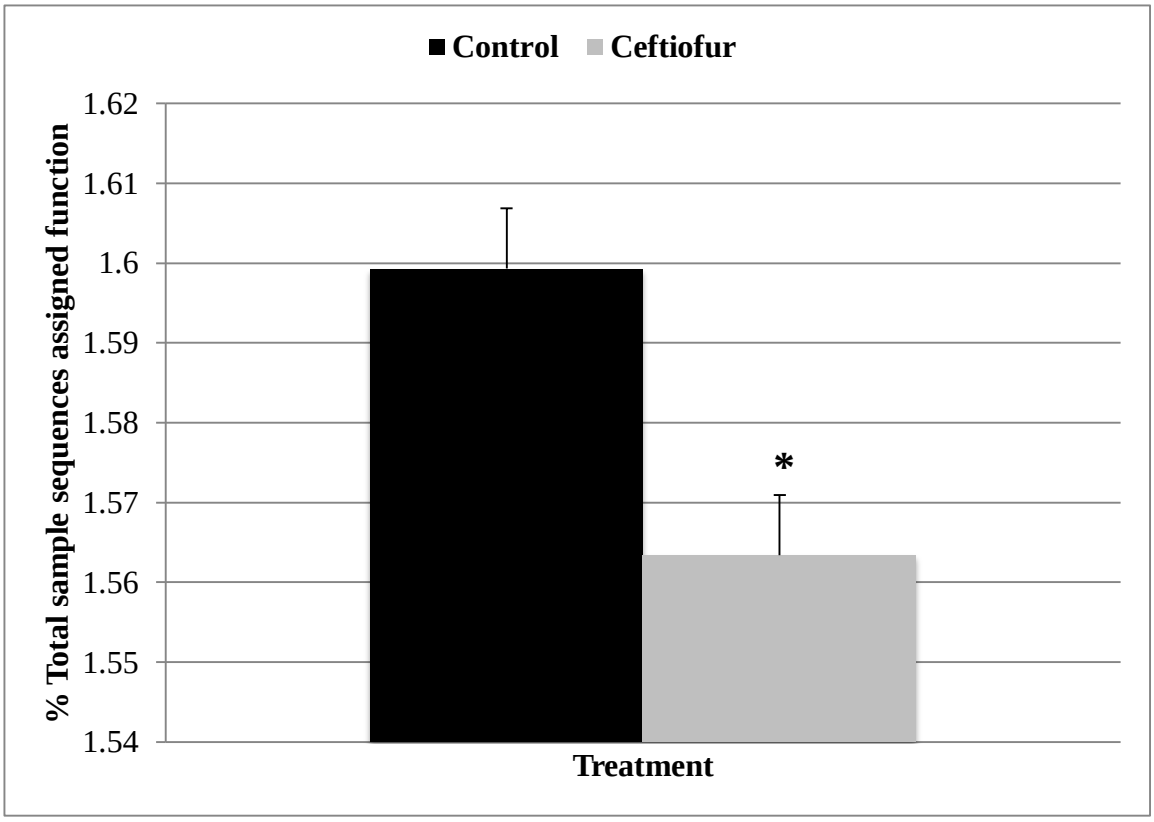


Figure 1. MG-RAST analysis: abundance of "cell division and cell cycle" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. Day -1 (pre-treatment) samples were used as a covariate. Values are expressed as a percentage of the total sample sequences assigned function. The symbol * indicates significant ($P < 0.06$). Tukey adjusted pairwise comparisons for treatment within day 3.

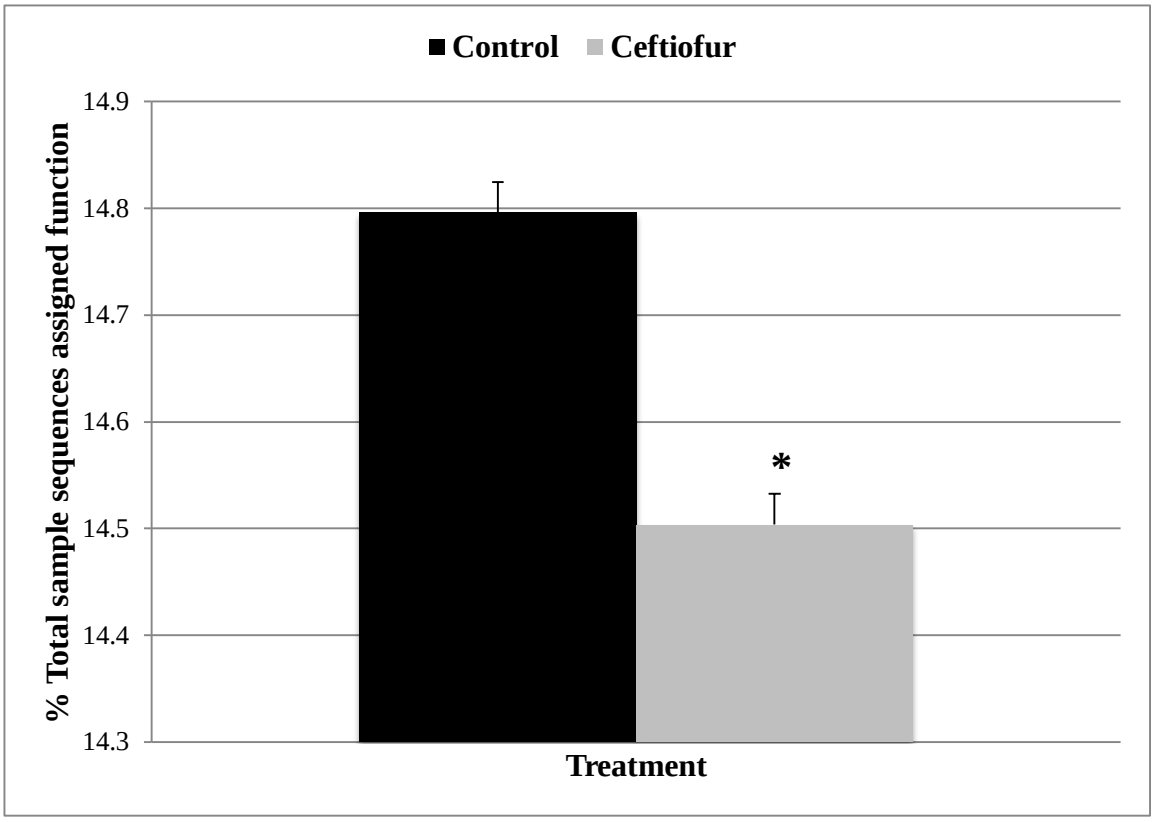


Figure 2. MG-RAST analysis: abundance of "clustering-based subsystems" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. Day -1 (pre-treatment) samples were used as a covariate. Values are expressed as a percentage of the total sample sequences assigned function. The symbol * indicates significant ($P < 0.01$). Tukey adjusted pairwise comparisons for treatment within day 3.

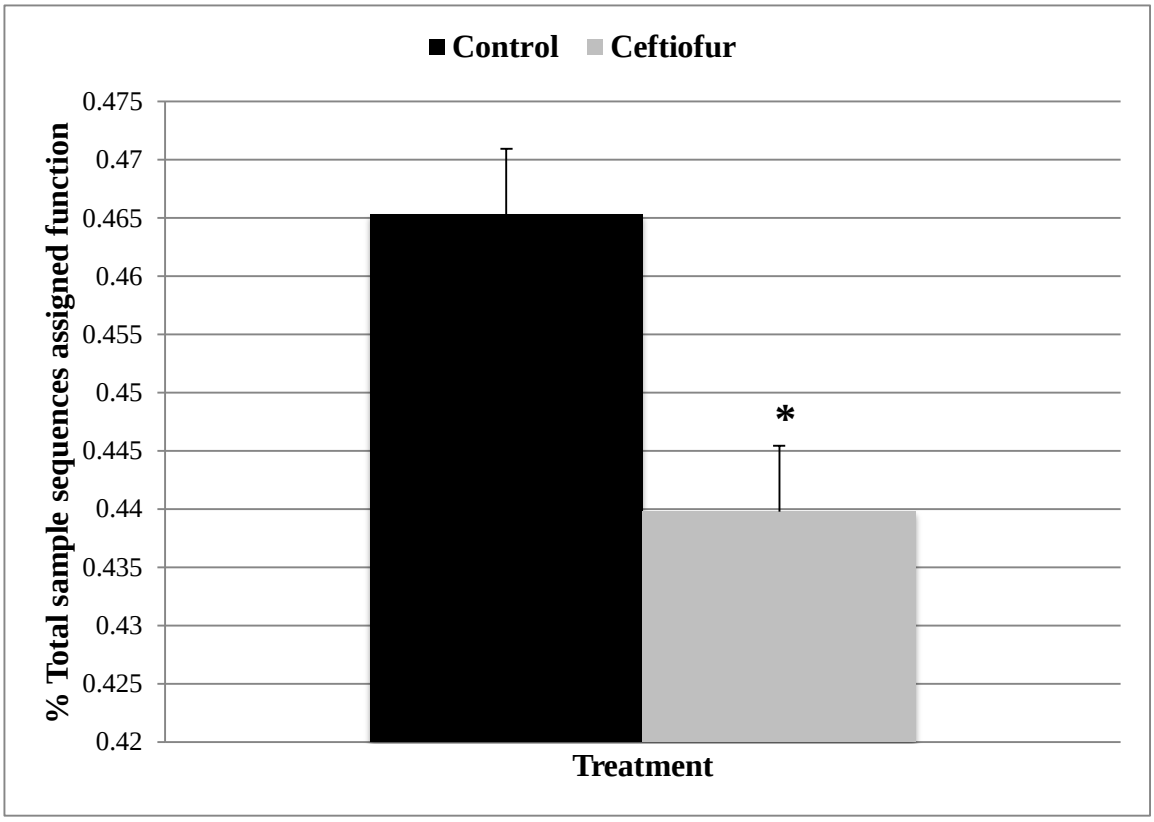


Figure 3. MG-RAST analysis: abundance of "metabolism of aromatic compounds" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. Day -1 (pre-treatment) samples were used as a covariate. Values are expressed as a percentage of the total sample sequences assigned function. The symbol * indicates significant ($P < 0.06$). Tukey adjusted pairwise comparisons for treatment within day 3.

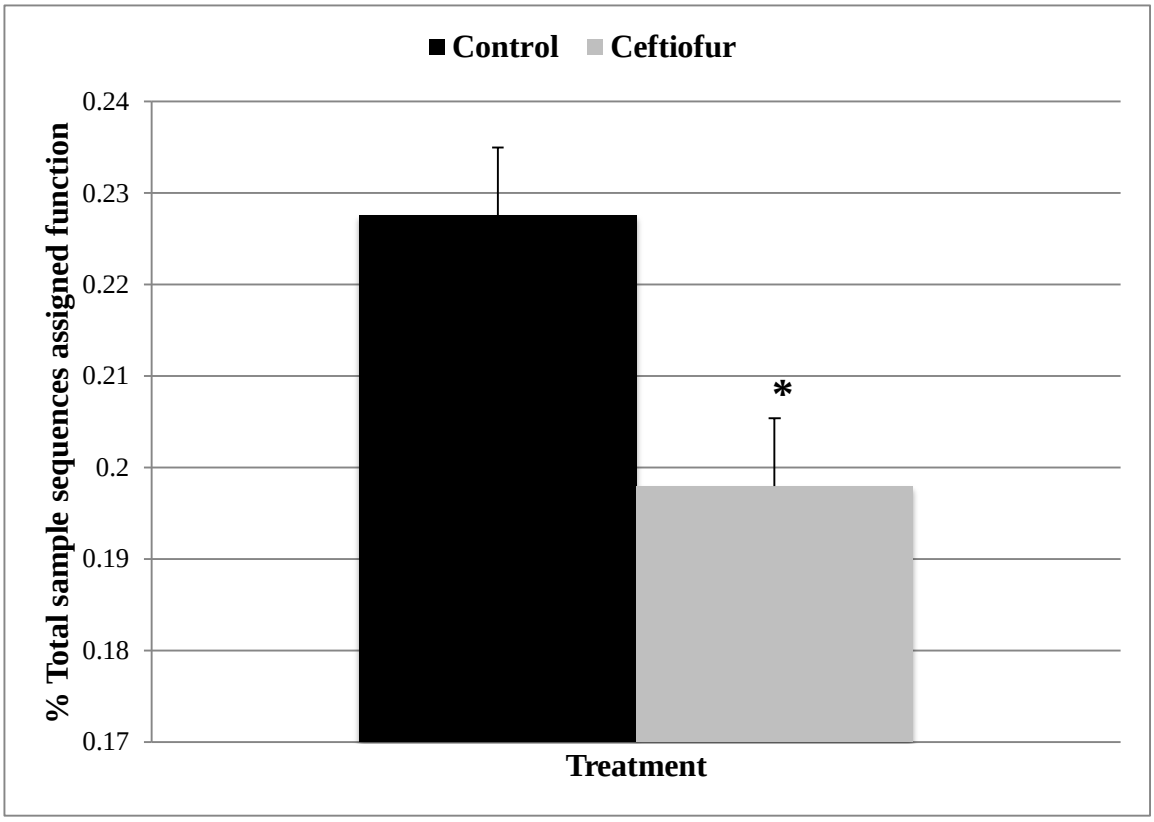


Figure 4. MG-RAST analysis: abundance of "secondary metabolism" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. Day -1 (pre-treatment) samples were used as a covariate. Values are expressed as a percentage of the total sample sequences assigned function. The symbol * indicates significant ($P < 0.07$). Tukey adjusted pairwise comparisons for treatment within day 3.

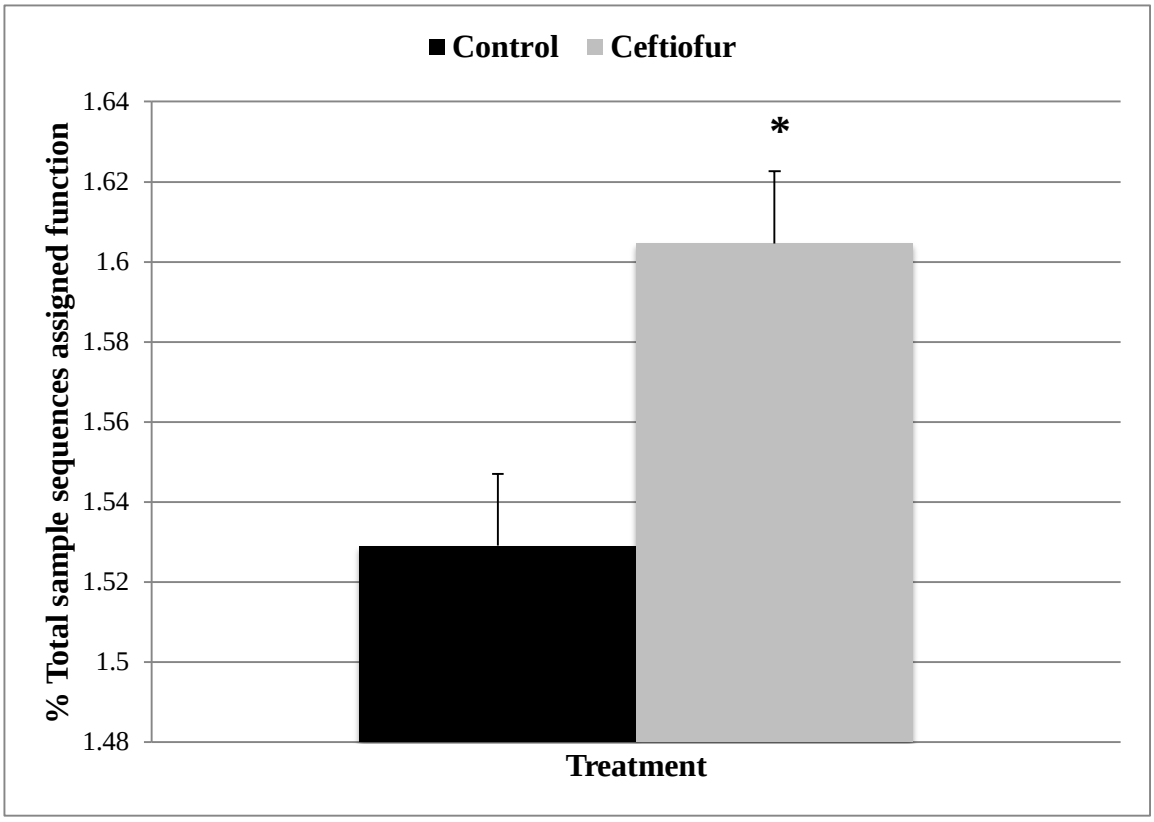


Figure 5. MG-RAST analysis: abundance of "stress response" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. Day -1 (pre-treatment) samples were used as a covariate. Values are expressed as a percentage of the total sample sequences assigned function. The symbol * indicates significant ($P < 0.07$). Tukey adjusted pairwise comparisons for treatment within day 3.

Table 4 MG-RAST analysis: effect of ceftiofur crystalline-free acid antibiotic treatment on the abundance of "phages, prophages, transposable elements, plasmids" sequences in dairy cow feces

Sequence abundance as a % of "phages, prophages, transposable elements, plasmids" total sequences					
	Antibiotic[1]		Control[2]		P = [3]
Category	LSM[4]	SEM[5]	LSM	SEM	Treatment
Gene Transfer Agent	0.0043	0.0009	0.0039	0.0009	NS
Pathogenicity Islands	11.60	0.61	14.41	0.61	< 0.06
Phages, Prophages	83.14	1.36	78.21	1.36	< 0.11
Plasmid Related Functions	0.0022	0.0004	0.0025	0.0004	NS
Transposable Elements	5.58	0.84	7.04	0.84	NS
Other	0.008	0.002	0.005	0.002	NS

[1] n = 3 subcutaneous antibiotic injection, 1.5 mL ceftiofur crystalline free acid sterile suspension (150 mg ceftiofur activity) per 45.4 kg body weight

[2] n = 3 no antibiotic treatment

[3] Significance. NS indicates $P > 0.10$

[4] Least squares means for treatment

[5] Standard error for LSM

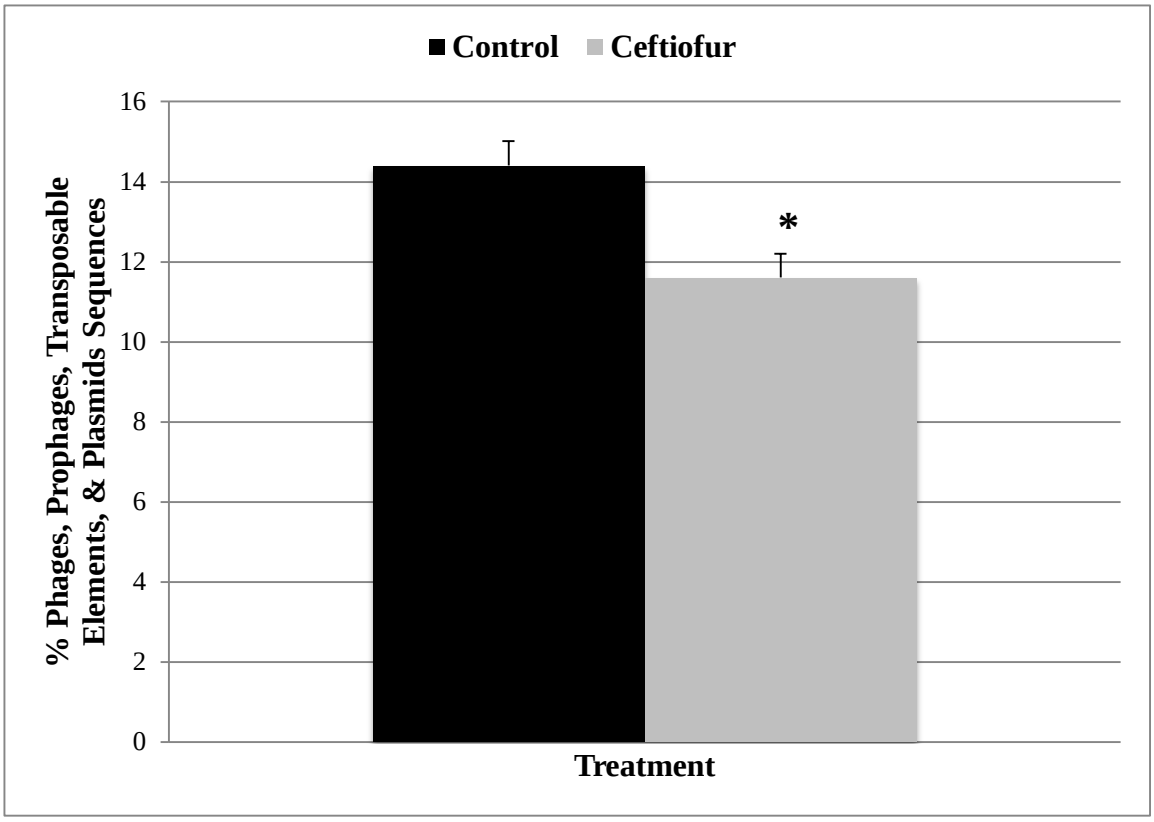


Figure 6. MG-RAST analysis: abundance of "pathogenicity islands" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. Day -1 (pre-treatment) samples were used as a covariate. Values are expressed as a percentage of the total "phages, prophages, transposable elements, plasmids" sequences. The symbol * indicates significant ($P < 0.06$). Tukey adjusted pairwise comparisons for treatment within day 3.

Table 5 MG-RAST analysis: effect of ceftiofur crystalline-free acid antibiotic treatment on the abundance of "virulence, disease, and defense" sequences in dairy cow feces

Sequence abundance as a % of "virulence, disease, and defense" total sequences					
	Antibiotic[1]		Control[2]		*P* = [3]
Category	LSM[4]	SEM[5]	LSM	SEM	Treatment
Adhesion	2.97	0.27	3.00	0.27	NS
Bacteriocins, Ribosomally Synthesized Antibacterial Peptides	0.07	0.005	0.08	0.005	NS
Detection	10.29	0.26	10.86	0.26	NS
Invasion and Intracellular Resistance	1.41	0.22	1.64	0.22	NS
Resistance to Antibiotics and Toxic Compounds (RATC)	73.85	0.10	71.92	0.10	< 0.01
Toxins and Superantigens	0.17	0.02	0.21	0.02	NS
Other	11.36	0.30	12.15	0.30	NS

[1] n = 3 subcutaneous antibiotic injection, 1.5 mL ceftiofur crystalline free acid sterile suspension (150 mg ceftiofur activity) per 45.4 kg body weight

[2] n = 3 no antibiotic treatment

[3] Significance. NS indicates $P > 0.10$

[4] Least squares means for treatment

[5] Standard error for LSM

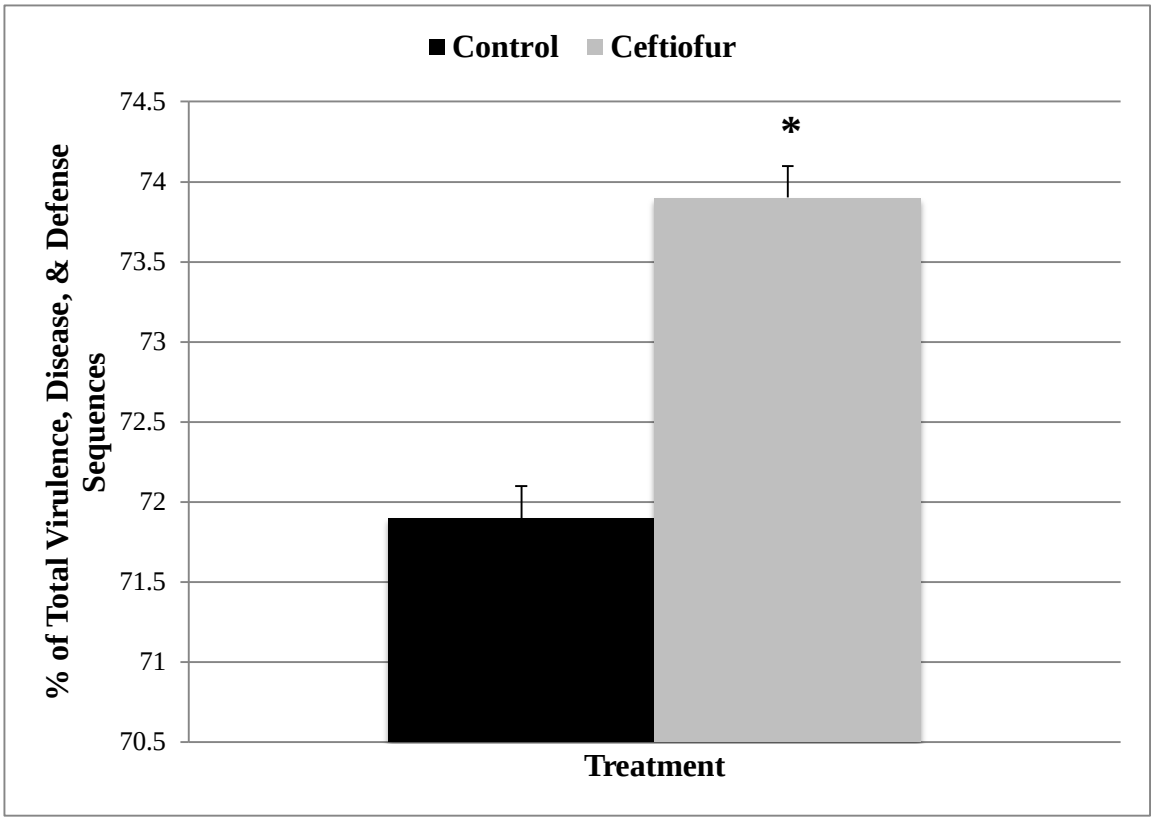


Figure 7. MG-RAST analysis: abundance of "resistance to antibiotics and toxic compounds (RATC)" sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. Day -1 (pre-treatment) samples were used as a covariate. Values are expressed as a percentage of the total "virulence, disease, and defense" sequences. The symbol * indicates significant ($P < 0.01$). Tukey adjusted pairwise comparisons for treatment within day 3.

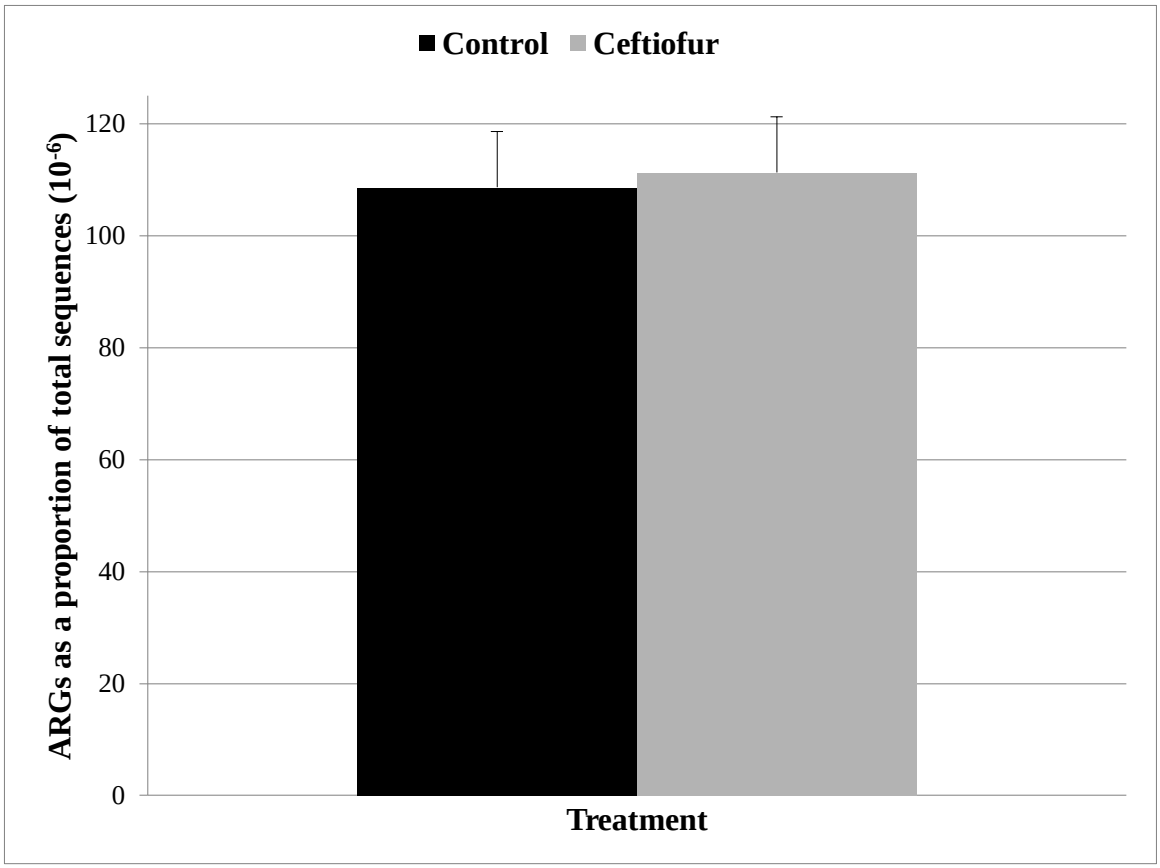


Figure 8. BLAST/ARDB analysis: abundance of antibiotic resistance gene (ARG)-like sequences in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. Day -1 (pre-treatment) samples were used as a covariate. Values are expressed as a proportion of the total sample sequences (x 10^{-6}). There was no effect of antibiotic treatment on the abundance of ARG-like sequences as a proportion of total sample sequences.

Table 6 BLAST/ARDB analysis: effect of ceftiofur crystalline free acid antibiotic treatment on the abundance of resistance gene sequences in dairy cow feces

Resistance gene sequences as a proportion of total sample sequences (x 10^6)

	Antibiotic[1]		Control[2]		P = [3]
Class	LSM[4]	SEM[5]	LSM	SEM	Treatment
Bacitracin	1.15	0.19	1.15	0.19	NS
β-lactam	12.74	0.38	6.78	0.38	< 0.01
MLS[6]	5.62	0.80	4.19	0.80	NS
Multidrug	8.24	0.71	3.24	0.71	< 0.02
Tetracycline	86.41	9.37	89.40	9.37	NS

[1] n = 3 subcutaneous antibiotic injection, 1.5 mL ceftiofur crystalline free acid sterile suspension (150 mg ceftiofur activity) per 45.4 kg body weight

[2] n = 3 no antibiotic treatment

[3] Significance. NS indicates $P > 0.10$

[4] Least squares means for treatment

[5] Standard error for LSM

[6] MLS = Macrolide-lincosamide-streptogramin

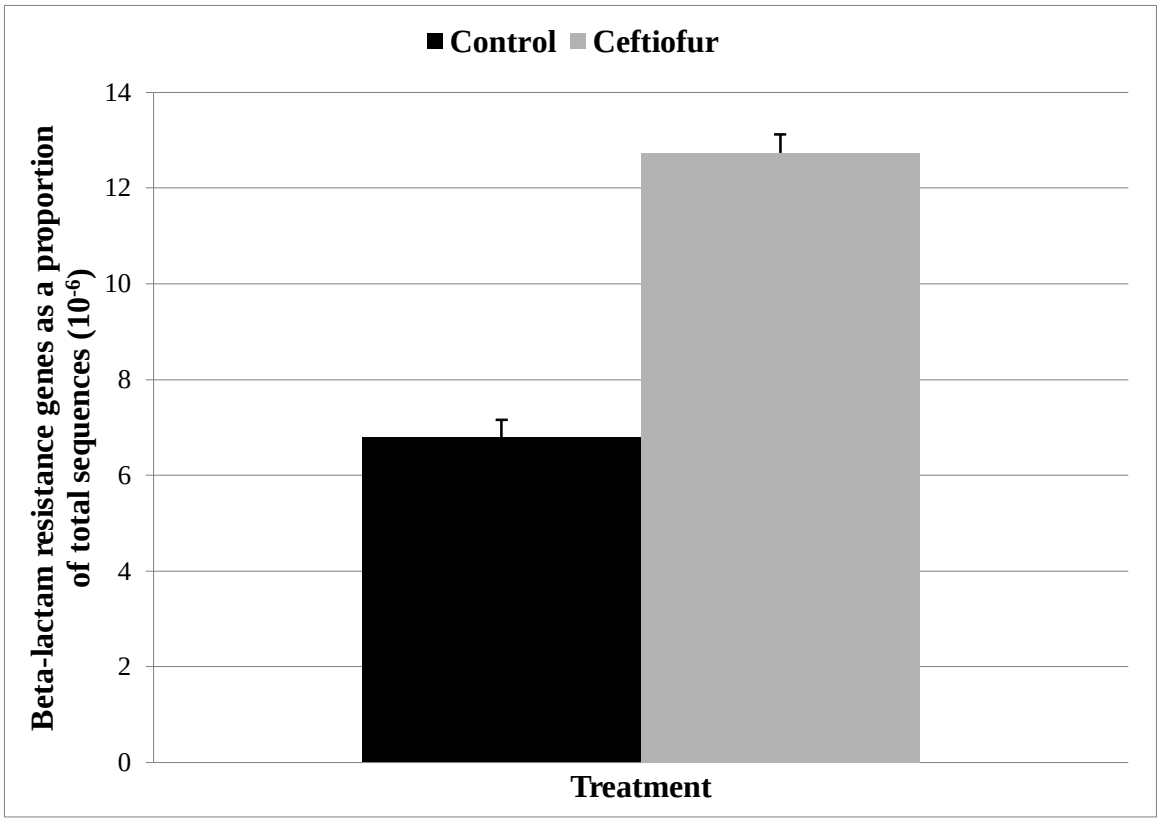


Figure 9. BLAST/ARDB analysis: abundance of beta-lactam resistance genes in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. Day -1 (pre-treatment) samples were used as a covariate. Values are expressed as a percentage of the total sample sequences (x 10^{-6}). The symbol * indicates significant ($P < 0.01$). Tukey adjusted pairwise comparisons for treatment within day 3.

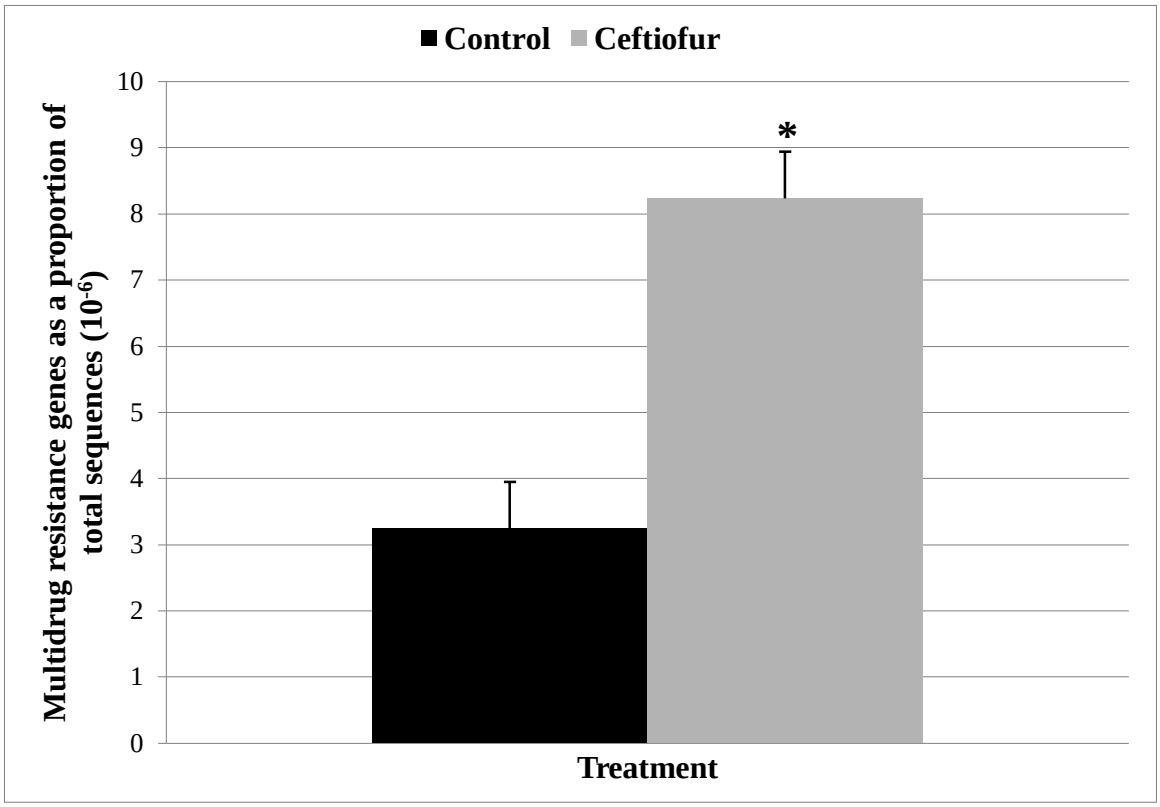


Figure 10. BLAST/ARDB analysis: abundance of multidrug resistance genes in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. Day -1 (pre-treatment) samples were used as a covariate. Values are expressed as a percentage of the total sample sequences (x 10^{-6}). The symbol * indicates significant ($P < 0.02$). Tukey adjusted pairwise comparisons for treatment within day 3.

Table 7 BLAST/ARDB analysis: effect of ceftiofur crystalline free acid antibiotic treatment on the proportion of resistance gene sequences in dairy cow feces

Resistance gene sequences as a % of total ARG-like sequences

	Antibiotic[1]		Control[2]		P = [3]
Class	LSM[4]	SEM[5]	LSM	SEM	Treatment
Bacitracin	1.00	0.14	1.08	0.14	NS
β-lactam	11.45	0.47	6.09	0.47	< 0.01
MLS[6]	4.82	0.66	4.12	0.66	NS
Multidrug	6.14	1.22	4.32	1.22	NS
Tetracycline	75.32	3.12	84.74	3.12	NS

[1] n = 3 subcutaneous antibiotic injection, 1.5 mL ceftiofur crystalline free acid sterile suspension (150 mg ceftiofur activity) per 45.4 kg body weight

[2] n = 3 no antibiotic treatment

[3] Significance. NS indicates $P > 0.10$

[4] Least squares means for treatment

[5] Standard error for LSM

[6] MLS = Macrolide-lincosamide-streptogramin

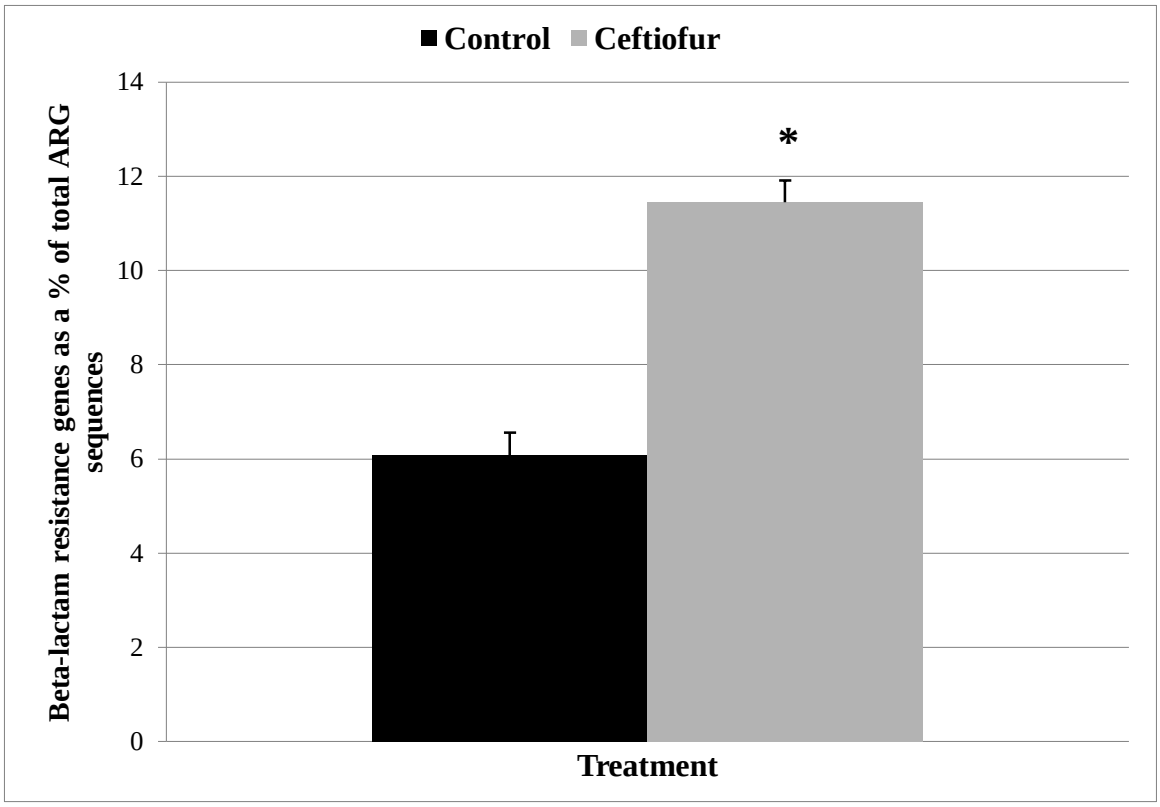


Figure 11. BLAST/ARDB analysis: abundance of beta-lactam resistance genes in fecal samples collected from control (n=3) and ceftiofur crystalline free acid treated (n=3) cows on day 3 post-treatment. Day -1 (pre-treatment) samples were used as a covariate. Values are expressed as a percentage of the total ARG-like sequences (x 10^{-6}). The symbol * indicates significant ($P < 0.01$). Tukey adjusted pairwise comparisons for treatment within day 3.

Table 8 qPCR analysis: effect of antibiotic treatment on antibiotic resistance gene absolute abundance in dairy cow feces

Gene copies (log10) per gram of feces

	Ceftiofur[1]		Pirlimycin[2]		Cephapirin[3]		Control[4]		*P* =[5]		
Gene	LSM[6]	SEM[7]	LSM	SEM	LSM	SEM	LSM	SEM	Treatment	Day	Interaction[8]
16S rRNA	11.18	0.10	10.94	0.10	11.48	0.11	11.40	0.10	< 0.03	< 0.08	NS
tetO	10.12	0.24	9.82	0.20	10.11	0.24	10.02	0.20	NS	NS	NS
tetW	10.11	0.14	10.29	0.16	10.25	0.16	10.09	0.14	NS	< 0.01	NS
ampC	7.78	0.20	7.82	0.20	8.18	0.21	8.13	0.20	NS	NS	NS
ermB	5.46	0.17	5.19	0.17	5.81	0.17	5.20	0.16	< 0.11	NS	NS

[1] n = 3 subcutaneous antibiotic injection, 1.5 mL ceftiofur crystalline free acid sterile suspension (150 mg ceftiofur activity) per 45.4 kg body weight

[2] n = 3 intramammary antibiotic infusion, 10 mL of pirlimycin hydrochloride (50 mg pirlimycin activity)

[3] n = 3 intramammary antibiotic infusion, 10 mL cephapirin benzathine (300 mg cephapirin activity)

[4] n = 3 no antibiotic treatment

[5] Significance. $P \leq 0.10$ indicates significance. $P \leq 0.15$ indicates trend. NS indicates $P > 0.15$

[6] Least squares means for treatment

[7] Standard error for treatment LSM

[8] Two way interaction of treatment and day

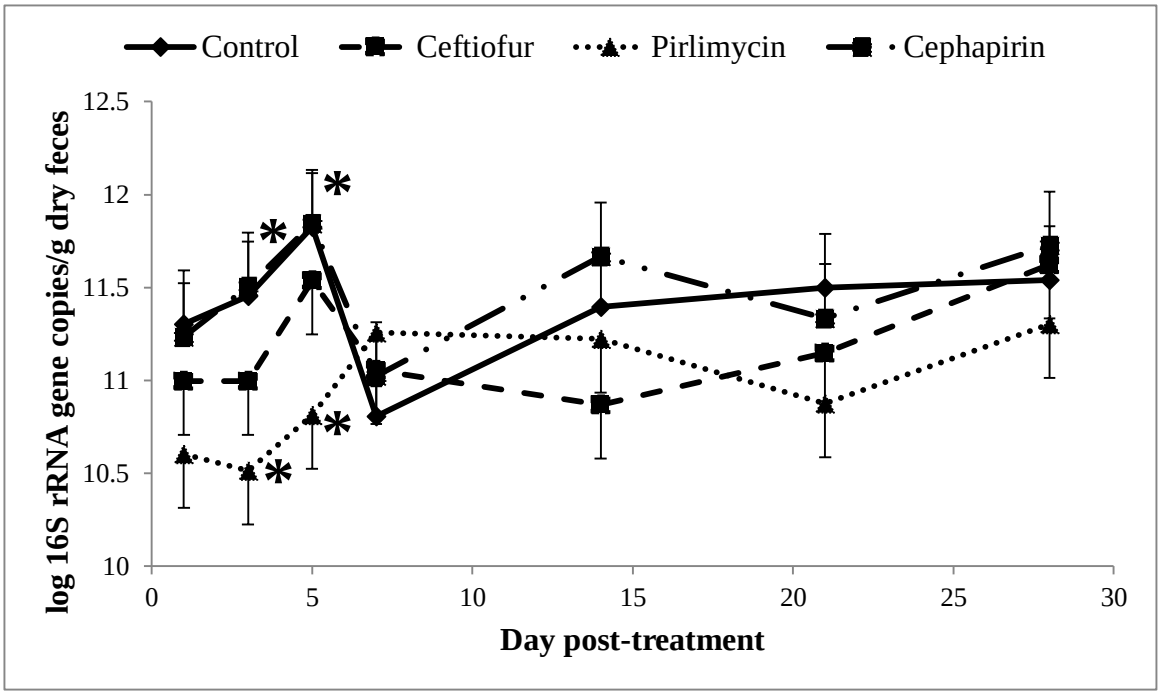


Figure 12. qPCR analysis: abundance of 16S rRNA (log10 gene copies per 1.0 g freeze-dried feces) in fecal samples. Samples were collected from control (n = 3), ceftiofur crystalline free acid treated (n = 3), pirlimycin hydrochloride treated (n = 3), and cephapirin benzathine treated (n = 3) cows. Day -1 (pre-treatment) samples were used as a covariate. The symbol * indicates significant ($P < 0.10$) Tukey adjusted pairwise comparisons for treatment within day.

Table 9 qPCR analysis: effect of antibiotic treatment on antibiotic resistance gene relative abundance in dairy cow feces

Gene copies per 16S rRNA

	Ceftiofur[1]		Pirlimycin[2]		Cephapirin[3]		Control[4]		*P* =[5]		
Gene	LSM[6]	SEM[7]	LSM	SEM	LSM	SEM	LSM	SEM	Treatment	Day	Interaction[8]
tetO	0.09	0.10	0.23	0.09	0.13	0.09	0.18	0.10	NS	NS	NS
tetW	0.13	0.05	0.25	0.05	0.09	0.05	0.06	0.05	NS	< 0.04	NS
ampC	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	NS	< 0.04	NS
ermB	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	NS	NS	NS

[1] n = 3 subcutaneous antibiotic injection, 1.5 mL ceftiofur crystalline free acid sterile suspension (150 mg ceftiofur activity) per 45.4 kg body weight

[2] n = 3 intramammary antibiotic infusion, 10 mL of pirlimycin hydrochloride (50 mg pirlimycin activity)

[3] n = 3 intramammary antibiotic infusion, 10 mL cephapirin benzathine (300 mg cephapirin activity)

[4] n = 3 no antibiotic treatment

[5] Significance. NS indicates $P > 0.10$

[6] Least squares means for treatment

[7] Standard error for treatment LSM

[8] Two way interaction of treatment and day

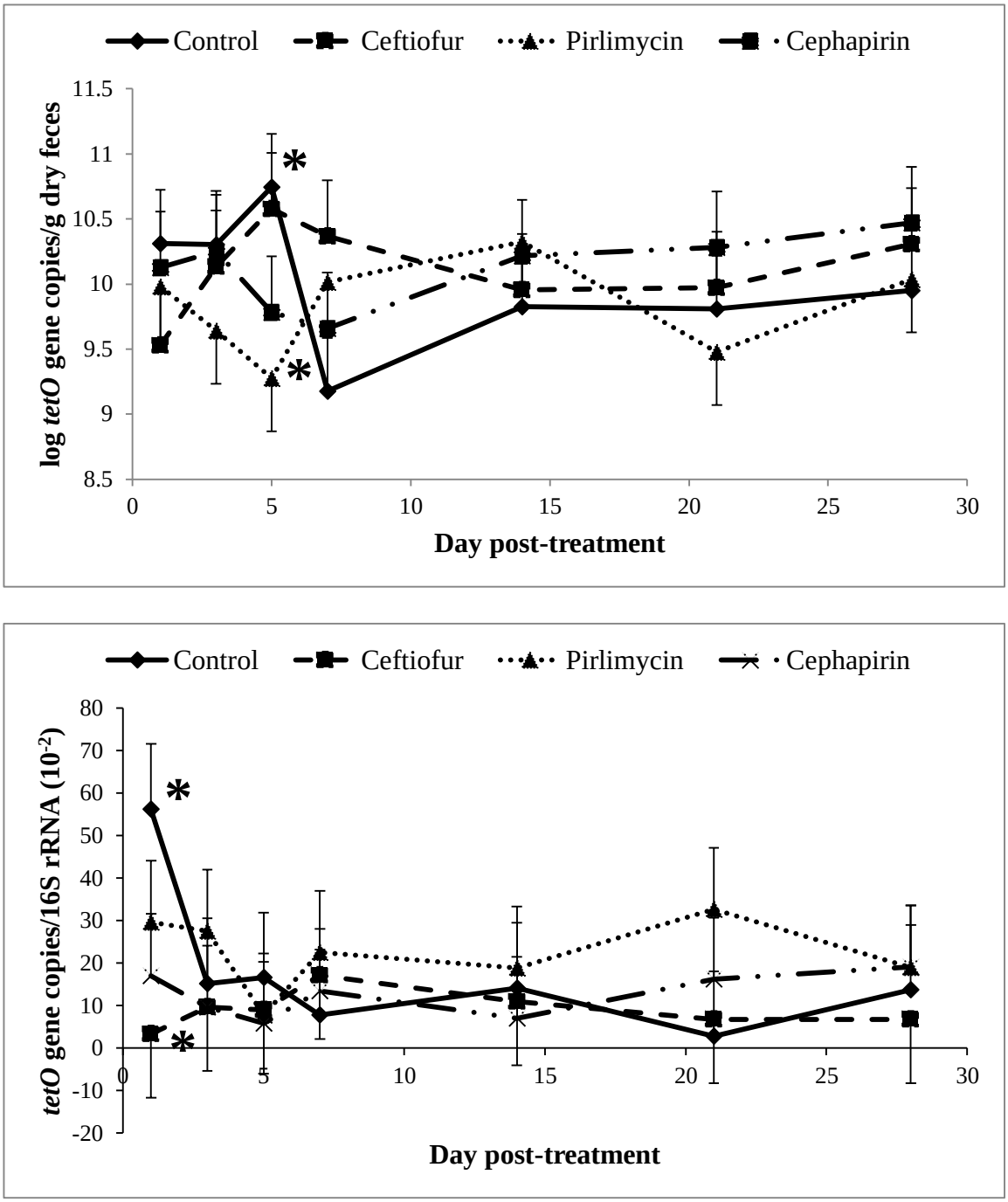
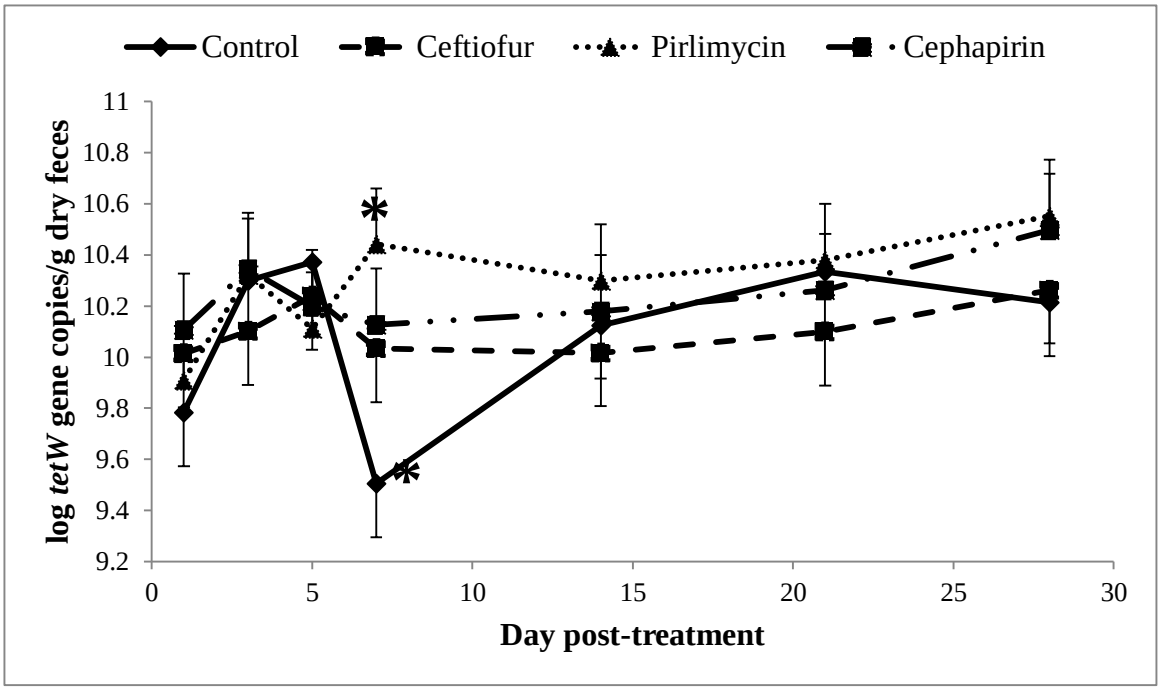


Figure 13. qPCR analysis: (A) Abundance of *tetO* (log10 gene copies per 1.0 g freeze-dried feces) in fecal samples. (B) Relative abundance of *tetO* (gene copies/ 16S rRNA) in fecal samples collected. (Values expressed as x 10^{-2}.) Samples were collected from control (n = 3), ceftiofur crystalline free acid treated (n = 3), pirlimycin hydrochloride

treated (n = 3), and cephapirin benzathine treated (n = 3) cows. Day -1 (pre-treatment) samples were used as a covariate. The symbol * indicates significant ($P < 0.10$) Tukey adjusted pairwise comparisons for treatment within day.



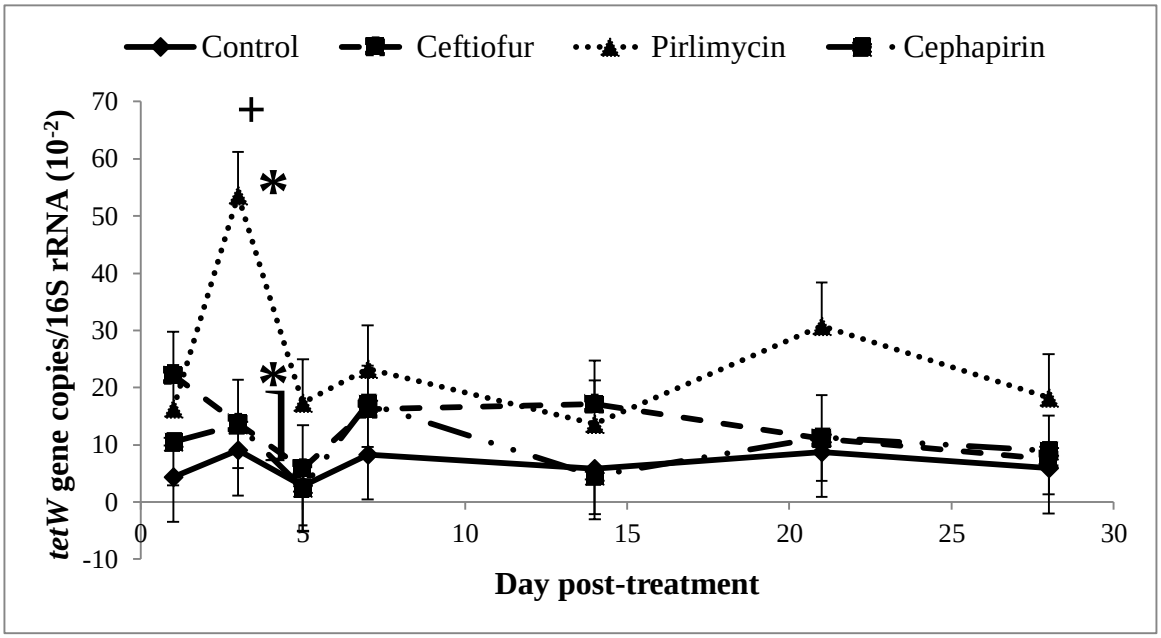


Figure 14. qPCR analysis: (A) Abundance of *tetW* (log10 gene copies per 1.0 g freeze-dried feces) in fecal samples. (B) Relative abundance of *tetW* (gene copies/ 16S rRNA) in fecal samples collected. (Values expressed as x 10^{-2}.) Samples were collected from control (n = 3), ceftiofur crystalline free acid treated (n = 3), pirlimycin hydrochloride treated (n = 3), and cephapirin benzathine treated (n = 3) cows. Day -1 (pre-treatment)

samples were used as a covariate. The symbol * indicates significant ($P < 0.05$) Tukey adjusted pairwise comparisons for treatment within day. The symbol + indicates significant ($P < 0.10$) Tukey adjusted pairwise comparisons for day across all treatments.

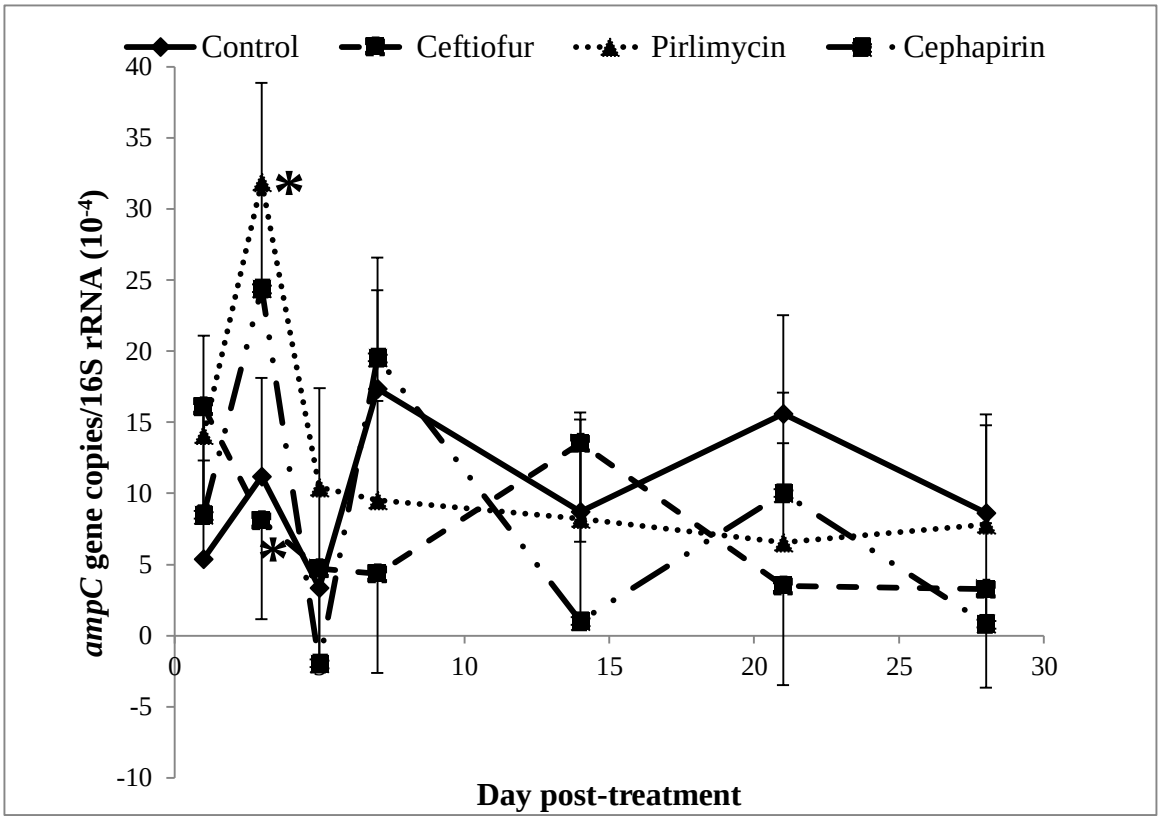


Figure 15. qPCR analysis: relative abundance of *ampC* (gene copies/ 16S rRNA) in collected fecal samples. (Values expressed as x 10^{-4}.) Samples were collected from control (n = 3), ceftiofur crystalline free acid treated (n = 3), pirlimycin hydrochloride treated (n = 3), and cephapirin benzathine treated (n = 3) cows. Day -1 (pre-treatment) samples were used as a covariate. The symbol * indicates significant ($P < 0.10$) Tukey adjusted pairwise comparisons for treatment within day.

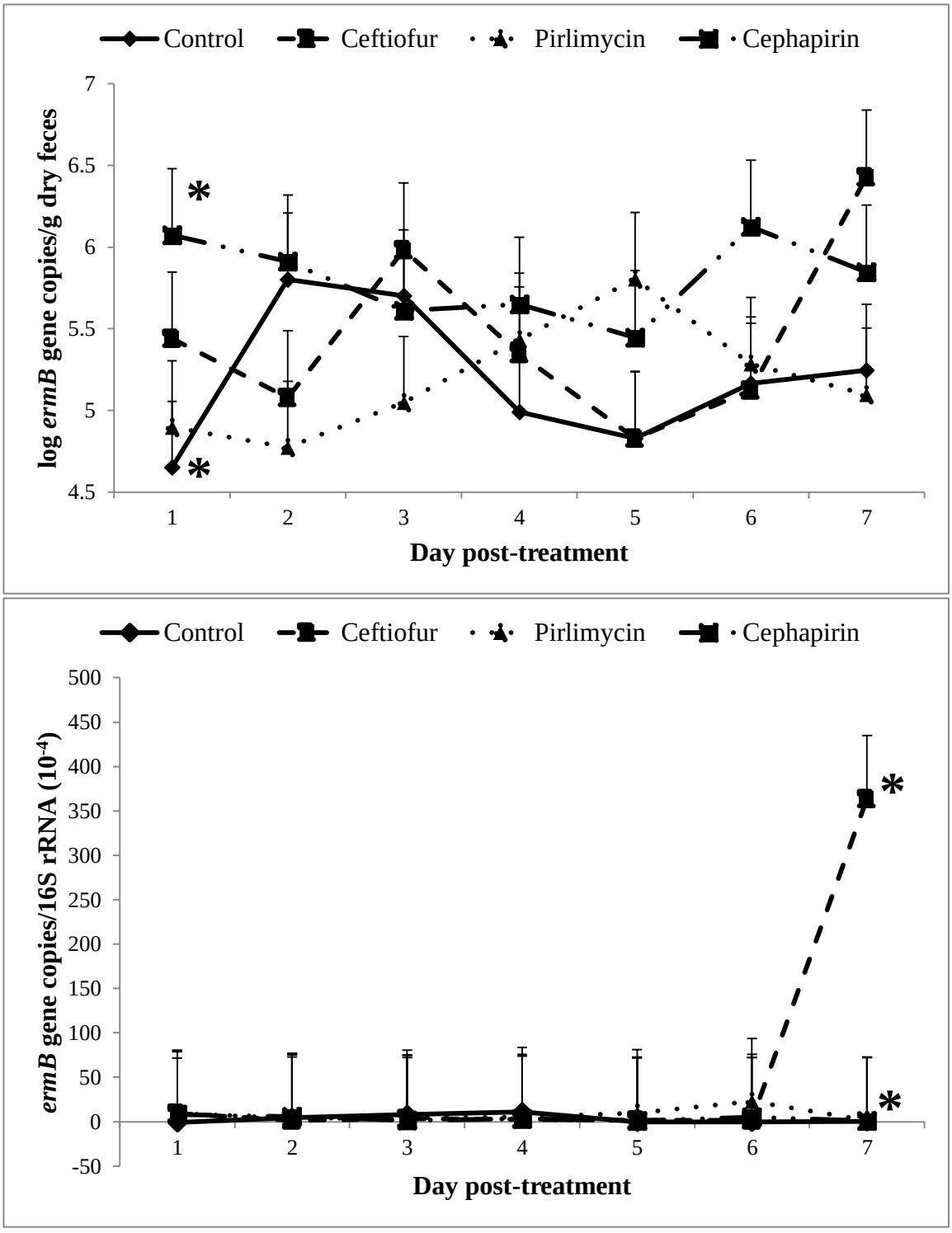


Figure 16. qPCR analysis: (A) Abundance of *ermB* (log10 gene copies per 1.0 g freeze-dried feces) in fecal samples. (B) Relative abundance of *ermB* (gene copies/ 16S rRNA) in collected fecal samples. (Values expressed as x 10^{-4}.) Samples were collected from

control (n = 3), ceftiofur crystalline free acid treated (n = 3), pirlimycin hydrochloride treated (n = 3), and cephapirin benzathine treated (n = 3) cows. Day -1 (pre-treatment) samples were used as a covariate. The symbol * indicates significant ($P < 0.10$) Tukey adjusted pairwise comparisons for treatment within day.

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APPENDIX

Forward and reverse primer sequences utilized in qPCR analysis

Primer	Target gene	Primer sequence 5' – 3'	Reference
1369F	16S rRNA	CGGTGAATACGTTCYCGG	(Suzuki et al., 2000)
1492R	16S rRNA	GGWTACCTTGTTACGACTT	
ampC-Fw	ampC	CCTCTTGCTCCACATTTGCT	(Shi et al., 2013)
ampC-Rv	ampC	ACAACGTTTGCTGTGTGACG	
tetO-Fw	tetO	ACGGARAGTTTATTGTATACC	(Aminov et al., 2001)
tetO-Rv	tetO	TGGCGTATCTATAATGTTGAC	
tetW-Fw	tetW	GAGAGCCTGCTATATGCCAGC	(Aminov et al., 2001)
tetW-Rv	tetW	GGGCGTATCCACAATGTTAAC	
ermB-Fw	ermB	GAATCCTTCTTCAACAATCA	(Jacob et al., 2008)
ermB-Rv	ermB	ACTGAACATTCGTGTCACTT	