

**Abundance of Antibiotic Resistance Genes in Feces Following Prophylactic and
Therapeutic Intramammary Antibiotic Infusion in Dairy Cattle**

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ABSTRACT

Abundance of Antibiotic Resistance Genes in Feces Following Prophylactic and Therapeutic Intramammary Antibiotic Infusion in Dairy Cattle

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Prophylactic and therapeutic antibiotic treatments have the potential to increase excretion of antibiotic resistance genes (ARGs) by dairy cattle through selection pressure on the gut microbiome. The objective of these studies was to evaluate the effect of cephalosporin benzathine administered prophylactically at the end of lactation and pirlimycin hydrochloride administered therapeutically during a clinical mastitis infection on the abundance and relative abundance of ARGs in dairy cow feces. For prophylactic treatment using cephalosporin benzathine, nineteen end-of-lactation cows were used. Treatment cows (n = 9) received cephalosporin benzathine as an intramammary infusion prior to dry-off, and control cows (n = 10) received no antibiotics. All cows received an internal non-antibiotic teat sealant. Fecal grab samples were collected for each cow on d -2 (baseline, used as covariate), d 1, 3, 5, 7, and once per week until d 49. Fecal samples were collected in sterile containers, then freeze-dried and subject to DNA extraction. The abundance of *ampC*, *bla_{CMY-2}*, *ermB*, *sulI*, *tetO*, *tetW*, integrase-specific gene *int1*, and 16S rRNA were quantified using quantitative polymerase chain reaction (qPCR). The genes *ampC* and *bla_{CMY-2}* encode resistance to β -lactam antibiotics, *ermB* to macrolides, *sulI* to sulfonamides, *tetO* and *tetW* to tetracyclines, and *int1* a class-1 integrase gene that facilitates horizontal transfer of ARGs across bacteria. The 16S rRNA gene was used as a representation of bacterial population. Absolute abundance was defined as number of ARG copies per gram of freeze-dried feces, while relative abundance was defined as ARG copy numbers per copy of 16S rRNA gene, which is indicative of the proportion of bacteria carrying ARGs. Non-normal data were

logarithmically transformed and were statistically analyzed using PROC GLIMMIX in SAS 9.2. Abundance and relative abundance of *sull* and *bla_{CMY-2}* were below the limit of quantification in most samples and therefore not suitable for statistical comparisons. The *int1* gene was not detectable in any sample. There were significant interactions between treatment and day for the abundance and relative abundance of *ampC*, *tetO*, and *tetW*. The abundance and relative abundance of *ampC* increased with time in control cows while remaining constant in antibiotic treated cows through the dry period. Antibiotics may act to stabilize the gut microbiome in response to diet and housing changes. There was a significant main effect of treatment for *ermB* with a significantly greater proportion of bacteria carrying *ermB* in control cows when compared to antibiotic treated cows. The tetracycline resistance genes *tetO* and *tetW* behaved similarly with a significant treatment by day interaction for the abundance and relative abundance of both genes. The relative abundance of both *tetO* and *tetW* were greater in control cows when compared to antibiotic treated cows on days 3, 5, 7, and 14. The abundance of both *tetO* and *tetW* resistance genes increased in antibiotic treated cows from day 1 to 49. There was also a significant increase in *tetW* relative abundance when comparing day 1 to 49. Administering long-acting antibiotics as intramammary dry treatment changed fecal bacteria composition during the dry period perhaps by stabilizing GI bacteria through dietary and housing changes. However, the use of prophylactic dry cow treatment does not uniformly or predictably lead to changes in fecal ARGs.

In a second study, after clinical mastitis detection and identification, 6 lactating dairy cows received therapeutic mastitis treatment (pirlimycin hydrochloride as an intramammary infusion). Fecal grab samples were collected from each cow on d 0, 3, 9, and 12. Collection and analytical methods were as previously described. Abundance and relative abundance of *sull* and

bla_{CMY-2} were again below the limit of quantification and therefore not suitable for statistical comparison. The *int1* gene was not detected in any sample. The abundance of 16S rRNA genes decreased with day and relative abundance *ermB*, *tetO*, and *tetW* increased with day. There was no significant effect of day on the relative abundance of *ampC* or the abundance of *ampC*, *ermB*, *tetO*, and *tetW* in feces of cows with clinical mastitis. Administering fast-acting antibiotics as therapeutic intramammary mastitis treatment to dairy cows increased the relative abundance (gene copies per 16S rRNA) of selected ARGs but not the total abundance of ARGs in feces. The use of antibiotics for prevention and treatment of bacterial infections does not uniformly or predictably increase ARGs.

Key words: antibiotic resistance gene, prophylactic, therapeutic

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Chapter 1 INTRODUCTION

Emerging concerns about antibiotic resistance and the genes encoding resistance have gained much interest in the past twenty years. The World Health Organization (WHO) has deemed antimicrobial resistance a global challenge and expressed a need for containment (WHO., 2000). The WHO calls antibiotic resistance “a natural, unstoppable phenomenon exacerbated by the abuse, overuse, and misuse of antimicrobials in the treatment of human illness and in animal husbandry, aquaculture, and agriculture.” The WHO’s five-point plan, Wisely and Widely Points for Action, calls for the reduction of antimicrobial use in animal agriculture. Antibiotic resistance genes (ARG) themselves are considered an emerging environmental contaminant because ARGs behave similarly to other environmental contaminants and can be perpetuated in the environment because they persist when the host dies and can be transferred to other bacteria (Pruden et al., 2006).

Antimicrobials have been used for disease treatment (therapeutic use), disease prevention (prophylactic use), and as growth promotants in animal agriculture since the late 1940s (Gustafson and Bowen, 1997; Aarestrup, 1999). Antimicrobial use can affect the composition of the gastrointestinal (GI) microbiome, as the GI tract is the largest reservoir of commensal bacteria. Antimicrobials can alter endogenous populations of bacteria by bactericidal or bacteriostatic properties and also facilitate change in the genetic composition of bacteria through selective pressure (Dibner and Richards, 2005). Such selective pressures increase the expression, horizontal gene transfer, persistence, and transport of ARGs (Van den Bogaard and Stobberingh, 2000). Antibiotic resistance genes may be carried by pathogenic and non-pathogenic bacteria, and by Gram-positive and Gram-negative bacteria. The selective pressures caused by

antimicrobials allow resistant bacteria to flourish in a less competitive environment (Van den Bogaard and Stobberingh, 2000; Kolář et al., 2001).

The proliferation of ARGs through bacteria is a major concern to human and animal health. Antibiotics may cause resistance in the normal flora and/or pathogenic bacterial strains relevant to human and animal medicine (Barbosa and Levy, 2000; Van den Bogaard and Stobberingh, 2000). Bacteria can assimilate ARGs from extracellular DNA in the environment and can transfer resistance genes among themselves, within, or across species of bacteria. Bacteria carrying resistance proliferate and transmit ARGs vertically through binary fission. ARGs can also be transferred horizontally through processes of transduction, transformation, and conjugation (Schwarz and Chaslus-Dancla, 2001). These mechanisms can facilitate multiple antibiotic resistances within a single organism.

In dairy cattle, the largest use of antibiotics is for the treatment and prevention of bacterial mastitis (Mitchell et al., 1998; Sawant et al., 2005). This disease is the most costly disease in the dairy industry (National Mastitis Council, 1982). The largest profit loss is due to clinical and subclinical milk loss, with other losses from discarded milk due to abnormality or antibiotic contamination (Janzen, 1970), veterinary services for acute and chronic mastitis, antibiotic cost, increased labor, decreased animal sale value, and increased replacement costs (Dobbins Jr, 1977). Little is known about the effect of intramammary therapeutic or prophylactic treatment on fecal ARG excretion by cows. Understanding this may provide beneficial information on ARGs in animal agriculture and help producers make better-informed decisions for the selection and use of antibiotics in production animal agriculture.

Chapter 2 REVIEW OF LITERATURE

Antibiotic Effect on Normal Flora

The endogenous GI flora forms a stable microbiome. In the gut microbiome, only a very small proportion of operational taxonomic units (measure of bacterial relatedness) defined by 16S rRNA are shared among individuals (Bäckhed et al., 2005) and approximately 70% are unique with no single operational taxonomic unit shared among all individuals (Turnbaugh et al., 2008). Microorganisms occupy all available niches and contribute to the biological needs of their host. The GI microflora are extremely important because they synthesize vitamins B12, K, (Tannock, 1995), B1, B2, B6, folic acid, biotin, and vitamin K (Clarke, 1977; Tortora et al., 2004). Transient bacteria from food or the oral cavity pass through the GI tract and typically do not colonize the gut. These bacteria are not considered to be part of the endogenous GI microflora (Berg, 1996).

Establishment of a stable GI microbiome is not spontaneous in neonates. Instead, microorganisms colonize the gut through bacterial succession. Typically the first colonization of the neonate occurs as the animal passes through the birth canal. In many species, lactic acid-producing bacteria and coliforms are the first bacteria predominant in the GI tract, although as the animal ages the microbiome begins to shift to obligate anaerobes. Subsequently obligate anaerobes proliferate and become the most predominant bacteria (Savage, 1977).

As the microbiome shifts with increasing animal age, it becomes beneficial to the host. The 'normal' GI microflora limits the colonization of pathogenic microorganisms in the gut by competitive exclusion. Through competitive exclusion the access of transient ingested pathogens to essential nutrients, other energy sources, and adhesion sites for colonization is limited (Van der Waaij et al., 1971). Therefore, the animal does not have a need for constant peristaltic rush.

Competitive exclusion can be overcome by pathogens if the animal is immunocompromised, has poor nutrition, or is given antibiotics that decrease the total bacterial population of the animal's GI tract.

The use of antibiotics can disrupt the animal's GI tract homeostasis causing instability among the normal gut microflora. Niches in the endogenous microflora will respond differently depending on the parameters of antibiotic administration (Looft et al., 2012). Oral and injected antibiotics can kill entire niches of bacteria depending on the bacteriostatic or bactericidal effects and the target pathogen of interest. This can cause a shift in the total microbial population and the diversity of bacteria which can result in overgrowth of other indigenous GI microorganisms (Berg, 1996), acquisition and colonization of pathogenic bacteria, and increased selection pressure for antibiotic resistance resulting in the dissemination of ARGs (Van der Waaij et al., 1986).

Antibiotic Resistance When Feeding Antibiotics

Increasing Resistance

Antibiotic compounds can be fed subtherapeutically in North America for prevention of animal disease and to increase production efficiency such as growth rate, milk production, and feed efficiency (Gustafson and Bowen, 1997).

Using antibiotics as feed additives has been shown to increase fecal antibiotic resistance measured phenotypically and the abundance of fecal ARGs (Alexander et al., 2010). Non-type specific *Escherichia coli* (NTSEC) isolates in feces of cattle receiving antibiotics had more phenotypic resistance than in feces of cattle not receiving subtherapeutic antibiotics in feed (Morley et al., 2011). Interpretation of these results are complicated because previous antibiotic exposure was unknown as animals were from different farms of origin. Also, the subtherapeutic

antibiotic fed was unknown in some groups of cattle. To further confound these results, it is unknown if the increase in antibiotic resistance can be attributed to feeding subtherapeutic antibiotics or if animals that became clinically ill (treated with enrofloxacin or florfenicol) were not removed from the study, biasing the results (Morley et al., 2011). It has also been reported that fecal *E. coli* had significantly more phenotypic resistance to ampicillin, streptomycin, kanamycin, gentamycin, chloramphenicol, tetracycline, and sulfamethoxazole on conventional dairy farms routinely using antibiotics compared to *E. coli* in feces from organic dairy farms (Sato et al., 2005).

Similarly, subtherapeutic antibiotics in cattle feed increased the abundance of ARGs in the feces (Sharma et al., 2008; Harvey et al., 2009). Harvey *et al.* (2009) determined fecal abundance of five of fourteen tetracycline resistance genes significantly increased in feces of beef feedlot steers and Holstein steers fed subtherapeutic antibiotics. Sharma *et al.* (2008) demonstrated increased resistance to tetracycline (mediated by a number of *tet* genes) when steers were fed the combination of chlortetracycline and sulfamethazine.

Antibiotic resistance is also present in cattle without antibiotic selection pressure (Mirzaagha et al., 2011). In the feces of beef feedlot steers, genes coding for resistance to ampicillin, tetracycline, and sulfonamides were discovered prior to feeding any subtherapeutic antibiotics (Sharma et al., 2008). Similarly, tetracycline and erythromycin resistance were found in beef feedlot cattle feces at initial time points for all treatments including control cows and resistance increased with antibiotic treatment (Inglis et al., 2005). Also, phenotypic resistance has been observed in fecal bacteria prior to antibiotic treatment in calves fed antibiotic-containing milk (Langford et al., 2003) and milk replacer (Berge et al., 2005; Pereira et al., 2011).

In the Inglis *et al.* (2005) study, increased erythromycin resistance was not observed with tylosin phosphate (a macrolide), but it was observed in response to chlortetracycline administration. This suggests that feeding one antibiotic can potentially increase resistance to another class of antibiotic (Inglis *et al.*, 2005; Sharma *et al.*, 2008).

Similar to when subtherapeutic antibiotics are included in grain, increasing phenotypic antibiotic resistance has also been observed in calves fed medicated milk replacer (Berge *et al.*, 2005; Berge *et al.*, 2006; Pereira *et al.*, 2011). Interestingly, no significant difference was observed in antibiotic susceptibility of fecal bacteria from calves fed medicated milk replacer at subtherapeutic and therapeutic levels of antibiotic administration (Berge *et al.*, 2006). Also, resistance to penicillin in the feces of calves fed antibiotic-containing milk increased as penicillin levels increased in milk (Langford *et al.*, 2003).

In addition to an increased phenotypic resistance, antibiotic containing milk replacer can increase the abundance of ARGs. Dairy calves fed a milk replacer diet for seven weeks also exhibited increased fecal ARGs (Thames *et al.*, 2012). Although, in contrast to the phenotypic resistance observed in Berge *et al.* (2006), one tetracycline resistance gene was significantly greater for calves fed therapeutic levels of antibiotic than for calves fed subtherapeutic antibiotic levels in milk replacer (Thames *et al.*, 2012).

After antibiotic administration there is a mandated withdrawal period before the animal or its products can be used for human consumption to allow antibiotics to be metabolized or excreted from the animal. Fecal antibiotic resistance tends to be persistent and stable throughout the withdrawal period for treated cattle (Sharma *et al.*, 2008; Harvey *et al.*, 2009; Alexander *et al.*, 2010). Beef feedlot steers fed an antibiotic for 151 days with a twenty-eight day withdrawal period had increased fecal antibiotic resistance in response to antibiotic treatment but resistance

remained unchanged through the antibiotic withdrawal period (Alexander et al., 2010).

Conversely, after removal of antibiotics from medicated milk replacer fed to dairy calves there was a significant decrease in phenotypic multi-drug resistance compared to animals that continually received antibiotics (Kaneene et al., 2009).

Temporal Shift in Antibiotic Resistance

Shifts in fecal ARGs are not always permanent and may respond in a temporal fashion to antibiotic treatment. It has previously been established that withdrawal period may not be associated with a change in fecal antibiotic resistance (Sharma et al., 2008; Alexander et al., 2010). Therefore, it can be hypothesized that completely removing antibiotics from feed may not reduce the incidence of multidrug resistance and bacterial resistance to antibiotics.

In a field study, after a three-month antibiotic adaptation period, ‘intervention’ herds were fed milk replacer without antibiotics while control animals continued to receive a medicated version of the same brand of milk replacer. After removal of antibiotics, tetracycline resistance decreased for three to four months and then returned to pre-intervention resistance levels within a few months (Kaneene et al., 2008). Similar results were observed with multi-drug resistance with the discontinued use of medicated milk replacer (Kaneene et al., 2009).

This phenomenon was also observed in a study by Berge *et al.* (2006). With the administration of antibiotics, phenotypic bacterial resistance increased although resistance eventually returned to initial resistance levels (Berge et al., 2006). These results suggest a transient increase in fecal antibiotic resistance after removal of antibiotic selection pressure followed by a return to initial resistance levels.

Similarly, ARGs responded in a temporal fashion in response to antibiotic treatment in beef cattle (Alexander et al., 2009; Alexander et al., 2011). Beef feedlot steers fed subtherapeutic

antibiotics for 197 d prior to fecal sampling had increased fecal ARG abundance until d fifty-six, and then fecal ARGs declined to an undetectable level by d 175 post-treatment. Some fecal resistance genes decreased to lower than baseline levels by d 175.

Over the lifetime of an animal, antibiotic resistance measured phenotypically decreased with age (Khachatryan et al., 2004). Resistance in fecal bacteria declined continually from calves less than three months, to heifers three to six months, to heifers greater than six months, to lactating cows, and ended with dry cows having had the lowest resistance in fecal bacteria. The highest resistances were seen in calves less than three months, possibly due to an antibiotic-containing milk supplement for pre-weaned calves (Khachatryan et al., 2004). Over an eight month period, fecal antibiotic resistance decreased regardless of antibiotic treatment in beef calves (Hoyle et al., 2004). The development of the GI and its endogenous microflora is associated with a decrease of fecal antibiotic resistance in the gut bacteria, and it appears that changes in fecal antibiotic resistance are temporal and strongly correlated with age.

Multiple Drug Resistance

Some fecal ARGs may travel together on the same genetic element (Van den Bogaard and Stobberingh, 2000). Phenotypically, *E. coli* displayed co-resistance to ampicillin and tetracycline antibiotics, and after their administration the multi-drug resistance significantly increased in the feces of beef feedlot steers (Alexander et al., 2008; Sharma et al., 2008) and dairy calves receiving antibiotics had significantly more multi-resistant *E. coli* than calves not receiving antibiotics (Berge et al., 2005).

The possibility of linked genes does not only pertain to ampicillin-tetracycline resistance. This type of relationship has also been observed between tetracycline and erythromycin resistance genes (Inglis et al., 2005) in bovine feces and erythromycin and pirlimycin resistance

genes in mastitic milk (Lüthje and Schwarz, 2006). This may suggest an acquisition of multi-drug resistance, because no macrolides were used in the antibiotic treatments, and a positive correlation was observed between tetracycline and erythromycin resistance.

Changes in Bacterial Resistance Profiles

Bacteria display different antibiotic resistance profiles depending on the antibiotic, dose, and duration of treatment. In a study by Inglis *et al.* (2005), the addition of virginiamycin, monensin, and tylosin phosphate to the diet of beef feedlot steers resulted in decreased fecal bacterial resistance to ampicillin, and steers fed chlortetracycline displayed a significant increase in erythromycin resistance in fecal *Campylobacter*. However, this effect was not observed with any other treatment including tylosin, another macrolide (Inglis *et al.*, 2005). Interestingly, a gene that encoded tetracycline resistance, *tetQ*, had significantly greater abundance in the feces of beef feedlot and Holstein steers not receiving antibiotics than antibiotic treated cattle (Harvey *et al.*, 2009). This may suggest feeding antibiotics can potentially decrease prevalence of some ARGs. Similar conflicting evidence about antibiotics fed and their effect on fecal antibiotic resistance has been shown in swine (Langlois *et al.*, 1984; Gellin *et al.*, 1989; Kalmokoff *et al.*, 2011). These findings suggest that exogenous factors could contribute to the acquisition, increase, or dissemination of fecal antibiotic resistance (Inglis *et al.*, 2005; Sharma *et al.*, 2008; Harvey *et al.*, 2009).

Ionophores

Ionophores, which improve feed and growth efficiency, are defined as antibiotics and are used extensively in production agriculture in cattle (Brandt, 1982; Goodrich *et al.*, 1984). Efficiency is improved because ionophores alter rumen fermentation characteristics through their impacts on the proton motive force in the bacterial cell. This causes a shift in rumen microbial

populations resulting in metabolic changes that improve animal efficiency (Dennis et al., 1981; Bergen and Bates, 1984; Russell and Strobel, 1988). Ionophores alter rumen microbial composition without decreasing the total bacterial population (Olumeyan et al., 1986). This suggests that bacteria resistant to ionophores multiply and occupy newly available niches not previously inhabited (Dawson and Boling, 1983).

Ionophore resistance is not a concern to human health because ionophores are not used in human medicine. It is important to note that various species of rumen bacteria are intrinsically resistant to ionophores but the mechanism of this resistance is not the same as acquired antibiotic resistance (Dawson and Boling, 1983). Of the major classes of rumen bacteria, lactic-, butyric-, and formic acid-producing bacteria tend to be susceptible to ionophores whereas succinate- and lactic acid- fermenting bacteria tend to be resistant (Nagaraja and Taylor, 1987). This effect could be due to the reduced outer membrane permeability of Gram-negative bacteria that allow them to be more resistant than their Gram-positive counterparts. (Watanabe et al., 1981). There is no evidence of genes coding for ionophore resistance that may be spread between bacteria (Dawson and Boling, 1983). Rather than being a result of horizontal gene transfer or mutation, resistance to ionophores appears to be a physiological selection facilitated by binary fission (Quintiliani Jr et al., 1999; Schwarz and Chaslus-Dancla, 2001). Therefore, ionophore resistant bacteria do not transfer ionophore resistance (Houlihan and Russell, 2003; Edrington et al., 2006; Jacob et al., 2008).

Ionophores do not increase resistance to other classes of antibiotics. Studies involving dairy and beef cattle have determined that bacteria cultured from the rumen (Dawson and Boling, 1983) and feces (Edrington et al., 2006; Jacob et al., 2008) of animals not fed ionophores had bacteria that were susceptible to ionophores. The bacteria *Enterococcus faecalis* and

Enterococcus faecium do not carry ionophore resistance and therefore the use of ionophores did not contribute to the acquisition or dissemination of vancomycin resistant *Enterococcus* (Nisbet et al., 2008).

Fecal Resistance in Response to Injected Antibiotics

Intramuscular therapeutic use of antibiotic has limited effect on the acquisition or amplification of antibiotic resistance. Fecal *E. coli* isolates from dairy cows treated with ceftiofur intramuscularly (5 injections, once per day consecutively) had a transient increase in phenotypic resistance during the time of antibiotic use and immediately after cessation of use but returned to a susceptible bacterial population (Singer et al., 2008). Similarly, ceftiofur antibiotic administration to beef feedlot cattle caused a transient increase in fecal phenotypic *E. coli* resistance although resistance levels returned to pre-treatment levels when ceftiofur was no longer administered (Schmidt et al., 2013). There also seemed to be dissemination of the *bla_{CMY-2}* gene but this was not attributed to horizontal gene transfer.

In contrast, dairy calves receiving five consecutive injections of ceftiofur hydrochloride displayed increased fecal phenotypic bacterial resistance to ceftriaxone for three consecutive days. No change was observed from the third injection day to thirteen days post-injection, although there was an increase in resistant bacteria observed on day 17 (Jiang et al., 2006). Also, there was detection of the cephalosporinase gene *bla_{CMY-2}* and class 1 integron *int1*. It was determined that these genes were transferred between Gram-positive and Gram-negative bacteria cultured from antibiotic treated calves. Diet composition was not reported in this study. It is possible that the effects observed in fecal bacteria could have been the result of a medicated milk replacer or antibiotic-containing milk.

Antibiotic Resistance Associated with Prevention and Treatment of Mastitis

Prophylactic Dry Cow Treatment

Antibiotics have historically been utilized prophylactically to reduce intramammary infections during the dry period and to reduce the occurrence of infection during the subsequent lactation. Prophylactic antibiotics are used to eliminate bacterial mastitis present at dry-off and to prevent the development of new infections (Neave et al., 1966). Curative rates with dry cow treatment range from seventy to ninety-eight percent (Natzke, 1981). If an infection arises or persists through the dry period, the infected quarter will produce less milk during the next lactation (Smith et al., 1968) and will be at increased risk for the development of a clinical bacterial infection (Berry and Hillerton, 2002; Green et al., 2002). This provides a financial incentive to eliminate and prevent intramammary infections during the dry period through prophylactic treatment (Blosser, 1979).

Of the 9.2 million dairy cows in the United States (USDA., 2012a; 2013), approximately 200,000 are housed on 1,800 organic dairy farms with the remaining cows housed on 49,000 conventional dairy farms (USDA., 2012b). Approximately 9 out of 10 conventional dairy farms use antimicrobial dry cow treatment with the majority using cephapirin or penicillin G with dihydrostreptomycin antibiotics (APHIS, 2007). Exact information on the use of individual antibiotics is not available, however estimates on the annual use of dry cow treatments can be made (Table 1).

Table 1: Dry cow treatment annual antibiotic use

Compound	% of cows ¹	# of cows ²	Mg antibiotic/dose ³	Annual use
Ceftiofur hydrochloride	7.0	630,000	500.0 mg ⁴	1,260.0 kg
Cephapirin benzathine	31.0	2,790,000	300.0 mg ⁵	3,348.0 kg
Cloxacillin benzathine	7.9	711,000	500.0 mg ⁶	1,422.0 kg

Erythromycin	0.3	27,000	300.0 mg ⁵	32.4 kg
Novobiocin	2.5	225,000	400.0 mg ⁵	360.0 kg
Penicillin G procaine	1.7	153,000	0.6 mg ⁵	3.6 kg
Penicillin G / Dihydrostreptomycin	36.9	3,321,000	600.0 mg ⁵ 1,000.0 mg ⁵	21,254.0 kg
Penicillin G / Novobiocin	13.2	1,188,000	120.0 mg ⁵ 400.0 mg ⁵	2,661.0 kg
Total				30,151.0 kg

¹ Percent of cows treated with antibiotic (APHIS, 2007)

² Number of cows determined from 9 million conventional dairy cows (USDA., 2012a; 2013)

³ Milligrams antibiotic activity per dose

⁴ (Pharmacia & Upjohn Co., 2005)

⁵ (FDA, 2012)

⁶ (Boehringer Ingelheim Vetmedica, 1975)

This table provides a good basis for understanding the dynamics of dry cow antibiotic use. However, this may be an overestimate of total antibiotic use because it is assumed that all cows have four functioning mammary glands and 100% of conventional farms utilize dry-cow treatment in all quarters with no selective dry cow treatment.

Literature on the development of antibiotic resistance caused by dry cow treatment is limited and there are no published studies evaluating the fecal abundance of ARGs in prophylactically treated dairy cows. However, two studies have attempted to elucidate the effect of dry cow treatment on fecal phenotypic antibiotic resistance. These studies show minimal effects on antibiotic resistance in fecal bacteria (Rollins et al., 1974; Mollenkopf et al., 2010).

However, dry cow treatment has caused an increase in phenotypic expression of antibiotic resistance in mastitis pathogens cultured from milk (Berghash et al., 1983; Rajala-

Schultz et al., 2004; Rajala-Schultz et al., 2009). Mastitis pathogens had significantly greater resistance to antibiotics after cephalosporin dry-cow treatment (Berghash et al., 1983; Rajala-Schultz et al., 2009). Over a 3-year period antibiotic susceptibility of mastitis pathogens significantly increased from bacteria cultured at dry-off and parturition (Schultze, 1983). Interestingly, pirlimycin resistance in mastitis pathogens decreased between dry-off and parturition, and was significantly lower in mastitis-causing bacteria cultured from multiparous cows than either primiparous or untreated low-risk cows (Rajala-Schultz et al., 2009). Conversely, in an earlier and similarly designed study antibiotic susceptibility was not different in mastitis pathogens cultured from primiparous and multiparous cows (Rajala-Schultz et al., 2004).

Antibiotic Resistance with Therapeutic Treatment of Mastitis

Antibiotic resistance in mastitis-causing bacteria has been extensively studied and reviewed (Erskine et al., 2002; Erskine et al., 2004; Oliver et al., 2011). In a retrospective survey of seven years of milk samples submitted for culture, resistance of mastitis pathogens to various antibiotics showed no evidence of a general trend for increasing resistance. Resistance to erythromycin, lincomycin, and pirlimycin increased in some bacterial strains, but the majority of mastitis pathogens cultured showed decreased resistance to β -lactams and other antibiotics over the seven year period (Makovec and Ruegg, 2003). The only significant change in resistance of mastitis-causing bacteria cultured from cows on organic and conventional farms was the increased presence of β -lactam resistant *S. aureus*, *S. uberis*, and *S. dysgalactiae* on conventional farms (Roesch et al., 2006).

While most long-term scientific evidence suggests no substantial increase in antibiotic resistance due to antibiotic therapy for mastitis, there is some evidence to suggest antibiotic

resistance in mastitis is increasing in response to time and antibiotic exposure. Over a two-year period antibiotic administration increased resistance in clinical mastitis isolates with repeated exposure to pirlimycin and other antibiotics (Pol and Ruegg, 2007). Also, in a survey, *E. coli* cultured from clinical mastitis were resistant to antibiotics used in both human and animal medicine and carried multiple drug resistance (Srinivasan et al., 2007). The *E. coli* isolates carried ampicillin, sulfonamide, and tetracycline resistance genes. However, more studies are needed to determine if the increases in antibiotic resistance in mastitis causing pathogens are true effects of treatment or the effects of temporal variation independent of antibiotic use which has been reported with subtherapeutic antibiotic feeding (Berge et al., 2005; Alexander et al., 2009; Alexander et al., 2011).

Pharmacokinetics/pharmacodynamics of Cephapirin and Pirlimycin

A distinct sequence of events occurs when administering antibiotics via intramammary infusion. These events can be broken down into three phases, the pharmaceutical, pharmacokinetic, and pharmacodynamic phases. The pharmaceutical phase begins after administration of the antibiotic. During this phase, the antibiotic begins to disintegrate and dissolve. The drug is then released into the milk. This leads to the second phase, the pharmacokinetic phase. The drug is absorbed via the milk: plasma barrier and then it is distributed locally, travels systemically, and is metabolized. The drug is then excreted through a local or systemic excretion. This phase is strongly dependent on the antibiotic's bioavailability. The final phase is the pharmacodynamic phase which is the effect of drug on the bacteria in the infection site (Ziv, 1980b).

The relationship between pharmacokinetics and pharmacodynamics is the association between the antibiotic concentration in blood, the biologically active concentration at the site of

infection, and the clinical outcome of infection (Levison, 2004). There are three parameters important for the pharmacokinetic and pharmacodynamic interaction. The first parameter is the amount of time in which the antibiotic concentration is greater than the minimum inhibitory concentration (MIC) for the bacteria of interest. This is associated with the half-life of the antibiotic. The second is the peak plasma concentration divided by the MIC, which is dependent on the dose of antibiotic administered. The third parameter is the area under the concentration/time curve divided by the MIC. This is dependent on total antibiotic dose given during a time period and is inversely related to antibiotic clearance (Van Bambeke et al., 2006).

The site of antibiotic accumulation (i.e. milk vs. mammary tissue) is extremely important in the treatment or prevention of mastitis (Erskine et al., 2003). After antibiotic release from the pharmaceutical phase the drug will passively diffuse into the lipid and aqueous fractions of milk. A water-soluble antibiotic will congregate in the hydrophilic portions of the mammary gland (e.g. the udder cisterns). Conversely, a lipid soluble compound will congregate towards the lipid-rich membranes (Gruet et al., 2001). Therapeutic mastitis treatments tend to be quick release (water soluble) vehicles to cure infection and minimize antibiotic withdrawal time. Antibiotics created for prophylactic use at dry-off usually are formulated as a slower, lipid-soluble dissemination method (Ziv, 1980b).

Cephalosporin antibiotics are semi-synthetic antibiotics in the β -lactam class derived from the fungus *Cephalosporium acremonium* (Papich, 1984). Cephapirin benzathine is a lipid-soluble, slow acting antibiotic used for the prevention of mastitis in dairy cows and has limited transfer across the milk: blood barrier. It is a first generation cephalosporin with broad spectrum antibiotic activity (Caprile, 1988). Its chemical nature is a weak organic acid with a pKa of 2.67 and 4.49. It has low to moderate lipid solubility and its milk to serum concentration ratio is 0.14

– 0.18 (Ziv, 1980a). Cephapirin is a chemical analog of the cross-linking structures used in the peptidoglycan cell wall structure in prokaryotes (Tipper and Strominger, 1965). β -lactams like cephapirin bind to the penicillin binding protein in the bacterial cell wall and inhibit cell wall synthesis through a bacteriostatic mechanism of action (Spratt, 1975). These findings make cephapirin a good antibiotic for mastitis prevention, although the antibiotic persists only in the early to mid-dry period or approximately twenty-one days (Oliver et al., 1990).

Pirlimycin is a semi-synthetic lincosamide antibiotic derived from lincomycin and clindamycin (Hornish et al., 1992). Pirlimycin hydrochloride is a water-soluble, fast acting antibiotic used for the therapeutic treatment for Gram-positive bacterial mastitis infections in lactating dairy cows. Pirlimycin is a weak organic base with moderate lipid-solubility and a pKa of 9.47 and 12.88 (Gehring and Smith, 2006). Pirlimycin has a sixty-eight percent unaltered excretion in milk, urine, and feces (Hornish et al., 1992). Four percent is altered by hepatic oxidation to form pirlimycin sulfoxide; this metabolite is excreted in urine (3%) as well as feces (1%) (Hornish et al., 1992). Resistance to pirlimycin has been observed in CNS (Lüthje and Schwarz, 2006), *Staphylococcus aureus*, and *Streptococcus spp.* cultured from the milk of cows subjected to pirlimycin (Pol and Ruegg, 2007).

Udder to Gastrointestinal Tract

The route of antibiotic administration can affect the bacterial resistance in the gut microbiome (Zhang, 2013). Antibiotics from an intramammary infusion can travel from the udder to the G.I. tract and may have an impact on gut microbial composition. The transfer of antibiotics begins with passage from milk to serum and is dependent on properties such as the antibiotic's pKa, the lipid solubility (non-ionized fraction), and percent of antibiotic binding to udder and milk proteins. Water-soluble compounds pass from milk to serum primarily through

protein channels, whereas lipid-soluble compounds pass through the lipoproteic regions of the membrane. The excretion of therapeutic and prophylactic antibiotics is also governed by the vehicle, dose, quantity of milk produced, molecular characteristics of the compound, health of mammary gland, efficiency of mammary tissue binding, and number of daily milkings (Ziv, 1980b). After crossing the epithelial barrier, the antibiotic can then be absorbed into systemic circulation or returned to the mammary gland. The amount of absorption varies but can be significant if there is damage to the epithelial tight junctions (Gehring and Smith, 2006). Damage to the tight junctions could be attributed to pressure in the mammary gland just after cessation of milking. After crossing the milk: blood barrier the antibiotic travels through the body via systemic circulation.

Antibiotics freely traveling through the systemic circulation can enter the G.I. tract similarly to how they exited the udder, i.e. transfer is largely dependent on the compounds chemical structure and physiochemical properties like pKa and lipid solubility (Gad, 2007). However, most compounds are transferred into the G.I. tract through local pH dependent diffusion (Gad, 2007). Once the antibiotic compounds have entered the G.I. tract they then can impact the gut microbial composition and provide an antibiotic selection pressure that may contribute to increased dissemination of ARGs.

Antibiotic Resistance Genes in Bovine

Tetracycline Resistance Genes tetO and tetW

The expressions of *tetO* and *tetW* resistance genes are highly regulated in bacteria; they encode the proteins TetO and TetW. These proteins allow for bacterial survival due to ribosomal protection by preventing tetracycline binding (Wang and Taylor, 1991; Aminov et al., 2001). Ribosomal protection proteins have been shown to be dependent on GTP-ase activity and GTP

binding which is an important factor in resistance (Taylor et al., 1998; McMurry and Levy, 2000).

Tetracycline resistance genes are the most common type found in nature (Levy, 1989). Animals such as swine (Patterson et al., 2007), bovine (Dogan et al., 2005), and even *Homo sapiens* (Scott et al., 2000) carry *tetO* and *tetW* ARGs in their commensal flora. These genes may be housed in the rumen (Billington et al., 2002; Billington and Jost, 2006) and transferred between facultative and obligate anaerobic bacteria in the rumen (Barbosa et al., 1999), supporting the notion of widespread dissemination of these genes across species of bacteria. In American bison, fecal *tetO* resistance genes were found in abundance on highly transmissible genetic elements (Anderson et al., 2008). Tetracycline resistance genes persist in cattle manure handling systems although there have been observations of marginal to significant declines over time and large variations dependent on seasonal persistence (Peak et al., 2007; Pei et al., 2007; Storteboom et al., 2007; McKinney et al., 2010).

These tetracycline resistance genes have been characterized in mastitic bovine milk from the pathogens *Streptococcus agalactiae* (Duarte et al., 2004), Group B *Streptococcus*, and *Trueperella pyogenes* (Zastempowska and Lassa, 2012). Also *Streptococcus agalactiae* isolates have been shown to carry pirlimycin resistance via the *tetO/ermB* genotype (Rato et al., 2010).

Erythromycin Resistance Gene, ermB

The erythromycin resistance gene, *ermB*, encodes the enzyme ribosomal RNA-methylase allowing it to compete for binding to the 23S rRNA. This is the site of action for erythromycin (Skinner et al., 1983; Arthur et al., 1987; Leclercq, 2002).

The *ermB* resistance gene has been identified in *Streptococcus uberis* (Schmitt-van de Leemput and Zadoks, 2007), CNS (Frey et al., 2013), *Streptococcus agalactiae*, and *Trueperella*

pyogenes (Zastempowska and Lassa, 2012) in the milk from cows with mastitis. The *ermB* resistance gene confers phenotypic resistance to erythromycin and the lincosamide pirlimycin in *S. uberis* (Loch et al., 2005; Schmitt-van de Leemput and Zadoks, 2007; Haenni et al., 2011) and *S. agalactiae* (Rato et al., 2010).

The *ermB* resistance gene is also shed fecally from the bovine (Anderson et al., 2008). In beef cattle fed tylosin (a macrolide-class antibiotic like erythromycin), the abundance and prevalence of *ermB* was significantly greater than any other erythromycin resistance gene (Chen et al., 2008). This gene persists in fecal excreta exposed to composting conditions for up to eleven weeks (Sharma et al., 2009).

β-lactam Antibiotic Resistance Genes, ampC and bla_{CMY-2}

The Class C cephalosporinases coded for by the genes *ampC* (Jaurin and Grundström, 1981) and *bla_{CMY-2}* (Folster et al., 2011) hydrolyze the β-lactam ring, the backbone to many antibiotics, and therefore cause bacterial resistance (Frère, 1995). These genes can be disseminated through the bovine GI tract or into the environment via horizontal gene transfer (Alcaine et al., 2005; Kang et al., 2006). The *bla_{CMY-2}* gene is common in *Salmonella* spp. (Daniels et al., 2009), *Klebsiella* spp., *Proteus mirabilis*, and *E. coli* strains in cattle (Daniels et al., 2009).

Sulfonamide Resistance Gene, sull

The sulfonamide resistance gene, *sull*, acts to retarget sulfonamide antibiotic binding to dihydropteroate synthase (Swift et al., 1981). This gene has been found in twenty-two different bacterial genera including genera clinically relevant to cattle such as *Enterobacter*, *Escherichia*, *Klebsiella*, *Mycobacterium*, *Proteus*, and *Salmonella* (Liu and Pop, 2009). It is the predominant sulfonamide resistance gene found in the commensal bacteria of cattle (Sáenz et al., 2004; Aslam

et al., 2010; Alexander et al., 2011) and it persists in the feces after excretion (Ho et al., 2009; McKinney et al., 2010).

Integrase Gene, *int1*

Integrations are composed of a two-component system that allows for the recognition and capture of mobile gene cassettes containing ARGs. They are not genetically transferrable alone, although they have components that allow for their transfer. Integrations are a prime way to quickly and effectively transfer drug resistance and also allow for the quick assimilation of multi-drug resistance (Ho et al., 2009). The gene *int1*, a component of the class one-integron, is an integrase gene with a recombinant site that allows for the uptake of gene cassettes. There is some evidence to suggest that subtherapeutic antibiotic treatment in cattle can select for horizontal gene transfer via the class one integron (Du et al., 2005; Ho et al., 2009; Wu et al., 2011).

Summary and Research Objectives

Considering the uniqueness and complexity of the GI microbiome, it may be challenging to predict how cattle fecal bacteria will respond to antibiotic selection pressure from therapeutic and prophylactic treatment. Also, little is known about the effect of intramammary therapeutic or prophylactic treatment on fecal abundance and relative abundance of ARGs in dairy cows. Literature on the development of antibiotic resistance caused by dry cow treatment is limited and there are no published studies evaluating fecal abundance of ARGs in dry cows following intramammary prophylactic treatment. While some research has documented effects of antibiotic treatment on the development of antibiotic resistance in mastitis pathogens (Sato et al., 2005; Roesch et al., 2006), no studies have been published evaluating fecal abundance and relative abundance of ARGs with antibiotic therapy in mastitic cows. Understanding the effect of common antibiotic treatments in the dairy industry may help to provide beneficial information

about the effect of animal agriculture on the further development of antibiotic resistant bacteria important to human health. Therefore, the objective of this research was to evaluate the effect of cephalosporin prophylactic dry cow treatment administered at the end of lactation and penicillin therapeutic mastitis treatment administered upon diagnosis of an IMI on the abundance and relative abundance of ARGs in dairy cow feces.

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Chapter 3 EFFECT OF PROPHYLACTIC DRY COW TREATMENT ON ABUNDANCE OF ANTIBIOTIC RESISTANCE GENES IN FECES

ABSTRACT

Brittany Faith Willing

Prophylactic antibiotic treatment may increase excretion of antibiotic resistance genes (ARGs) by dairy cattle through selection pressure on the gut microbiome. The objective of this study was to evaluate the effect of a prophylactic cephapirin benzathine administered at the end of lactation on the abundance and relative abundance of ARGs in dairy cow feces during the dry period. Cephapirin is a β -lactam antibiotic that prevents peptidoglycan cross-linking in bacteria. Nineteen cows nearing dry-off were used in the study. Treatment cows (n = 9) received cephapirin benzathine as an intramammary infusion prior to dry-off and control cows (n = 10) were dried off with no antibiotics. All cows received an internal non-antibiotic teat sealant. Fecal grab samples were collected from each cow on d -2 (baseline, used as covariate), d 1, 3, 5, 7, and once per week until d 49 relative to dry-off. Fecal samples were collected in sterile containers, then freeze-dried and subject to DNA extraction. The abundance *ampC*, *bla_{CMY-2}*, *ermB*, *sull*, *tetO*, *tetW*, *int1*, and the 16S rRNA gene were quantified using qPCR. The genes *ampC* and *bla_{CMY-2}* encode resistance to β -lactam antibiotics, *ermB* to macrolides, *sull* to sulfonamides, *tetO* and *tetW* to tetracyclines, and *int1* a class-1 integrase gene that facilitates horizontal transfer of ARGs across bacteria. The 16S rRNA gene was used as a representation of bacterial population. Absolute abundance was defined as number of ARG copies per gram of freeze-dried feces, while relative abundance was defined as ARG copy numbers per copy of 16S rRNA gene, which is indicative of the proportion of bacteria carrying ARGs. Non-normal data were logarithmically

transformed and were statistically analyzed using PROC GLIMMIX in SAS 9.2. Abundance and relative abundance of *sull1* and *bla_{CMY-2}* were below the limit of quantification in most samples and therefore not suitable for statistical comparisons. The *int1* gene was not detectable in any sample. No significant effects of treatment or time were observed on the abundance the *ermB* gene in feces. However, the relative abundance of bacteria carrying *ermB* was greater in control cows when compared to antibiotic treated cows. There were significant interactions between treatment and day for the abundance and relative abundance of *ampC*, *tetO*, and *tetW*. The abundance and relative abundance of *ampC* was significantly greater in control cows than antibiotic treated cows and increased with time. The tetracycline resistance genes *tetO* and *tetW* behaved similarly with a significant treatment by day interaction for the abundance and relative abundance of both genes. The relative abundance of both *tetO* and *tetW* were greater in control cows when compared to antibiotic treated cows on days 3, 5, 7, and 14. The abundance of both *tetO* and *tetW* resistance genes increased in antibiotic treated cows from day 1 to 49. There was also a significant increase in *tetW* relative abundance when comparing day 1 to 49.

Administering long-acting antibiotics as intramammary dry treatment changed fecal bacterial composition during the dry period perhaps by stabilizing GI bacteria through dietary and housing changes. However, the use of prophylactic dry cow treatment does not uniformly or predictably increase fecal ARGs.

Key words: antibiotic resistance gene, cephalosporin, prophylactic

INTRODUCTION

The World Health Organization (WHO) has deemed antimicrobial resistance a global challenge, calling it “a natural, unstoppable phenomenon exacerbated by the abuse, overuse, and misuse of antimicrobials in the treatment of human illness and in animal husbandry, aquaculture,

and agriculture” (WHO., 2000). The WHO’s five-point plan, Wisely and Widely Points for Action, call for the reduction of antimicrobial use in animal agriculture. Antibiotic resistance genes (ARG) themselves are considered an emerging environmental contaminant because they are a fundamental driver of antibiotic resistance and can be shared among bacteria (Pruden et al., 2006).

In dairy cattle, the largest use of antibiotics is for the treatment and prevention of bacterial mastitis (Mitchell et al., 1998; Sawant et al., 2005). This disease is the most costly disease in the dairy industry (Blosser, 1979). The largest profit loss is due to clinical and subclinical milk loss, with other losses from discarded milk due to abnormality or antibiotic contamination (Janzen, 1970), veterinary services for acute and chronic mastitis, antibiotic cost, increased labor, decreased animal sale value, and increased replacement costs (Dobbins Jr, 1977). There are 9.2 million dairy cows in the United States (USDA., 2012; 2013), with approximately 2.7 million receiving cephalosporin benzathine treatment at dry-off (APHIS, 2007). From this information it can be estimated that cephalosporin benzathine administered to dry cows contributed 3,348 kg to the total amount of antibiotics used in animal agriculture per year.

Little is known about the effect of prophylactic intramammary antibiotic treatment on ARG excretion by cows. The development of genetic approaches to measure antibiotic resistance offers the opportunity to advance knowledge in this area, because traditional measures of phenotypic expression are useful only in culture-able bacteria. Literature on the development of antibiotic resistance caused by dry cow treatment is limited and there are no published studies evaluating fecal abundance of ARGs in dry cows following intramammary prophylactic treatment. Understanding this may help producers make better-informed decisions for the selection and use of antibiotics in production animal agriculture. The objective of this study was

to determine the abundance and relative abundance of ARGs in feces after prophylactic antibiotic treatment of end-of lactation dairy cattle.

MATERIALS AND METHODS

Animals and Experimental Treatments

Holstein (n = 12), Jersey (n = 3), and crossbred (n = 4) cows in their second or later lactation were dried-off using standard criteria of the Virginia Tech Dairy Center (Blacksburg, Virginia). The standard criteria were pregnancy status, stocking density in the low milk production pen, and milk yield (< 14 kg/d milk). Selected cows all had a SCC of less than 200,000 cells/ mL (DHIA laboratory, Blacksburg, VA) to avoid confounding of treatment with mammary gland health. Prior to dry-off, cows were housed in a free-stall barn containing rubber mattresses and bedded with sawdust and received a total mixed ration containing the ionophore, monensin. At dry-off cows were moved from the free-stalls to a pasture. Between 21 and 28 days-dry cows were moved into a pack barn and remained there until calving. On pasture and in the pack barn, cows were fed a total mixed ration with anionic salt in accordance with dietary needs. The feed bunk was of sufficient length in the free-stalls, pasture, and pack barn to allow all individuals to feed at once. Cows were fed once daily and had *ad libitum* access to water.

At dry-off, cows in the treatment group (n = 9) were treated with the antibiotic cephalosporin benzathine (ToMorrow[®], Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO). Animals were milked completely and aseptically treated. Teat ends were cleaned with 4 x 4 gauze pads soaked in 70% isopropyl alcohol and 10 mL cephalosporin benzathine (300 mg cephalosporin activity) per quarter was infused. Teats were then sealed with (Orbeseal[®], Zoetis, Madison, NJ), a mixture of bismuth subnitrate, colloidal silicon dioxide, and liquid paraffin, to prevent bacterial entry.

Procedures regarding aseptic milk sampling and microbiological culture were as

previously described (Hogan et al., 1999). Samples were evaluated for SCC with control cow samples also evaluated for microbiological status to determine quarter-level infection status. Control cows (n = 10) were subject to three bacterial cultures on -3, -2, and 0 d relative to dry-off to ensure no preexisting subclinical infection went undetected for cows not receiving antibiotics. A cow was considered infected if at least one quarter isolated the same mastitis-causing pathogen (<3 cfu/10 μ L of milk) in two of the three samples.

In brief, bacterial identification was determined using MacConkey agar plates divided in half (2 quarters per plate; 100 μ L of milk from each quarter) and blood agar plates divided in four (1 quarter per section), with 10 μ L of milk from each quarter streaked using a disposable sterile loop. Following incubation (37°C, 18 h and 48 h), a presumptive identification of isolated bacteria was made and biochemical tests were performed. If a cow developed an IMI during the pre-treatment period then she would have been removed from the study and treated with the appropriate antibiotic.

Control animals meeting the established criteria were milked completely, their teats aseptically cleaned with 4 x 4 gauze pads soaked in 70% isopropyl alcohol, and were administered Orbeseal[®] (non-antibiotic teat sealant). Control cows were comingled with treated cows in the pasture and pack barns until parturition. All protocols and procedures were approved by The Virginia Tech Institutional Animal Care and Use Committee.

Sample Collection and Preparation

Fecal samples were obtained by rectal palpation with lubricant (Jorgensen Laboratories, Loveland, CO), with a new palpation sleeve used for each sample collected. Approximately 500-1,000 g of feces was discarded to minimize glove and lubrication contamination. Fecal samples were collected into sterile snap cap vials, frozen immediately (-20°C), and stored until analysis.

Fecal samples were obtained just prior to dry-off, and on d 1, 3, 5, and 7 and then weekly until parturition. The dry period of cows used in this study ranged from 34 to 89 d (median = 50 d).

Fecal samples were thawed at room temperature and mixed thoroughly with a flame-sterilized spatula. One gram of feces was weighed into 50 mL conical tubes and tubes were covered with a square of aluminum foil. Samples were frozen for 30 min in an -80°C freezer in preparation for freeze-drying. Fecal samples were freeze-dried to minimize variation in fecal dry matter and to standardize the amount of fecal material used for DNA extraction (FreezeZone Benchtop, Labconco, Kansas City, MO; (Alexander et al., 2011). Freeze-dried samples were mixed uniformly with a flame-sterilized spatula in preparation for DNA extraction.

DNA Extraction

DNA was extracted from 0.05 g of freeze-dried feces using the FastDNA[®] Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) in accordance to manufacturer instructions including the use of the FastPrep[®] instrument (MP Biomedical, Santa Ana, CA). All DNA extracts were incubated for five minutes in a 55°C water bath to increase DNA yield then the DNA was immediately stored in a -80°C freezer.

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Previously reported qPCR protocols were utilized to quantify the genes 16S rRNA (Suzuki et al., 2000), *ampC* (Shi et al., 2012), *bla_{CMY-2}* (Boyer and Singer, 2012), *ermB* (Jacob et al., 2008), *tetO* and *tetW* (Aminov et al., 2001), *sulI* (Pei et al., 2006), and *intI* (Hardwick et al., 2008). Primer sequences are provided in the appendix (Table 4).

Q-PCR quantifications were performed in analytical triplicate using a CFX96[™] Real-Time System (Bio-Rad, Hercules, CA). The 30 µL reaction mixture contained 5 µL SsoFast EvaGreen (Bio-Rad, Hercules, CA), with 0.6 µL (5M) of each forward and reverse primer, 2.8

μL of molecular grade water, and 1 μL of 1:75 diluted DNA per well. Temperature specifications were 98°C for 2 min; 39 cycles of 98°C then respective annealing temperatures [60.0°C for 16S rRNA, *int1*, and *tetW*, 50.3°C for *tetO*, 57.8°C for *ampC*, 69.9°C for *sul1*] 45 cycles of 98°C [57.8°C for *ermB*, and 70.0°C for *bla_{CMY-2}*]. For graphical representation, the absolute abundance was defined as number of ARG copies per gram of freeze-dried feces, while relative abundance was defined as ARG copy numbers per copy of 16S rRNA gene, which is indicative of the proportion of bacteria carrying ARGs.

Q-PCR Calibration Curves and Quantification

Extracted fecal DNA was amplified using traditional PCR and cloned using TOPA TA Cloning Kit (Invitrogen, Carlsbad, CA). Gene copies was determined by quantification through gel electrophoresis (1.5% agarose, 1:50 TAE buffer) and QuantityOne[®] Software (Bio-Rad, Hercules, CA). Log gene copies per mL of DNA was determined with methods described previously (Pei et al., 2006). Calibration curves were prepared for each gene with serial dilutions of positive controls over seven orders of magnitude.

Data Management and Statistical Analysis

Q-PCR wells with values below the limit of quantification (LOQ) were excluded from data analysis. Samples with two of three wells above the LOQ were included for statistical analysis. LOQ for *ampC* was 1×10^4 and for all other genes were 1×10^2 .

All statistical tests were performed using the GLIMMIX procedure of SAS 9.2 (SAS Institute Inc. Cary, NC). Non-normal data were logarithmically transformed to achieve normality prior to statistical analyses. Log-transformed data were used to calculate LSM and SEM. The statistical model included fixed effects of treatment, day, and the treatment by day interaction with cow nested within treatment as the random effect. The repeated statement was applied to the

day of sampling using cow nested within treatment as the subject. Samples from d -2 were used as covariate and when covariate was non-significant it was removed from the model. Various error structures were tested; Akaike produced the lowest information criterion and was chosen for analysis. When the treatment by day interaction was significant, pairwise comparisons were used to determine treatment differences on individual days and differences by day within each treatment. Significance was declared at $P \leq 0.05$.

RESULTS

16S rRNA

The abundance of the 16S rRNA gene was analyzed for treatment, day, and interaction, with covariate (-2 d) in the model (Table 2). There was a significant main effect of treatment ($P < 0.01$, Figure 1). Cephapirin benzathine treated cows had greater abundance of 16S rRNA when compared to control cows (Figure 1).

β -lactam Resistance Genes

There was no effect of covariate (an observed continuous control variable) fecal abundance of *ampC* therefore it was not included in the model. There was a significant treatment by day interaction for the fecal abundance of *ampC* ($P < 0.02$, Table 2). Control cows had a significantly greater abundance of *ampC* on d 28 when compared to antibiotic treated cows (Figure 2a). Also, there was a significant increase in the fecal abundance of *ampC* between day 1 and 28 (Figure 2a).

There was no effect of covariate on the relative abundance of *ampC* therefore it was not included in the model. The interaction of treatment and day significantly affected the relative abundance of *ampC* ($P < 0.01$, Table 3). The proportion of bacteria carrying the *ampC* resistance gene was significantly greater in control cows when compared to antibiotic treated cows on d 7

and remained greater through d 49 (Figure 2b). Also, the relative abundance of *ampC* in control cows increased from d 1 compared to d 14, 28, and 49. In contrast there were no significant differences between days for antibiotic treated cows (Figure 2b).

Data on abundance of *bla_{CMY-2}* were not statistically analyzed due to a large number of samples below the LOQ. The *bla_{CMY-2}* gene was detectable in some animals with no apparent effect of treatment.

Macrolide Resistance Gene

There was no effect of covariate on the abundance of *ermB*, so it was not included in the model. Day 49 was not included in the statistical analysis because there were too few samples above the LOQ for control cows. There was no significant effect of the interaction of treatment and day and no significant main effects of treatment or day for the abundance of *ermB* (Table 2).

The covariate did not affect the relative abundance of *ermB*, so it was not included in the model (Table 3). There was no significant interaction of treatment and time or the main effect of day, however the relative abundance of *ermB* was significantly affected by treatment ($P < 0.04$, Figure 3). Control cows had a significantly greater proportion of bacteria carrying the *ermB* resistance gene than antibiotic treated cows.

Tetracycline Resistance Genes

The abundance of *tetO* was analyzed with covariate (d -2) in the model. The abundance of *tetO* was significantly affected by the interaction of treatment and day ($P < 0.01$, Table 2). The fecal abundance of *tetO* was similar in control cows and antibiotic treated cows from d 1 through 21. However, the fecal abundance of *tetO* in control cows was significantly less on d 28 and 35 compared to antibiotic treated cows (Figure 4a). Control cows had greater abundance of *tetO* on

both d 1 and 49 compared to d 28 (Figure 4a). There was a significant increase in *tetO* abundance in treated cows between d 1 and 49.

The relative abundance of *tetO* adjusted to 16s rRNA was analyzed without covariate in the model (Table 3). There was a significant interaction between treatment and day ($P < 0.01$, Figure 4b). Also, in control cows there was a greater relative abundance of *tetO* compared to antibiotic treated cows from d 3 through 14. However, d 21 to 49 had no significant differences between antibiotic treated cows and control cows. Within the control treatment there was a greater relative abundance of *tetO* on d 14 and 49 compared to d 28 (Figure 4b).

The tetracycline resistance gene *tetW* was analyzed with covariate in the model (Table 2). The interaction of treatment and day significantly affected the abundance of *tetW* ($P < 0.01$, Figure 5a). There was greater fecal abundance of *tetO* in control cows on d 1 and 5 when compared to antibiotic treated cows. Also, there was a significant increase in the fecal abundance of *tetW* in antibiotic treated cows from d 1 to d 14, 28, and 49 (Figure 5a).

The relative abundance of *tetW* was analyzed with covariate in the model (Table 3). The treatment by day interaction significantly affected the relative abundance for *tetW* ($P < 0.01$, Table 3). Relative abundance was significantly greater in control cows on d 3 through 14 when compared to antibiotic treated cows, although relative abundance was similar in the two groups on d 21 through 49 (Figure 5b). In antibiotic treated cows there was a significant increase in the relative abundance of *tetW* between d 1 and 49.

Sulfonamide Resistance Gene

Data on abundance of *sull* were not statistically analyzed due to a large number of samples below the LOQ. The *sull* gene was detectable in some animals with no apparent effect of treatment.

Class-1 Integrase

The *int1* gene was not detected in any sample.

DISCUSSION

This study's aim was to evaluate the prevalence and persistence of antibiotic resistance genes in the feces of dairy cattle treated with the dry cow treatment cephalosporin benzathine.

While the SCC criteria for treated and control cows were the same, control cows were also subject to culturing prior to inclusion on the study to minimize the risk of drying off cows with a subclinical mastitis infection without antibiotics. If any treated cows had an undetected IMI on dry-off, we may have inadvertently selected for animals having different 16S rRNA concentrations in the gut because illness may cause changes in the gut microbial composition (Khafipour et al., 2009; Macpherson et al., 2009; Wang et al., 2012). However, this seems unlikely given that all cows aside from three control cows had a SCC of less than 100,000 cells/mL, and the three control cows had a SCC of less than 200,000 cells/mL, meeting the selection criteria (102,000 cells/mL, 103,000 cells/mL, and 163,000 cells/mL). The National Mastitis Council defines a normal SCC to be $\leq 200,000$ cell/mL, therefore a SCC above 200,000 is indicative of udder inflammation (Harmon, 2001), and potentially a bacterial infection.

Normalization to the 16S rRNA gene is used as a way to quantify the abundance of the bacterial population, adjust resistance genes to the total bacterial population (Patel, 2001), and correct for minor sample processing variation (McKinney et al., 2010). After adjustment for the significant covariate, fecal abundance of the 16S rRNA gene was greater in antibiotic treated cows when compared to control cows. This could be a result of an increased microbial population or the effect of antibiotic changing the species composition. This change in species composition could be explained by antibiotic killing of bacteria with low copy numbers of 16S

rRNA per bacteria; the resistant bacteria that grew to occupy the newly empty niche may have had more copies of 16S rRNA. For instance, cephalosporins have a bactericidal effect on bacteria from the *Streptococcus* and *Staphylococcus* genera (average 5 copies of 16S rRNA per genome) (Liu and Pop, 2009); this niche could have been filled with bacteria from the *Bacillus* genus (average 11 copies of 16S rRNA per genome). This could explain the increased number of 16S rRNA genes in feces from antibiotic treated cows versus control cows (Klappenbach et al., 2001). These effects would influence all data presented as normalized to the 16S rRNA gene.

During the transition from lactating to non-lactating the animal's diet also changes, and this change can affect the diversity (Pitta et al., 2010), composition, and function of the gut microbiome (Karasov et al., 2011). Lactating cows are typically fed a high-energy ration to support milk production and dry cows are fed a low-energy, high fiber ration to meet maintenance and pregnancy requirements without excessive weight gain. Diets containing low-energy and high fiber favor cellulolytic bacteria and decrease the need for lactate- and propionate-utilizing bacteria (Dirksen et al., 1985). Also, during the shift from a high to low-energy ration the total gut length decreases (Mashek and Beede, 2001), therefore potentially decreasing the total bacterial population. In this study all cows were moved abruptly from a lactating ration to a dry cow ration with *ad libitum* access to grass, probably decreasing the total microbial population as well as the diversity of microorganisms in the GI tract (Warner, 1962). These effects may have influenced abundance and relative abundance of antibiotic resistance genes.

Also, dry cows on pasture could have consumed a large amount of grass which house bacteria from the *Streptococcus* genus (Langston et al., 1960; Lopez-Benavides et al., 2007). Both tetracycline resistance genes and the erythromycin resistance gene have been associated

with the *Streptococcus* genus (Liu and Pop, 2009). This may have altered the abundance and relative abundance of erythromycin and tetracycline resistance genes in the feces of cows.

In this study, prior to dry-off cows were fed a total mixed ration containing the ionophore monensin. Currently, there is no monensin withdrawal period because the concentration of monensin in milk and tissues is close to zero and far below safe consumption concentrations (FDA, 2005). However, monensin does impact gut microbial composition and therefore there may be a period of bacterial adjustment after its removal (bacterial monensin withdrawal). Though there is evidence to suggest monensin is cleared rapidly from the feces (Herberg, 1978), this may provide some insight into the fluctuations of ARGs in control cows while cows fed antibiotics may have been buffered to the effects of monensin withdrawal from the gut.

Between 21 and 28 days after dry-off the cows were moved from pasture to a pack barn where they were fed a ration containing greater amounts of concentrate in preparation for calving. Higher-concentrate diets select for starch, propionate, and lactate-utilizing bacteria and typically reduce rumen pH (Goff and Horst, 1997; Bačić et al., 2007). These changes in the diet and rumen microbial concentration are stabilized after approximately 10 days (Warner, 1962). During housing and ration changes, unfamiliar surroundings and establishment of a new hierarchy may cause stress for the cow. During this transition, DMI may be reduced, which would impact the rumen microbial population (Warner, 1962).

The *ampC* resistance gene behaved counterintuitively with increases in both abundance and relative abundance with time in control cows but no changes these measures in antibiotic treated cows. This may be due to a competitive advantage for bacteria containing the antibiotic resistance gene *ampC*. Furthermore, it has been observed that not all antibiotic resistance genes convey a fitness cost on bacteria and some may help bacterial proliferation in the presence of

heat stress and low glucose levels (Rodríguez-Verdugo et al., 2013). However, not all ARGs convey a competitive advantage (Khachatryan et al., 2006). Also, *ampC* is considered a cryptic (non-expressive) antibiotic resistance gene that is not being expressed phenotypically (Barlow, 2002; Salipante, 2003; Hall, 2004). Therefore, it is possible that bacteria carrying the *ampC* resistance gene proliferated and were competitive in control cows. However, bacteria carrying the *ampC* gene in treated cows may have been killed due to the cryptic nature of the *ampC* resistance gene, thus allowing cephalosporins to kill bacteria containing this gene. The genes may not have been quantified by qPCR in the feces because the genes could have been degraded in the abomasum due to the low pH.

In this study, the temporal resistance patterns were similar in the two tetracycline resistance genes. ARGs can be carried together on the same genetically transferable elements (Van den Bogaard and Stobberingh, 2000). Previous studies with cattle have suggested a possible link between tetracycline and erythromycin resistance genes (Inglis et al., 2005; Alexander et al., 2008). The tetracycline resistance genes and erythromycin resistance gene may be contributing to multiple drug resistance and they may be linked together on the same transferable element.

Control cows tended to have greater ARG relative abundance from days 1 through 21 and then decreased to a similar or lower level than antibiotic treated cows. In antibiotic treated cows, the abundance and relative abundance of ARGs remained fairly stable and persistent through the dry period.

CONCLUSIONS

There may be a benefit to using prophylactic dry cow treatment to stabilize the proportion of bacteria carrying ARGs and the abundance of ARGs in feces. Antibiotic treated cows

appeared have a more stable fecal ARG population than control cows, which may be attributed to cephalosporin use moderating the effects of monensin withdrawal at dry-off. Thus it is possible that intramammary dry cow treatment provides a more stable gut microbiome to reduce fluctuations in microbial population due to housing and dietary changes during the dry period. The use of prophylactic dry cow treatment does not uniformly or predictably increase ARGs. However, this study did not examine an exhaustive set of ARGs. The ARGs selected may not have responded in a representative fashion and a metagenomic analysis would be beneficial for future research.

Table 2 Effect of cephalosporin benzathine antibiotic and day on abundance of antibiotic resistance genes in the feces of dry dairy cattle

Log ₁₀ gene copies per gram of freeze-dried feces							
Gene	Antibiotic ¹		Control ²		<i>P</i> = ⁵		
	LSM ³	SEM ⁴	LSM	SEM	Treatment	Day	Interaction ⁶
16S rRNA	9.44	0.03	9.13	0.05	< 0.01	NS	NS
<i>ampC</i>	8.05	0.05	8.46	0.09	< 0.01	< 0.03	< 0.02
<i>ermB</i>	5.81	0.04	5.72	0.07	NS	NS	NS
<i>tetO</i>	8.18	0.03	7.96	0.04	< 0.01	< 0.01	< 0.01
<i>tetW</i>	8.73	0.02	8.76	0.04	NS	< 0.01	< 0.01

¹ n = 9 intramammary antibiotic infusion, 10 mL cephalosporin benzathine (300 mg cephalosporin activity)

² n = 10 control cows received no antibiotic infusion

³ Least squares means for treatment

⁴ Standard error for treatment LSM

⁵ Significance. NS indicates *P* > 0.05

⁶ Two way interaction of treatment and day

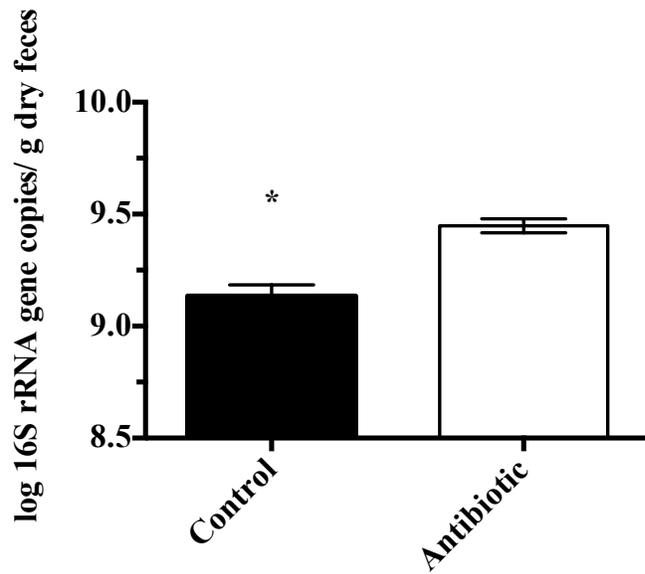


Figure 1 Abundance of 16S rRNA (log₁₀ gene copies per 1.0 g freeze-dried feces) in fecal samples collected from control (n = 10) and antibiotic treated cows (n = 9). Antibiotic treatment was 10 mL cephalosporin benzathine (300 mg cephalosporin activity). The symbol * indicates significant ($P < 0.05$) main effect of treatment.

Table 3 Effect of cephalosporin benzathine antibiotic and control on the relative abundance of antibiotic resistance genes in the feces of dry dairy cattle

Gene	Gene copies per 16S rRNA						
	Antibiotic ¹		Control ²		<i>P</i> = ⁵		
	LSM ³	SEM ⁴	LSM	SEM	Treatment	Day	Interaction ⁶
<i>ampC</i>	0.05	0.01	0.29	0.01	< 0.01	< 0.01	< 0.01
<i>ermB</i>	0.01	0.01	0.01	0.01	< 0.04	NS	NS
<i>tetO</i>	0.05	0.01	0.10	0.01	< 0.01	< 0.01	< 0.01
<i>tetW</i>	0.27	0.02	0.47	0.04	< 0.01	NS	< 0.01

¹ n = 9 intramammary antibiotic infusion, 10 mL cephalosporin benzathine (300 mg cephalosporin activity)

² n = 10 control cows received no antibiotic infusion

³ Least squares means for treatment

⁴ Standard error for treatment LSM

⁵ Significance. NS indicates *P* > 0.05

⁶ Two way interaction of treatment and day

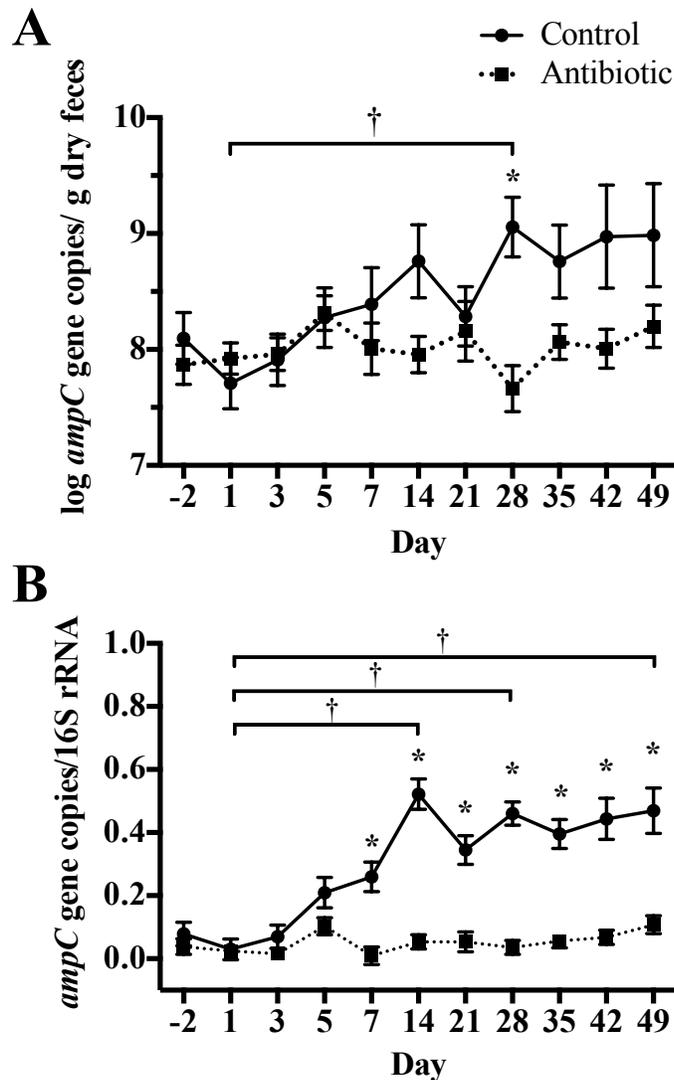


Figure 2 (A) Abundance of *ampC* (log₁₀ gene copies per 1.0 g freeze-dried feces) in fecal samples. (B) Relative abundance of *ampC* (gene copies/ 16S rRNA) in fecal samples collected from control (n = 10) and antibiotic treated cows (n = 9). Antibiotic treatment was 10 mL cephalosporin benzathine (300 mg cephalosporin activity). The symbol * indicates significant ($P < 0.05$) Tukey adjusted pairwise comparisons for treatment within day. The symbol † indicates significant ($P < 0.05$) Tukey adjusted pairwise comparisons for day within treatment.

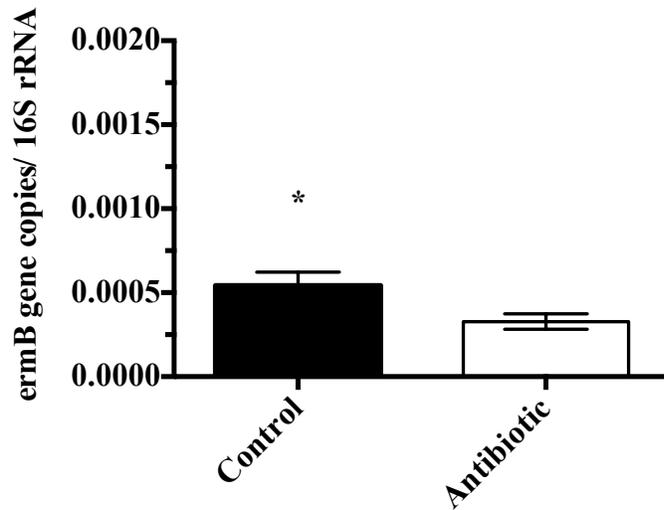


Figure 3 Relative abundance of *ermB* (gene copies per 16S rRNA) in fecal samples collected from control (n = 10) and antibiotic treated cows (n= 9). Antibiotic treatment was 10 mL cephalixin benzathine (300 mg cephalixin activity). The symbol * indicates significant ($P < 0.05$) main effect of treatment.

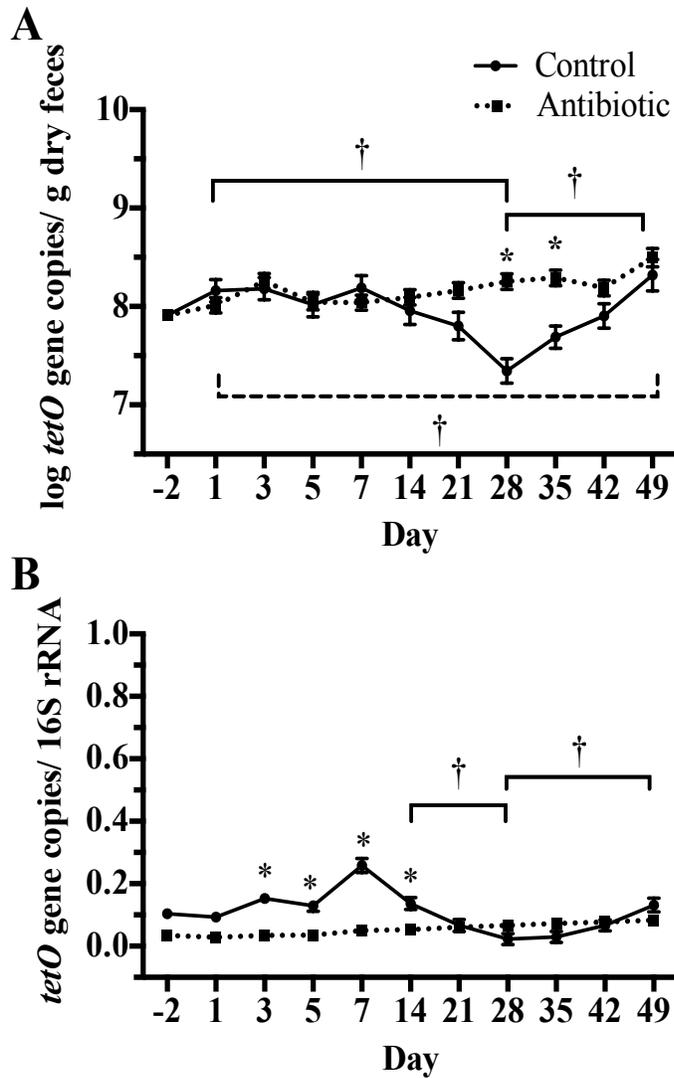


Figure 4 (A) Abundance of *tetO* (log₁₀ gene copies per 1.0 g freeze-dried feces) in fecal samples. (B) Relative abundance of *tetO* (gene copies per 16S rRNA) in fecal samples collected from control (n = 10) and antibiotic treated cows (n = 9). Antibiotic treatment was 10 mL cephalosporin benzathine (300 mg cephalosporin activity). The symbol * indicates significant ($P < 0.05$) Tukey adjusted pairwise comparisons for treatment within day. The symbol † indicates significant ($P < 0.05$) Tukey adjusted pairwise comparisons for day within treatment.

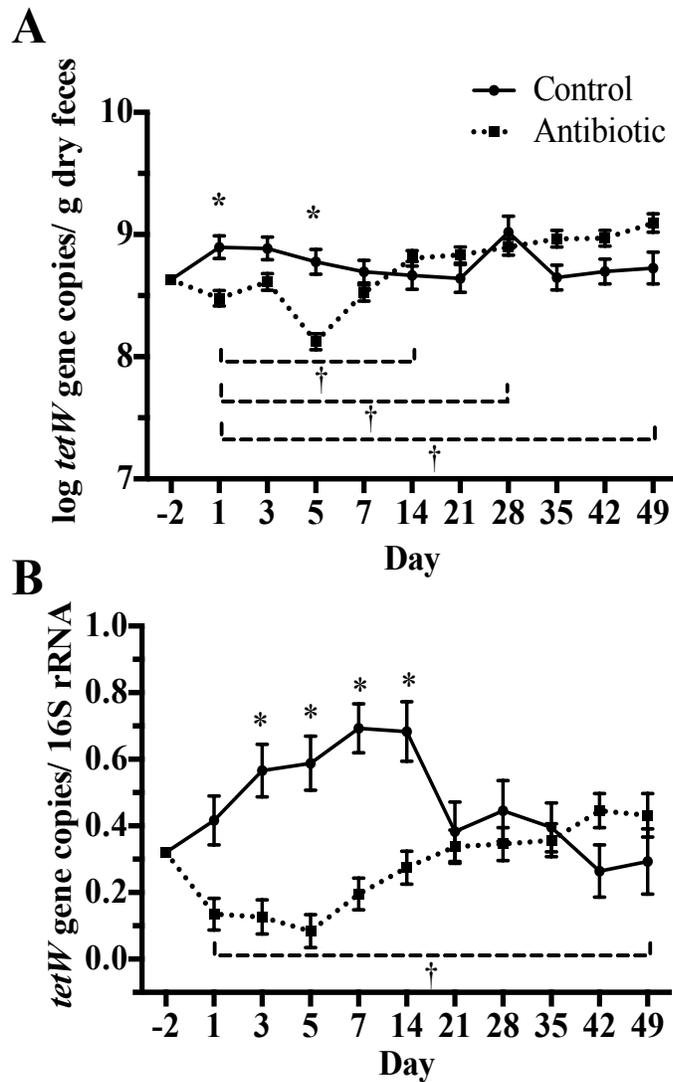


Figure 5 (A) Abundance of *tetW* (log₁₀ gene copies per 1.0 g freeze-dried feces) in fecal samples. (B) Relative abundance of *tetW* (gene copies per 16S rRNA) in fecal samples collected from control (n = 10) and antibiotic treated cows (n = 9). Antibiotic treatment was 10 mL cephalosporin benzathine (300 mg cephalosporin activity). The symbol * indicates significant ($P < 0.05$) Tukey adjusted pairwise comparisons for treatment within day. The symbol † indicates significant ($P < 0.05$) Tukey adjusted pairwise comparisons for day within treatment.

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APPENDIX

Table 4 Primers and annealing temperatures used for this study

Primer	Target gene	Primer Sequence 5'-3'	Reference
1369F	16S rRNA	CGGTGAATACGTTCYCGG	(Suzuki et al., 2000)
1492R	16S rRNA	GGWTACCTTGTTACGACTT	
ampC-Fw	<i>ampC</i>	CCTCTTGCTCCACATTTGCT	(Shi et al., 2013)
ampC-Rv	<i>ampC</i>	ACAACGTTTGCTGTGTGACG	
bla _{CMY-2} -Fw	<i>bla_{CMY-2}</i>	CAGCATCTCCCAGCCTAATC	(Boyer and Singer, 2012)
bla _{CMY-2} -Rv	<i>bla_{CMY-2}</i>	GAAGCCCGTACACGTTTCTC	
ermB-Fw	<i>ermB</i>	GAATCCTTCTTCAACAATCA	(Jacob et al., 2008)
ermB-Rv	<i>ermB</i>	ACTGAACATTCGTGTCACTT	
HS463a	<i>int1</i>	CTGGATTTTCGATCACGGCACG	(Hardwick et al., 2008)
HS464	<i>int1</i>	ACATGCGTGTAATCATCGTCG	
sul1-Fw	<i>sul1</i>	CGCACCGGAAACATCGCTGCAC	(Pei et al., 2006)
sul1-Rv	<i>sul1</i>	TGAAGTTCCGCCGCAAGGCTCG	
tetO-Fw	<i>tetO</i>	ACGGARAGTTTATTGTATACC	(Aminov et al., 2001)
tetO-Rv	<i>tetO</i>	TGGCGTATCTATAATGTTGAC	
tetW-Fw	<i>tetW</i>	GAGAGCCTGCTATATGCCAGC	(Aminov et al., 2001)
tetW-Rv	<i>tetW</i>	GGGCGTATCCACAATGTTAAC	

**Chapter 4 EFFECT OF THERAPEUTIC MASTITIS TREATMENT ON ABUNDANCE
AND ABUNDANCE OF ANTIBIOTIC RESISTANCE GENES IN FECES**

ABSTRACT

Brittany Faith Willing

Intramammary antibiotic mastitis therapy may increase excretion of antibiotic resistance genes (ARGs) by dairy cattle through selection pressure on the gut microbiome. The objective of this study was to evaluate the effect of intramammary pirlimycin on the abundance and relative abundance of ARGs in dairy cattle feces. Upon diagnosis of an intramammary infection (IMI) by bacterial culture, cows (n = 6) received pirlimycin hydrochloride as an intramammary infusion. Fecal grab samples were collected from each cow on d 0, 3, 9, and 12. Fecal samples were collected in sterile containers, then freeze-dried. The abundance and relative abundance of ARGs *ampC*, *bla_{CMY-2}*, *ermB*, *sulI*, *tetO*, *tetW*, *intI*, and 16S rRNA were quantified using qPCR. The genes *ampC* and *bla_{CMY-2}* encode resistance to β -lactam antibiotics, *ermB* to macrolides, *sulI* to sulfonamides, *tetO* and *tetW* to tetracyclines, and *intI* a class-1 integrase gene that facilitates horizontal transfer of ARGs across bacteria. The 16S rRNA gene was used as a representation of bacterial population. Absolute abundance was defined as number of ARG copies per gram of freeze-dried feces, while relative abundance was defined as ARG copy numbers per copy of 16S rRNA gene, which is indicative of the proportion of bacteria carrying ARGs. Non-normal data were logarithmically transformed and were statistically analyzed using PROC GLIMMIX in SAS 9.2. Abundance and relative abundance of *sulI* and *bla_{CMY-2}* were below the limit of quantification in most samples and therefore not suitable for statistical comparisons. The *intI* gene was not detectable in any sample. There was a significant main effect of day on the abundance of 16S rRNA (decreased with day) and relative abundance (increased with day) for

ermB, *tetO*, and *tetW*. There was no effect of day on the relative abundance of *ampC* or the abundance of *ampC*, *ermB*, *tetO*, or *tetW* in feces of cows with clinical mastitis. Administering fast-acting antibiotics as therapeutic intramammary mastitis treatment to dairy cows may increase the relative abundance of ARGs (gene copies per 16S rRNA) but not the total abundance of ARGs in feces.

Key words: antibiotic resistance gene, pirlimycin, therapeutic

INTRODUCTION

The World Health Organization (WHO) has deemed antimicrobial resistance a global challenge and expressed the need for containment (WHO., 2000). The WHO calls antibiotic resistance “a natural, unstoppable phenomenon exacerbated by the abuse, overuse, and misuse of antimicrobials in the treatment of human illness and in animal husbandry, aquaculture, and agriculture.” The WHO’s five-point plan, Wisely and Widely Points for Action, call for the reduction of antimicrobial use in animal agriculture. Antibiotic resistance genes (ARG) themselves are considered an emerging environmental contaminant because ARGs behave similarly to other environmental contaminants and can be perpetuated in the environment (Pruden et al., 2006).

Antimicrobials have been used for disease treatment (therapeutic use), disease prevention (prophylactic use), and as growth promotants in animal agriculture since the late 1940s. (Gustafson and Bowen, 1997; Aarestrup, 1999). Antimicrobial use can affect the composition of the gastrointestinal (GI) microbiome, and the GI tract is the largest reservoir of commensal bacteria. Antimicrobials can alter populations of endogenous bacteria by bactericidal or bacteriostatic methods. They can also facilitate change in the genetic composition of bacteria through selective pressure (Dibner and Richards, 2005). Such selective pressures increase the

expression and spread of ARGs (Van den Bogaard and Stobberingh, 2000). Antibiotic resistance genes may be carried by pathogenic and non-pathogenic bacteria, and by Gram-positive and Gram-negative bacteria. The selective pressures caused by antimicrobials allow resistant bacteria to flourish in a less competitive environment (Van den Bogaard and Stobberingh, 2000; Kolář et al., 2001).

In dairy cattle, the largest use of antibiotics is for the treatment and prevention of bacterial mastitis (Mitchell et al., 1998; Sawant et al., 2005), the most costly disease in the dairy industry (Blosser, 1979). The largest economic cost is milk loss due to clinical and subclinical mastitis (Janzen, 1970). Other losses include those from discarded milk due to abnormality or antibiotic contamination, veterinary services for acute and chronic mastitis, antibiotic cost, increased labor, decreased animal sale value, and increased replacement costs (Dobbins Jr, 1977).

No published research has evaluated the effect of intramammary antibiotic mastitis therapy on the abundance or relative abundance of fecal ARGs, though several have documented increased phenotypic expression of antibiotic resistance in mastitis pathogens. Resistance to pirlimycin has been observed in CNS (Lüthje and Schwarz, 2006), *Staphylococcus aureus*, and *Streptococcus spp.* cultured from the milk of cows subjected to pirlimycin (Pol and Ruegg, 2007). Also, pirlimycin resistance has increased over a seven-year period in *Streptococcus uberis*. (Erskine et al., 2002).

MATERIALS AND METHODS

Animals and Experimental Treatments

Holstein (n = 4), Jersey (n = 1), and crossbred (n = 1) cows, in the second or later lactation, that contracted clinical mastitis at Virginia Tech Dairy Center (Blacksburg, Virginia) were used in this study. Cows were housed in a free stall barn and fed a total mixed ration with monensin in accordance with dietary needs. The feed bunk was of sufficient length to allow all individuals to feed at once. Cows were fed once daily and had *ad libitum* access to water.

Procedures regarding aseptic milk sampling and microbiological culture were as previously described (Hogan et al., 1999). Trained milking personnel detected clinical mastitis at the time of milking. Clinical mastitis was defined as the change in milk appearance with or without redness and/or swelling of the associated gland. Upon detection of mastitis, an aseptically collected milk sample was taken to determine quarter-level infection status. In brief, bacterial identification was determined using MacConkey agar plates divided in half (2 quarters per plate; 100 μ L of milk from each quarter) and blood agar plates divided in four (1 quarter per section), with 10 μ L of milk from each quarter streaked using a disposable sterile loop. Following incubation (37°C, 18 h and 48 h), a presumptive identification of isolated bacteria was made and biochemical tests were performed.

When an intramammary infection (IMI) was diagnosed, cows (n = 6) were treated (3 consecutive days) with 10 mL pirlimycin hydrochloride (50 mg pirlimycin activity); (Pirsue[®] Zoetis, Madison, NJ), per quarter. However, there were no paired control cows. Cultured mastitis pathogens are listed in the appendix (Table 7). All protocols and procedures were approved by The Virginia Tech Institutional Animal Care and Use Committee.

Sample Collection and Preparation

Fecal samples were obtained by rectal palpation with lubricant (Jorgensen Laboratories, Loveland, CO), and a new palpation sleeve used for each sample collected. Approximately 500-1,000 g of feces were discarded to minimize glove and lubricant contamination. Fecal samples were collected into sterile snap cap vials, frozen immediately (-20°C), and stored until analysis. Fecal samples were obtained just prior to treatment d 0, and on d 3, 9, and 12. Cows used in this study ranged from 32 to 91 DIM (median = 60 d).

Fecal samples were thawed at room temperature and mixed thoroughly with a flame-sterilized spatula. One gram of feces was weighed into 50 mL conical tubes and tubes were covered with a square of aluminum foil. Samples were frozen for 30 min in an -80°C freezer in preparation for freeze-drying. Fecal samples were freeze-dried to minimize variation in fecal dry matter and to standardize the amount of fecal material used for DNA extraction (FreezeZone Benchtop, Labconco, Kansas City, MO; (Alexander et al., 2011). Freeze-dried samples were mixed uniformly with a flame-sterilized spatula in preparation for DNA extraction.

DNA Extraction

DNA was extracted from 0.05 g of freeze-dried feces using the FastDNA[®] Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) in accordance to manufacturer instructions including the use of the FastPrep[®] instrument (MP Biomedical, Santa Ana, CA). All DNA extracts were incubated for five minutes in a 55°C water bath to increase DNA yield then DNA was immediately stored at -80°C.

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Previously reported qPCR protocols were utilized to quantify the genes 16S rRNA (Suzuki et al., 2000), *ampC* (Shi et al., 2013), *bla_{CMY-2}* (Boyer and Singer, 2012), *ermB* (Jacob et

al., 2008), *tetO* and *tetW* (Aminov et al., 2001), *sull* (Pei et al., 2006), and *int1* (Hardwick et al., 2008). Primer sequences are provided in the appendix (Table 8).

Q-PCR quantifications were performed in analytical triplicate using a CFX96™ Real-Time System (Bio-Rad, Hercules, CA). The 30 μL reaction mixture contained 5 μL SsoFast EvaGreen (Bio-Rad, Hercules, CA), with 0.6 μL (5M) of each forward and reverse primer, 2.8 μL of molecular grade water, and 1 μL of 1:75 diluted DNA per well. Temperature specifications were 98°C for 2 min; 39 cycles of 98°C then respective annealing temperatures [60.0°C for 16S rRNA, *int1*, and *tetW*, 50.3°C for *tetO*, 57.8°C for *ampC*, 69.9°C for *sull*] 45 cycles of 98°C [57.8°C for *ermB*, and 70.0°C for *bla_{CMY-2}*]. For graphical representation, absolute abundance was defined as number of ARG copies per gram of freeze-dried feces, while relative abundance was defined as ARG copy numbers per copy of 16S rRNA gene, which is indicative of the proportion of bacteria carrying ARGs.

Q-PCR Calibration Curves and Quantification

Extracted fecal DNA was amplified using traditional PCR and cloned using TOPA TA Cloning Kit (Invitrogen, Carlsbad, CA). Gene copies were determined by quantification through gel electrophoresis (1.5% agarose, 1:50 TAE buffer) and QuantityOne® Software (Bio-Rad, Hercules, CA). Log gene copies per mL of DNA were determined with previously described methods (Pei et al., 2006). Calibration curves were prepared for each gene with serial dilutions of positive controls over seven orders of magnitude.

Data Management and Statistical Analysis

Q-PCR wells with values below the limit of quantification (LOQ) were excluded from data analysis. Samples with two of three wells above the LOQ were included for statistical analysis. LOQ for *ampC* was 1×10^4 and for all other genes were 1×10^2 .

All statistical tests were performed using the GLIMMIX procedure of SAS 9.2 (SAS Institute Inc. Cary, NC). Non-normal data were log₁₀ transformed to achieve normality prior to statistical analysis. Log-transformed data were used to calculate LSM ± SEM. The statistical model included the fixed effect of day with cow as the random effect. The repeated statement was applied to the day of sampling using cow as the subject. Various error structures were tested; Akaike produced the lowest information criterion and was chosen for analysis. Goodness-of-fit *P* values were determined for the logistic and quadratic regression. Significance was declared at *P* < 0.05.

RESULTS and DISCUSSION

16S rRNA

The fecal abundance of 16S rRNA was defined as gene copies per 1.0 g freeze-dried feces in this study; this value is an indicator of bacterial population in the feces of cows administered therapeutic intramammary antibiotic treatment. The abundance of 16S rRNA was affected by day following antibiotic therapy (*P* < 0.01, Table 5), and declined in a linear and quadratic fashion (Figure 6). In this study, pirlimycin was administered for three consecutive days after culture identification of a clinical IMI. The decline in fecal abundance of 16s rRNA genes for the 9 days following treatment (a time period coinciding with the meat withdrawal period) was likely due to the bacteriostatic antibiotic effect of pirlimycin.

Resistance Genes

The relative abundance of *ermB*, *tetO*, and *tetW* increased with day (*P* < 0.01, *P* < 0.01, *P* < 0.01 respectively, Table 6) and in a linear and quadratic fashion (Figure 7, Figure 8, Figure 9 respectively). There was no effect of day on the fecal relative abundance of *ampC* or the abundance of *ampC*, *ermB*, *tetO*, and *tetW*.

The *bla*_{CMY2} and *sul1* genes were detected in too few fecal samples to be evaluated statistically, although both genes were detectable in some animals with no apparent effect of treatment. The *int1* gene was not detected in any sample.

There was a change in relative abundance of resistance genes following therapeutic pirlimycin use during a time frame that aligns with the meat withdrawal period. This finding could be contradictory to previous findings where antibiotic resistance measured phenotypically remains unchanged through the withdrawal period (Sharma et al., 2008; Alexander et al., 2010). The observed change in the relative abundance suggests an increase in the proportion of bacteria carrying antibiotic resistance. This could be due to the acquisition of ARGs in non-resistant bacteria or the spread of ARGs through a resistant bacterial population.

Following antibiotic therapy, the change in the relative abundance of the resistance genes *ermB*, *tetO*, and *tetW* had a similar quadratic goodness-of-fit, which may suggest co-selection on the same genetic element and/or multiple drug resistance in the GI tract of these cows. Others have reported multiple drug resistance exists in the feces of bovine fed subtherapeutic levels of antibiotics (Inglis et al., 2005; Sharma et al., 2008; Alali et al., 2009). The current study provides further evidence that low levels of antibiotics reaching the gut microflora may provide selection pressure to facilitate single and multi-drug resistance.

CONCLUSIONS

Therapeutic mastitis treatment may increase the proportion of bacteria carrying ARGs in the feces of lactating dairy cows. However, more studies are needed to determine if these are true effects of treatment or the effects of temporal variation independent of antibiotic treatment. Also, this study did not examine an exhaustive set of ARGs. The ARGs selected may not have

responded in a representative fashion and a metagenomic analysis would be beneficial for future research.

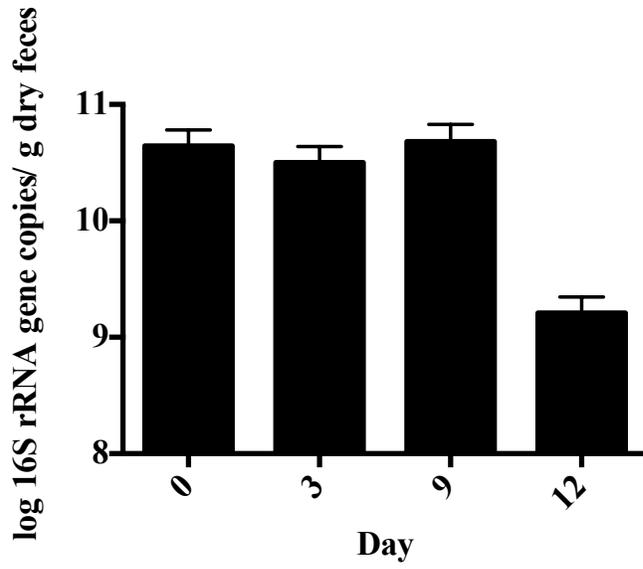


Figure 6 Abundance of 16S rRNA genes (log₁₀ gene copies per 1.0 g freeze-dried feces) in fecal samples from 10 mL pirlimycin hydrochloride (50 mg pirlimycin activity) treated cows (n = 6).

Table 5 Effect of day on the abundance of genes in the feces of mastitic dairy cattle treated with pirlimycin hydrochloride.

Gene	Day								$P =^3$		
	0		3		9		12		Day ⁴	Linear ⁵	Quadratic ⁶
	LSM ¹	SEM ²	LSM	SEM	LSM	SEM	LSM	SEM			
16S rRNA	10.64	0.08	10.50	0.16	10.52	0.22	9.21	0.15	< 0.01	< 0.01	< 0.01
<i>ampC</i>	8.26	0.18	8.10	0.18	8.39	0.19	8.07	0.19	NS	NS	NS
<i>ermB</i>	6.26	0.20	6.37	0.20	6.22	0.17	6.51	0.17	NS	NS	NS
<i>tetO</i>	7.60	0.12	7.61	0.21	7.52	0.24	7.80	0.18	NS	NS	NS
<i>tetW</i>	8.45	0.05	8.52	0.13	8.44	0.23	8.56	0.10	NS	NS	NS

¹ Least squares means for treatment

² Standard error for treatment LSM

³ Significance. NS indicates $P > 0.05$

⁴ Main effect of day

⁵ Goodness-of-fit linear

⁶ Goodness-of-fit quadratic

Table 6 Effect of day on the relative abundance (ARG copies/ 16S rRNA) of antibiotic resistance genes in the feces of mastitic dairy cattle treated with pirlimycin hydrochloride.

Gene	Day								$P =^3$		
	0		3		9		12		Day ⁴	Linear ⁵	Quadratic ⁶
	LSM ¹	SEM ²	LSM	SEM	LSM	SEM	LSM	SEM			
<i>ampC</i>	0.01	0.01	0.01	0.01	0.01	0.01	0.10	0.04	NS	NS	NS
<i>ermB</i>	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	< 0.01	< 0.01	< 0.04
<i>tetO</i>	0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.01	< 0.01	< 0.01	< 0.01
<i>tetW</i>	0.01	0.01	0.01	0.01	0.01	0.01	0.24	0.03	< 0.01	< 0.01	< 0.01

¹ Least squares means for treatment

² Standard error for treatment LSM

³ Significance. NS indicates $P > 0.05$

⁴ Main effect of day

⁵ Goodness-of-fit linear

⁶ Goodness-of-fit quadratic

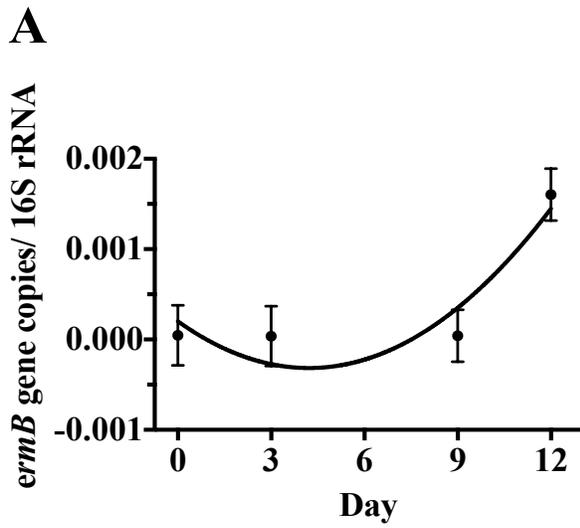


Figure 7 Relative abundance of *ermB* (gene copies per 16S rRNA) in fecal samples from 10 mL pirlimycin hydrochloride (50 mg pirlimycin activity) treated cows (n = 6). Significant goodness-of-fit for the quadratic regression ($P < 0.05$).

B

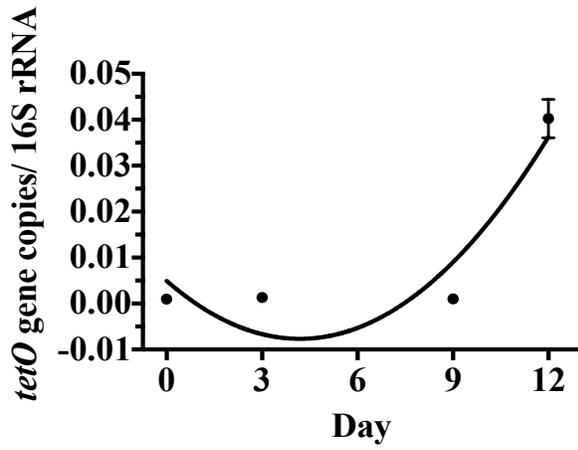


Figure 8 Relative abundance of *tetO* (gene copies per 16S rRNA) in fecal samples from 10 mL pirlimycin hydrochloride (50 mg pirlimycin activity) treated cows (n = 6). Significant goodness-of-fit for the quadratic regression ($P < 0.05$).

C

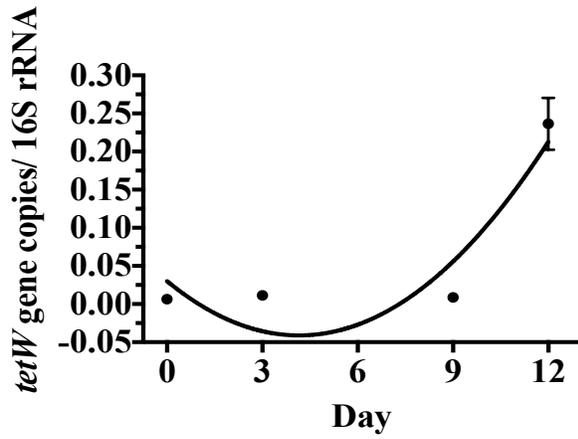


Figure 9 Relative abundance of *tetW* (gene copies per 16S rRNA) in fecal samples from 10 mL pirlimycin hydrochloride (50 mg pirlimycin activity) treated cows (n = 6). Significant goodness-of-fit for the quadratic regression ($P < 0.05$).

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APPENDIX

Table 7 Mastitis pathogens and cow information for six cows treated for intramammary infection with pirlimycin hydrochloride

Cow	DIM ¹	Quarter ²	Mastitis Pathogen
4479	75	Left Front	Undetermined Gram-negative Bacteria
4571	42	Left Front	Esculin-positive <i>Streptococcus</i>
4591	24	Right Rear	<i>Klebsiella</i>
4605	96	Right Front	Yeast and CNS ³
4609	32	Left Front	Esculin-positive <i>Streptococcus</i>
4719	91	Right Rear	Esculin-positive <i>Streptococcus</i>

¹ Days in milk

² Mammary gland with infection

³ Coagulase-negative *Staphylococcus*

Table 8 Primers and annealing temperatures used for this study

Primer	Target gene	Primer Sequence 5'-3'	Reference
1369F	16S rRNA	CGGTGAATACGTTCYCGG	(Suzuki et al., 2000)
1492R	16S rRNA	GGWTACCTTGTTACGACTT	
ampC-Fw	<i>ampC</i>	CCTCTTGCTCCACATTTGCT	(Shi et al., 2013)
ampC-Rv	<i>ampC</i>	ACAACGTTTGCTGTGTGACG	
bla _{CMY-2} -Fw	<i>bla_{CMY-2}</i>	CAGCATCTCCCAGCCTAATC	(Boyer and Singer, 2012)
bla _{CMY-2} -Rv	<i>bla_{CMY-2}</i>	GAAGCCCGTACACGTTTCTC	
ermB-Fw	<i>ermB</i>	GAATCCTTCTTCAACAATCA	(Jacob et al., 2008)
ermB-Rv	<i>ermB</i>	ACTGAACATTCGTGTCACTT	
HS463a	<i>int1</i>	CTGGATTTTCGATCACGGCACG	(Hardwick et al., 2008)
HS464	<i>int1</i>	ACATGCGTGTAATCATCGTCG	
sul1-Fw	<i>sul1</i>	CGCACCGGAAACATCGCTGCAC	(Pei et al., 2006)
sul1-Rv	<i>sul1</i>	TGAAGTTCCGCCGCAAGGCTCG	
tetO-Fw	<i>tetO</i>	ACGGARAGTTTATTGTATACC	(Aminov et al., 2001)
tetO-Rv	<i>tetO</i>	TGGCGTATCTATAATGTTGAC	
tetW-Fw	<i>tetW</i>	GAGAGCCTGCTATATGCCAGC	(Aminov et al., 2001)
tetW-Rv	<i>tetW</i>	GGGCGTATCCACAATGTTAAC	

Chapter 5 IMPLICATIONS

Antibiotic resistance and its associated genes are a complex problem with no clear solution. ARGs and their dissemination can be exacerbated or diminished under various conditions. In this research, ARGs remained similar through the dry period in cows administered prophylactic dry cow therapy whereas cows not receiving antibiotics seemed to fluctuate in both the number of ARGs and the proportion of bacteria carrying them. In this research, while using prophylactic antibiotic treatment tetracycline resistance genes (*tetO* and *tetW*) were one-fold higher in non-antibiotic treated cows than antibiotic treated cows. Therefore, the actual administration of antibiotics may be a method to control and reduce antibiotic resistance genes in dairy cow feces during the dry period. In contrast, using therapeutic antibiotics perpetuated certain ARGs.

Efforts to contain antibiotic resistance should focus on the DNA and not whole organisms. Other methods have also been tested for the reduction of ARGs in fecal material such as composting manure under high and low-intensity management strategies (Storteboom et al., 2007), biologically treating manure to reduce ARGs (Pei et al., 2007), and testing different temperature and size configurations of waste lagoons to reduce ARGs (McKinney et al., 2010). All methods have shown that treatment of manure can reduce certain ARGs. However, more research is needed to pinpoint an effective and efficient way to eliminate ARGs. Understanding and controlling the dissemination ARGs is the key to controlling antibiotic resistance.

The experiment with lactating cows (chapter 4) was a useful preliminary study but the lack of untreated controls and the prolonged time frame limit interpretation. The next step for this type of research is to conduct a microbial challenge study and control for the microorganism causing mastitis. For example, a challenge trial with the organism *Staphylococcus aureus* may be

extremely beneficial to provide insight into the effect of therapeutic antibiotics on the gut microbiome during a clinical mastitis infection since antibiotics can have an impact on gut microbial composition. The use of a wild-type strain of *S. aureus* would be beneficial to simulate a true mastitis infection and it should be characterized to determine preexisting ARGs. Also, a *S. aureus* infection is slow acting and curable if caught early. To determine the effect of a *S. aureus* infection on the gut microbial composition, fecal samples would need to be collected prior to bacterial challenge to act as a covariate in the model. Fecal samples would also need to be collected prior to antibiotic administration to analyze effects of disease on the gut microbiome independent of antibiotic effects. After infection with *S. aureus* an antibiotic suitable for the killing of this organism should be administered into the udder and fecal sampling should progress.

For this study and future research building on the dry cow study, a metagenomic analysis will provide a more complete understanding of resistance genes present in the feces of mastitic dairy cattle. With a metagenomic blueprint for ARGs, qPCR can be more effectively utilized to determine the amount of ARGs present before and after treatment. ARGs in dairy cattle is a new and exciting area for research. This research area may provide more information to veterinarians and producers about the use of antibiotics and the potential negative impacts of overusing and misusing them in animal agriculture and the benefits of manure management practices to reduce ARGs.