Antibiotic resistance gene abundance in feces of calves fed pirlimycin-dosed whole milk

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ABSTRACT

Exposure to antibiotics has the potential to increase the incidence and proliferation of antibiotic resistance genes (ARG) in the gut and fecal microbiome. Non-saleable, antibiotic-containing milk from cows treated with antibiotics (waste milk) is commonly fed to dairy calves but the effects of ingestion of antibiotics at an early age on the gut microbiome and the development of ARG in the naïve gut are not well understood. Pirlimycin, a lincosamide antibiotic acting against Gram positive bacteria through inhibiting protein synthesis by binding to the 50S ribosome, is commonly used as mastitis therapy. Lincosamides are also considered highly important in human medicine, often used against *Staphylococcus aureus* and *Clostridium difficile* infections. Emerging microbial resistance to pirlimycin is of concern for both animal and human health. The objective of this study was to determine the effect of early lincosamide antibiotic exposure on the abundance of ARG in feces of milk-fed calves. Eight female Holstein calves were blocked by age, paired by block, and randomly assigned to pasteurized whole milk (control; n = 4) or milk containing 0.2 mg/L of pirlimycin (treatment; n = 4). Calves were enrolled after receiving two colostrum feedings and were fed 5.68 L of pasteurized whole milk, treatment, or control, divided into two daily feedings, from d 1 to d 50 of age. After weaning calves were fed non-medicated starter grain *ad libitum*. Fecal samples were collected weekly until 85 d of age and freeze-dried. DNA was extracted using QiaAmp® Fast DNA Stool Mini Kit and qPCR was used to quantify the absolute abundance (gene copies/g of wet feces) and relative abundance (gene copies/copies of 16S rRNA genes) of *erm*(B), *tet*(O), *tet*(W) and 16S rRNA genes. Data was analyzed using PROC GLIMMIX in SAS. Abundance of 16S rRNA

genes, *tet*(O) and *tet*(W) were not different between control and pirlimycin-fed calves nor were the relative abundance of *tet*(O) (mean = 0.050 *tet*(O) copies/16S rRNA genes) or *tet*(W) (0.561 *tet*(W) copies/16S rRNA genes). While abundance of *erm*(B) was higher in pirlimycin-fed calves compared to control calves (6.46 and 6.04 log gene copies/g wet feces; $P = 0.04$) the relative abundance of *erm*(B) (0.273 gene copies/16S rRNA genes) in feces of calves was not influenced by treatment. There was an effect of day ($P < 0.10$) for absolute abundance of *tet*(O), *tet*(W), and *erm*(B) indicating that the levels change with time as the fecal microbiome develops. This study suggests that feeding pirlimycin-containing non-saleable milk to growing calves may increase environmental loading of e*rm*(B), which codes for resistance to highly important macrolide and lincosamide antibiotics. Additional research is needed on effects of feeding waste milk to calves on other fecal ARG and on the post-excretion and post-application fate of these genes.

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CHAPTER 1: INTRODUCTION

The development of antibiotics has changed the face of medicine by successfully combating diseases that were previously untreatable. However, resistance to antibiotics developed almost as soon as antibiotics were discovered (Abraham and Chain, 1940). Antibiotic resistance is becoming increasingly common in the United States due to their overuse and misuse, costing the US health system $21-$34 billion annually (World Health Organization, 2014). Commonly acquired infections, such as *Staphylococc*us *aureus* and *Escherichia coli* are becoming increasingly resistant to even the most potent antibiotics, deeming antibiotic resistance an issue of critical concern (Tan et al., 2014; World Health Organization, 2014). The possibility of a post-antibiotic era, where antibiotics are no longer able to treat even minor infections, is becoming increasingly realistic (World Health Organization, 2014). Consequently, this rise in antibiotic resistance must be addressed.

The World Health Organization has classified animal agriculture as one of the main human actions contributing to the acceleration of the emergence of antibiotic resistance, as more than half of antibiotic use in the US is agricultural (World Health Organization, 2012). In 2010, approximately 13.3 million kg of antimicrobials were sold in the United States for use in food-producing animals, while 3.28 million kg of antibacterial drugs were sold for human use. The estimated global average of antibiotic consumption for livestock animals is 45 mg/kg for cattle. Assuming the weight of the average Holstein weighing is about 680 kg, the estimated annual consumption per Holstein cow is .031 kg while the average person in the United States is administered approximately 1.061 kg of antibiotic annually (Van Boeckel et al., 2015; World Health Organization, 2014). Thus, livestock production's portion of antibiotic usage is actually closer to 80% of total domestic use, in terms of number of kilograms used (FDA, 2010; 2012b).

This administration of antibiotics to livestock can lead to antibiotic resistance in the microbial communities of the animal as well as those in the soil and water, due to the dissemination of residual antibiotic, antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARG) in livestock feces (McEwen and Fedorka-Cray, 2002).

There are three main mechanisms of bacterial transfer of antibiotic resistance, which can result in its rapid spread. These mechanisms include 1) transformation, where short fragments of DNA containing ARG are taken in by the cell, 2) conjugation where DNA coding for antibiotic resistance is transferred via cell to cell contact, and 3) transduction, where DNA is transferred from one bacterium to another via bacteriophages (Schwarz and Chaslus-Dancla, 2001). Traditionally, culture-based methods are used to identify and quantify antibiotic resistant bacteria. However, newer methods, like quantitative polymerase chain reaction (qPCR), are able to assess the incidence of ARG quantitatively for all bacteria present in a sample, rather than just bacterial isolates (Steffan and Atlas, 1991). Additionally, metagenomic analysis of environmental samples is becoming increasingly relevant to antibiotic resistance research, and methods like 454 pyrosequencing and Illumina sequencing are able to provide intricate detail for entire bacterial metagenomes. These next-generation sequencing methods are analyzed through the use of publicly available sequencing databases including SEED and MG-RAST (Wooley et al., 2010). However, it is important to note that while these technologies provide a wealth of information on the presence of ARG, the presence of ARG does not necessarily mean these ARG are being expressed (Fouhy et al., 2015). Therefore, detection of ARG does not always mean that resistance to antibiotic is occurring.

The effect of antibiotic exposure on the fecal bacteria of dairy calves has been studied for many years, but the results yielded are inconclusive due to utilizing a primarily culture-based

approach. Feeding antibiotic containing non-saleable milk to dairy calves is a common practice within the dairy industry. The digestive tract of calves is inhabited by an initially homogeneous population of bacteria that increases in diversity as the calf ages. When the naïve calf gut is exposed to antibiotics, the bacterial population becomes different from the population present in calves with no antibiotic exposure (Aust et al., 2012; Langford et al., 2003; Thames et al., 2012). While it is known that exposure to antibiotics can lead to the proliferation of ARG, studies of fecal ARG dissemination in dairy calves with oral exposure to antibiotics yield conflicting results, possibly due to the limitations of the primarily culture-based methods employed (Aust et al., 2012; Pereira et al., 2014; Wray et al., 1990). However, it is known that waterways and cropland located proximally to agricultural areas tend to have higher incidences of antibiotic resistant bacteria and ARG than other areas, and this higher incidence of ARG can contaminate crops that are for human consumption (Marti et al., 2013; Shi et al., 2013). Further research is needed to understand the role of early antibiotic exposure on ARG proliferation in the naïve gut microbiome of the dairy calf. The current study aims to address the limitations of previous culture-based research through the use of molecular techniques to better quantify the ARG abundance in feces of calves exposed to pirlimycin antibiotic.

CHAPTER 2: REVIEW OF LITERATURE

ANTIBIOTICS AND THE DAIRY INDUSTRY

Antibiotics are naturally occurring, synthetic, or semi-synthetic substances that prevent or inhibit microbial growth. Many antibiotics are produced naturally by specific strains of fungi and bacteria (Allen et al., 2010; Thomas et al., 2012) The first antibiotic, penicillin, was discovered by Alexander Fleming, in 1928 (Fleming, 1929). By 1940, the survival of bacteria that were previously susceptible to penicillin was observed, and the modern issue of antibiotic resistance (AR) began to emerge (Abraham and Chain, 1940). Antibiotic resistance, the ability of a microbe to survive, or even thrive, in the presence of an antibiotics, has since become a major global concern (World Health Organization, 2012).

Livestock operations are often considered reservoirs for antibiotic resistance, as antibiotic use is prevalent in animal agriculture (Khachatourians, 1998). It is estimated that more than half of global antibiotic use can be attributed to livestock production (Teuber, 2001). In 2010, approximately 13.3 million kg of antimicrobials were sold in the United States for use in food-producing animals, while 3.28 million kg of antibacterial drugs were sold for human use. The estimated global average of antibiotic consumption for livestock animals is 45 mg/kg for cattle. Assuming the weight of the average Holstein weighing is about 680 kg, the estimated annual consumption per Holstein cow is .031 kg while the average person in the United States is administered approximately 1.061 kg of antibiotic annually (Van Boeckel et al., 2015; World Health Organization, 2014). Thus, livestock production's portion of antibiotic usage is actually closer to 80% of total domestic use (FDA, 2010; 2012b). Antibiotic misuse and overuse is a continuing issue; the total amount of antimicrobials sold for use in food-producing animals

increased by 16% from 2009 to 2012, despite recent legislation focusing on minimizing the development of antibiotic resistance by reducing antibiotic use (FDA, 2012a; c).

Within the livestock industry, antibiotics are administered both therapeutically and prophylactically, and can also be used for growth promotion, although a recent FDA document calls for pharmaceutical companies to voluntarily eliminate the production of antibiotics for the latter use (FDA, 2013). Within the dairy industry, antibiotics are most commonly administered to treat or prevent pneumonia, enteritis and other diarrheal diseases in calves or to prevent or treat mastitis in cows (Sawant et al., 2005). The most commonly administered antibiotics on dairy farms - penicillins, cephalosporins, and tetracyclines - are mainly used to treat respiratory disease, mastitis, metritis, and foot maladies (Zwald et al., 2004). Most medications administered to livestock as growth promoters, such as bacitracin, are not ones that have human applications (Phillips et al., 2004). However, this is not the case for many common therapeutic antibiotics such as β-lactams and lincosamides, whose use in animal agriculture could be linked to the emergence of antibiotic resistance currently affecting humans (Phillips et al., 2004; Teuber, 2001).

Another common source of antibiotic exposure within the dairy industry is via liquid feed, such as milk replacer, bulk milk, and non-saleable milk, to dairy calves. The most commonly fed liquid feed, milk replacer, often contains a combination of oxytetracycline and neomycin Zwald et al. (2004). According to the USDA Dairy Heifer Raiser, published in 2011, milk replacer was fed on 85.9% of farms, with 62.8% of these farms utilizing medicated milk replacer. The most commonly fed medication was a 1:1 mixture of oxytetracycline and neomycin (USDA, 2011).

Calves are fed non-saleable (waste) milk, either solely or in combination with milk replacer, on approximately 28% of US dairy farms (USDA, 2011). Non-saleable milk is an otherwise unusable byproduct from antibiotic-treated cows, whose milk is unable to be sold due to its high somatic cell count and residual antibiotic or other medication present in the milk (Aust et al., 2012). This milk can be fed either pasteurized or unpasteurized (raw), although it is recommended that pasteurized non-saleable milk be fed to reduce the risk of infectious disease proliferation in the calf (Godden and Eastridge, 2011). Feeding this milk instead of milk replacer is beneficial to the producer, as it reduces the input cost of raising heifers by $34 per calf from birth to weaning (Godden et al., 2005). Feeding pasteurized non-saleable milk also results in higher growth rates and lower morbidity and mortality rates compared to calves fed milk replacer (Godden et al., 2005). This higher rate of growth is generally attributed to a decreased incidence of disease and a generally higher energy and protein intake (Godden et al., 2005). However, these benefits are not observed in calves fed raw (unpasteurized) milk (El-jack and Ahmed, 2012).

While there are numerous benefits to feeding pasteurized non-saleable milk there are also associated risks that must be considered, including potential for exposure of the naïve calf gut to functional antibiotics and pathogens. The bacterial log colony forming units (cfu) per mL is higher in raw non-saleable milk (6.7 log cfu/mL) than in milk replacer (3.9 log cfu/mL) (Elizondo-Salazar et al., 2010). Even after following pasteurization guidelines with non-saleable milk, it is possible that pathogens may still be viable in the milk, especially if the cfu in the raw milk is high (Godden and Eastridge, 2011). Additionally, most standard dairy industry pasteurization treatments of raw milk can result in as little as 0.01% degradation of antibiotics in milk. While certain temperature treatments have been found to be more effective at degrading

antibiotics, no treatment is able to completely remove the presence of the antibiotic residue (Zorraquino et al., 2008). Therefore when calves are fed pasteurized non-saleable milk, they, and their gut microbiome, are being exposed to whole, functional antibiotic.

MECHANISMS OF ANTIBIOTIC RESISTANCE

The development of AR can occur naturally as a response to selection pressure and other host-related factors, but is exacerbated by the use of antimicrobial drugs (Allen et al., 2010; Schwarz and Chaslus-Dancla, 2001). Bacterial resistance to antibiotics occurs via several different mechanisms. Resistance to antimicrobial agents has developed in as little as one year, demonstrating the ability of bacteria to quickly and efficiently adapt to overcome the effects of antimicrobials (Schwarz and Chaslus-Dancla, 2001).

Antibiotic resistance may be innate or induced (Anderson and O'Toole, 2008). Innate resistance is the natural ability of a bacteria to protect itself from the action of the antibiotic, due to the presence of an impermeable membrane or the absence of the specific target of the antibiotic (Alekshun and Levy, 2007). DNA segments coding for innate resistance are present on the chromosome within the bacteria (Perry et al., 2014). This innate resistance is governed by the presence of antibiotic resistance genes (ARG) and is transmitted vertically through cellular proliferation (Allen et al., 2010). Acquired resistance, or resistance that is not natively present, occurs several different ways: by utilizing efflux pumps to remove antibiotic from the cell before it can have an effect; by mutating the antibiotic's target protein or binding site, thus rendering it unable to act; and finally, by inactivating the antibiotic, causing it to be benign to the target cell (Allen et al., 2010; Perry et al., 2014). The genes that code for these actions are also referred to as ARG and may be present in plasmids or the host chromosome (Allen et al., 2010).

ARG present within a bacterial community can be vertically transferred via proliferation or can be transferred to bacteria of varied taxonomic and ecological groups through horizontal gene transfer (HGT). HGT mechanisms include transformation, conjugation, and transduction (Khachatourians, 1998). Most ARG are known to be located on transposons, integrons, and plasmids (Alekshun and Levy, 2007).

Antibiotic resistance genes exchanged by HGT are most commonly located on plasmids, extra-chromosomal, covalently closed, circular elements of double stranded DNA (Perry et al., 2014). These pieces of DNA can replicate independently of their host cell, and multiple plasmids can exist within one bacterium (Alekshun and Levy, 2007). Plasmid-mediated mechanisms of resistance can include efflux pumps and antibiotic enzyme modification or degradation (Khachatourians, 1998). Transposons are mobile segments of DNA that can relocate from one chromosome or plasmid to another, promoting the spread of ARG (Perry et al., 2014). Integrons, mobile elements commonly associated with transposons, contain gene cassettes which usually contain a single gene in a linear form. These gene cassettes are especially relevant, as they are the location of many ARG (Hall and Collis, 1998). The presence of plasmids and transposons usually indicates a higher level of resistance and they are easily conferrable between bacteria via HGT (Alekshun and Levy, 2007; Allen et al., 2010; Levy and Marshall, 2004).

Conjugation, the most prevalent mechanism of HGT, relies on direct cell-to-cell contact to transfer chromosomal or extrachromosomal DNA between cells. Conjugation can occur via plasmids or transposons (Alekshun and Levy, 2007). Transformation is the cellular uptake of naked DNA from the environment (Barbosa and Levy, 2000). Most often this DNA is remnant segments from lysed or dead bacteria. Conjugation and transformation require proximity but a third mechanism, transduction can occur between remotely located organisms. Transduction is

the movement of genes from one host to another via bacteriophage mediated transfer; phages can persist in the environment for up to a decade (Perry et al., 2014; Zhaxybayeva and Doolittle, 2011). These common mechanisms of HGT can introduce AR phenotypes that may not have occurred by natural selection and mutation (Zhaxybayeva and Doolittle, 2011).

No matter the mode of acquisition, the known genes that confer AR are collectively referred to as the "antibiotic resistome" (Wright, 2007). This group includes all genes whose presence enables an organism to survive upon exposure to an antibiotic that would otherwise be fatal (Perry et al., 2014). In addition to phenotypic and expressed resistance, silent and proto-resistance also contribute to the resistance potential of an organism. Organisms possessing silent resistance are susceptible to the antibiotic, but contain functional, unexpressed ARG. These genes become relevant if they become expressed by the cell. Proto-resistance genes do not typically have activity against antibiotics, but can become active if mutations occur. These mutations are thought to be the cause of the introduction of novel ARG (Perry et al., 2014).

CLASSES OF ANTIBIOTICS AND CORRESPONDING BACTERIAL MODES OF RESISTANCE

β-Lactams and Cephalosporins

Penicillins and cephalosporins are structurally similar compounds belonging to the β-lactam class of antibiotics (Seiffert et al., 2013). They are the most commonly sold antibiotic class, amounting for over $11.9 billion in sales in 2009 and comprising 28% of the antibiotic market (Hamad, 2010). First and second generation cephalosporins (e. g. cephalothin, cefuroxime) are considered narrow-spectrum cephalosporins, while third and fourth generation cephalosporins (e. g. ceftuifyrm, cefepime) are considered broad-spectrum cephalosporins (Seiffert et al., 2013).

β-lactams act by covalently binding to peptidoglycan, targeting a group of proteins known as the penicillin-binding proteins (PBP) (Mc Dermott et al., 2003). Peptidoglycan, or murein, comprises much of the cell wall in gram-positive bacteria, and is synthesized by assembling precursors in the cell's cytoplasm to be transported across the cytoplasmic membrane. To reach the PBP in gram negative cells, which contain much less peptidoglycan, β-lactams pass through cell wall channels called porins. The precursor glycan units are then inserted into the cell wall and are linked via transpeptidation (Mc Dermott et al., 2003). β-lactams most commonly act on the cell in the final stages of peptidoglycan formation, when the glycan units are being inserted into the cell wall and linked together (Mc Dermott et al., 2003). Once the antibiotic binds to the PBP, cell division may be inhibited or the cell wall structure may be affected, leading to cell lysis (Mc Dermott et al., 2003).

Resistance to β-lactams can occur in several different ways: 1) through mutations on the target PBP; 2) through production of β-lactamases; 3) by modifying cell wall porins; and 4) with efflux pumps to remove the drug from the bacteria (Mc Dermott et al., 2003). The most common of these is the production of β-lactamases, which function by hydrolyzing the β-lactam ring of the drug (Majiduddin et al., 2002; Mc Dermott et al., 2003). β-lactamases are globular proteins classified by the substrate they inhibit or by amino acid sequence similarity (Babic et al., 2006). The genes responsible for regulating the production of β-lactamases, *bla* genes, can reside on the chromosome, plasmid, transposons, or integrons (Babic et al., 2006). These genetically encoded β-lactamases can be either constitutive (active at all times) or inducible (active only in the presence of antibiotic) and the types of β-lactamases produced are often unique to certain species (Kong et al., 2010).

β-lactamases of importance include extended-spectrum β-lactamases (ESBL) which are considered the most clinically important β-lactamases. The most prevalent of these bla_{EBSL} genes are the CTX-M-types (Seiffert et al., 2013). The bla_{EBSL} genes confer resistance to broad-spectrum β-lactams such as third and fourth generation cephalosporins. Another gene of relevance, *ampC*, is responsible for the ability to resist third-generation cephalosporins. These *ampC* genes can be located on the chromosome (*cAmpC*) or on the plasmid (*pAmpC*) (Seiffert et al., 2013). These genes may be transferred to other bacteria, conferring resistance to β-lactams. Additionally, the *bla* genes have demonstrated the ability to adapt to and hydrolyze newly developed, novel, extended-spectrum β-lactams (Majiduddin et al., 2002).

Macrolides and Lincosamides

Macrolides and lincosamides (ML), including erythromycin, lincomycin, and pirlimycin, are broad-spectrum antibiotics that share a mode of action but differ chemically (Leclercq, 2002). ML account for $4.8 billion in sales annually in the United States (Hamad, 2010). These classes of antibiotics are often discussed together because they share an overlapping binding site on the 50S subunit of the ribosome. When ML bind to this site, peptide elongation is prevented, inhibiting growth of the affected bacteria (Roberts, 2004; Wilson, 2014). ML are generally considered bacteriostatic and tend to be long-acting and effective at penetrating deep into tissues (Pyörälä et al., 2014). This mechanism of action is temporary, and is reversible upon removal of the antibiotic (Roberts, 2004). ML are especially effective at treating infections caused by *S. aureus* because they increase the susceptibility of the invasive pathogen to phagocytosis by leukocytes, specifically polymorphonuclear leukocytes (Whittem, 1999). They are also effective at combating infections caused by many different species of anaerobic bacteria(Whittem, 1999).

Some bacteria, such as *Streptomycetes*, *E. coli*, and *Salmonella*, are intrinsically resistant to ML due to self-protective mechanisms such as poor membrane permeability (Pyörälä et al., 2014). Others acquire resistance to ML via drug inactivation, antibiotic efflux, and, most commonly, target site modification (Pyörälä et al., 2014; Roberts, 2004). Target site modification, also referred to as ribosomal methylation, is mediated by at least 36 known *erm* genes, most commonly *erm*(A), *erm*(B), *erm*(C), and *erm*(F). These genes of interest are often found in streptococci and enterococci *erm*(B), staphylococci (*erm*(A)and *erm*(C)), and anerobic bacteria (*erm*(F)) and are often subject to HGT, as they are commonly associated with mobile genetic elements (Leclercq, 2002). These genes function by demethylating an adenine in the 23S portion of the 50S ribosomal subunit, preventing the binding of ML to the ribosome (Leclercq, 2002; Roberts, 2004).

The second most common mechanism of resistance to ML is antibiotic efflux by transporter proteins by nine adenosine triphosphate (ATP) transporters that confer resistance to ML by pumping the antibiotic out of the cell or cellular membrane (Roberts, 2004). These efflux proteins are generally members of either the ATP-binding-cassette transporter superfamily or the major facilitator superfamily (Leclercq, 2002). The action of the efflux pumps maintains a low intracellular concentration of ML enabling the ribosome to continue to function (Roberts, 2004). Efflux pumps provide intrinsic resistance in many gram-negative bacteria due to their ability to remove hydrophobic compounds, like ML, from the bacterium (Leclercq, 2002).

The final mechanism of resistance to ML is enzyme-catalyzed drug modification or degradation, via phosphorylation, glycosylation, and esterases (Wilson, 2014). These mechanisms are less common than those previously described, but could become increasingly

relevant, as inactivation of ML is governed by a group of *mph* genes that are often detected in pathogens found in food animals (Leclercq, 2002; Pyörälä et al., 2014).

Fluoroquinolones

The third most prevalent drug class, fluoroquinolones (e.g. ciprofloxacin), accounted for 17% of US antibiotic sales in 2009 (Hamad, 2010). Flouroquinolones are synthetic substances and are among the most common treatment options utilized for respiratory diseases by heifer producers (Hooper, 2001; USDA, 2011). These antibiotics function by forming a physical barrier that disrupts nucleic acid synthesis, primarily targeting the enzymes DNA gyrase or topoisomerase IV, resulting in fragmentation of chromosomal DNA and bacterial cell death (Hooper, 2001; Redgrave et al., 2014). Fluoroquinolones have a quick acting bactericidal effect, are able to penetrate into mammalian tissues and cells, and are effective at killing both Gram-positive and Gram-negative bacteria (Appelbaum and Hunter, 2000). Because of this, they are heavily used in both human and veterinary medicine, leading to a rapid increase in fluoroquinolone resistance (Redgrave et al., 2014). Bacteria develop resistance to fluoroquinolones by either altering the target enzymes or by reducing the ability of the drug to penetrate the cell's cytoplasm (Hooper, 2001). Enzyme modification occurs through spontaneous, stepwise chromosomal mutations in the genes that code for enzyme production while drug permeation reduction occurs by the overexpression of naturally present multidrug resistance (MDR) pumps (Hooper, 2001).

Tetracyclines

Tetracyclines are broad-spectrum, bacteriostatic antibiotics that function by blocking aminoacyl-tRNA binding at the 30S subunit of the ribosome and inhibiting protein synthesis (Chopra and Roberts, 2001; Mc Dermott et al., 2003). They are commonly used in both human

and veterinary medicine, because they function against a wide range of pathogens with minimal adverse side effects (Chopra and Roberts, 2001). Additionally, tetracyclines are commonly fed to animals at subtherapeutic levels to promote growth, and are fed on about 45% of US dairy farms in dairy milk replacer (Mc Dermott et al., 2003; USDA, 2011). Tetracyclines are most commonly administered to dairy heifers to prevent or treat respiratory infections, lameness, and scours. They are fed to dairy heifers subtherapeutically on more than 30% of US dairy farms (USDA, 2011). Tetracycline's broad-spectrum characteristics and heavy use in both humans and animals has led to widespread bacterial resistance to the antibiotic (Chopra and Roberts, 2001).

Resistance to tetracyclines occurs via three main mechanisms – drug efflux, protection of the ribosome, and enzymatic inactivation of the antibiotic. The group of genes coding for efflux govern the production of membrane-associated proteins that remove tetracyclines from the cell, reducing the intercellular concentration and protecting the ribosome from the drug's mechanism of action (Chopra and Roberts, 2001). Protection of the ribosome by the binding of ribosomal protection proteins provides resistance to a broader range of tetracyclines when compared to efflux pumps. The shape of the ribosome is changed upon protein binding, preventing tetracycline from acting on the ribosome while still allowing the ribosome to function normally. This action is most commonly controlled by the genes *tet*(O) and *tet*(M). (Chopra and Roberts, 2001). Enzymatic inactivation is rarely observed, and is governed by the gene *tet*(X), which is closely linked *erm*(F), as both genes are often found on the same transposon with the macrolide resisting gene *erm*(F), as both genes are often found on the same transposon (Chopra and Roberts, 2001).

Sulfonamides

Sulfonamides, synthetic antibiotics that are structurally similar to *p*-aminobenzoic acid (PABA), a substrate of tetrahydrofolic acid, function by blocking the formation of nucleotide precursors by competing with the PABA to bind to the dihydopteroate synthetase (DHPS) enzyme binding site, preventing the synthesis of folic acid (Mc Dermott et al., 2003). Unlike mammalian cells, who do not need to synthesize folic acid and therefore lack DHPS, bacteria must synthesize their own folic acid. Therefore, when sulfonamides block this synthesis pathway, bacteria die from lack of folic acid and mammalian cells remain unaffected (Sköld, 2000). Sulfonamides are rarely used in human medicine for three main reasons: 1) a high incidence of resistance to the antibiotic; 2) relatively low efficacy when compared with more modern antibiotics; and 3) the common incidence of negative side effects (Sköld, 2000). However, sulfonamides are administered more commonly in dairy heifers to treat diarrhea, respiratory infections, navel infections, and general lameness (USDA, 2011). Resistance to sulfonamides develops through chromosomal mutations in the $DHPS_{folP}$ gene, which can occur with as little as one nucleotide base pair substitution (Huovinen et al., 1995). Plasmid-mediated resistance to sulfonamides, governed by the genes *sul1* and *sul2*, is a common sulfonamide resistance mechanism. These genes produce distinct DHPS, decreasing bacterial sensitivity to sulfonamides (Sköld, 2000).

METABOLISM AND EXCRETION OF ANTIBIOTICS IN THE DAIRY COW

When antibiotics are administered to dairy cattle, only a fraction of the antibiotic is absorbed and utilized (Sarmah et al., 2006). The remaining antibiotic residue, whole and functional or partially degraded, is excreted in the urine (up to 90%), feces (up to 75%), or milk (Sarmah et al., 2006). While some of this excreted antibiotic is degraded, much of it is disseminated into the environment via feces and urine in the parental, functional form. When this

antibiotic-containing manure is land applied, environmental bacteria are exposed to the excreted antibiotic, offering opportunity to acquire and maintain AR (Sarmah et al., 2006).

Treatment for mastitis is the most common use of antibiotics in the dairy industry, with approximately 15% of lactating cows being treated with antibiotics each year (USDA, 2008). It is estimated that up to 55% of dairy lactations will have an incidence of mastitis, with the majority of these cases treated with intramammary (IMM) antibiotics (Langford et al., 2003). The most common antibiotics administered to treat mastitis are cephalosporins (53.2%), noncephalosporin β-lactams (19.1%), and lincosamides (19.4%) (USDA, 2008). Additionally, calves are exposed to these when the resulting non-saleable milk is fed. This has been shown to increase the incidence of AR bacteria in their feces (Langford et al., 2003). Therefore, antibiotic metabolism and excretion is an issue of health and environmental concern.

Metabolism and Excretion of Cephalosporins and Noncephalosporin β-Lactams

Cephalosporin and noncephalosporin β-Lactams are structurally similar, and therefore the metabolism of these compounds can be discussed in combination. Noncephalosporin β-lactams are the most common antibiotic administered to treat naval infections and respiratory disease in dairy heifers (USDA, 2009). They are also used to treat approximately 15% of reproductive diseases, 17% of mastitis events, and 14% of lameness in dairy cows (USDA, 2009). The two most commonly administered noncephalosporin β-lactams are forms of penicillin G and amoxicillin. These are weak organic acids that have low lipid-solubility and are easily ionized in acidic conditions (Gehring and Smith, 2006). Amoxicillin is a semi-synthetic penicillin that has broad-spectrum activity and is susceptible to β-lactamases (Belmar-Liberato et al., 2011). These antibiotics are effective at combating pathogens such as *S. agalactiae*, *S. uberis*, *S. dysgalactiae*,

and other *Streptococcus* and *Staphylococcus* spp., but are not effective against *S. aureus* (Owens et al., 1997).

Cephalosporins, semisynthetic antibiotics derived from a compound naturally produced by *Cephalosporium acremonium*, are commonly administered to treat clinical mastitis caused by both Gram positive and negative pathogens, such as *S. aureus* and *E. coli* (Alcaine et al., 2005; Cagnardi et al., 2014). Cephalosporins can be administered IMM (cephapirin) or parenterally (ceftiofur) (Gruet et al., 2001). They are quickly metabolized to a desacatyl form which remains biologically active, but is less potent than the parental compound. Often, these metabolized forms of cephalosporins are used in combination drug therapy to increase antibiotic effectiveness through synergistic actions (Jones, 1989).

Cephapirin sodium, sold under the trade name ToDay®, is the IMM antibiotic most commonly administered to treat clinical mastitis in dairy cows. ToDay® has a milk withholding time of 96 h (Gorden et al., 2013; USDA, 2008). Cephapirin is a weak organic acid that is poorly absorbed from the mammary gland into systemic circulation, due to its poor lipid solubility (Gehring and Smith, 2006; Gorden et al., 2013). When administered IMM, most cephapirin is excreted in milk. Consequently, it is the second most common residue found in commercial milk and excretion in feces and urine is generally low (Moats et al., 2000; Ray et al., 2014).

Parenterally administered cephalosporins such as cefitofur crystalline free acid sterile suspension (CCFA-SS) are administered to treat respiratory infections like bovine respiratory disease (Washburn et al., 2005). These third-generation cephalosporins are administered via a subcutaneous injection in the neck or behind the ear (Washburn et al., 2005). Much of the excreted CCFA-SS is found in the urine, as glomerular filtration is the main contributor to its metabolism (El-Gendy et al., 2007). In cattle, approximately 65% of excreted parenteral ceftiofur

is in urine and about 35% is in the bile, and therefore excreted in feces. Approximately 37% of IM CCFA-SS is detected in bovine feces by 12 h post administration (Volkova et al., 2012). However, much of the administered CCFA-SS is well absorbed and utilized and it is unlikely that significant amounts of parenteral antibiotic crosses the udder membrane/blood barrier (El-Gendy et al., 2007).

Metabolism and Excretion of Lincosamides

Pirlimycin, a semi-synthetic lincosamide derived from lincomycin and clindamycin, is commonly administered therapeutically to treat bovine mastitis, specifically mastitis caused by *S. aureus* or other Gram positive bacteria (Hornish et al., 1992; Whittem, 1999). It is administered to the mammary gland via infusion into the teat cistern in an aqueous gel formulation under the trade name Pirsue® (Whittem, 1999). After infusion of Pirsue®, milk must be discarded for 36 h, as half of the dose of antibiotic is transported systemically after passing through the udder membrane/blood barrier. The other half remains unabsorbed in the udder until mechanical removal by milking (Hornish et al., 1992; Whittem, 1999). A total of 89 percent of administered pirlimycin is excreted by the cow – 68% in the unchanged parental form (Hornish et al., 1992). Of excreted pirlimycin, approximately 10% is excreted in the urine, 25% in the feces, and over 50% in the milk (Hornish et al., 1992).

MEASURES OF ANTIBIOTIC RESISTANCE

Culture-based Methods

Conventional culture-based methods for the quantification of AR are frequently used to diagnose and quantify the incidence of AR in laboratory settings (Smith et al., 1994). The two main types of assay utilized to quantify AR are disc diffusion and dilution methods (Smith et al., 1994). Both of these tests rely on the fact that AR phenotypes are highly selected for and are

easily detected using growth inhibition (Schmieder and Edwards, 2012). These assays require the isolation and growth of pathogens from the sample of interest. The isolated pathogens are exposed to varying concentrations of antibiotic, and growth (or inhibition of growth) is measured (Cockerill, 1999). There are published standards provided by the National Committee for Clinical Laboratory Standards (NCCLS) that must be followed for preparation of media, which include incubation temperatures and guidelines for interpreting data measured (Cockerill, 1999).

Agar disc diffusion, also known as the Kirby-Bauer test, uses small filter paper discs saturated with antibiotic and placed on a gel agar media plate containing the isolated pathogen of interest and incubated according to the NCCLS recommendations (Herrmann et al., 1960; Papich, 2013). The agar used is selected and prepared to provide optimal growing conditions for the pathogen (Smith et al., 1994). The antibiotic present within the disc will seep through the agar, and bacterial resistance is quantified by measuring the zone of no growth, referred to as the zone of inhibition, present on the agar plate. These results are normally recorded as the diameter of the zone of inhibition, and are used to estimate the minimum inhibitory concentration (MIC) of the antibiotic agent and the pathogen (Smith et al., 1994). This mathematically calculated MIC is the lowest concentration of antibiotic that visibly inhibits bacterial growth (Papich, 2013). The larger the zone of inhibition, the more susceptible the pathogen is to the corresponding antibiotic (Papich, 2013). Disc diffusion provides the ability to qualitatively test a pathogen's sensitivity to many different antibiotics simultaneously while requiring a limited quantity of sample (Smith et al., 1994).

Culture-based dilution methods utilize either solid or liquid media to directly determine MIC. These are more accurate than agar disc diffusion (Smith et al., 1994). This test is conducted by inoculating wells of a plate or individual tubes containing serial dilutions of antibiotic with

known quantities of isolated bacterial culture (Papich, 2013). After inoculation, plates or tubes are incubated according to NCCLS protocols and the turbidity or color change of the resulting suspension is assessed (Luber et al., 2003; Sarker et al., 2007). The MIC is established in terms of the concentration of antibiotic (Papich, 2013).

Culture-dependent methods are well-studied and test the susceptibility of pathogens to multiple antibiotics simultaneously using very little sample (Smith et al., 1994). Reliable results are fairly simple to achieve, due to the well-established and maintained guidelines of the NCCLS (Cockerill, 1999). However, there are many disadvantages of culture-based methods. Only a small fraction of bacteria – less than 1% – are culturable, and therefore the pathogen of interest may not be a candidate for these assays (Schmieder and Edwards, 2012). Additionally, for some pathogens growing the culture can be a process that can take weeks of careful management (Schmieder and Edwards, 2012). Because of this, newer culture-independent techniques have been developed quickly provide previously unattainable information about AR.

Genetic Based Methods

Polymerase chain reaction (PCR) is a method that allows for the detection of ARG isolated from the sample of interest (Schmieder and Edwards, 2012). This assay consists of three steps: 1. DNA isolated from the sample of interest is melted to convert it from double-stranded DNA to single stranded DNA (high temperature); 2. Oligonucleotide primers specific to the gene of interest anneal to the target DNA sequence (low temperature) and 3. DNA polymerase is used to elongate the DNA by adding complementary nucleotides (intermediate temperature). These three steps are repeated several times, resulting in an exponential increase in the target sequence (Steffan and Atlas, 1991). It is possible to amplify more than one gene of interest at the same time using multiplex PCR, where multiple primer pairs are added to the PCR reaction mixture

(Steffan and Atlas, 1991). After completion of PCR, the product can be subject to post-PCR analysis, such as restriction fragment length polymorphism (RFLP) or gel electrophoresis to confirm that the resulting amplicon is the desired target (Cockerill, 1999). With PCR the primers used must be designed to detect a known resistance gene, therefore PCR is not a method for discovering novel resistance genes (Schmieder and Edwards, 2012). Additionally, while PCR is somewhat quantitative, any results gathered are an estimate of the abundance of the gene of interest rather than a known amount (Steffan and Atlas, 1991). However, PCR and multiplex PCR are relatively simple, quick, and inexpensive assays, and are therefore commonly used to detect the presence of ARG (Cockerill, 1999).

Real-time PCR, also referred to as quantitative PCR (qPCR), is a method to quickly and reliably quantitatively measure accumulated PCR product through use of fluorescence (Heid et al., 1996). The initial concentration of the sequence of interest can be determined from the information gathered during the qPCR run (Wilhelm and Pingoud, 2003). The qPCR assay uses hybridization probes (e.g. SYBR Green) that emit fluorescence each time a new amplicon copy is created. This fluorescence is measured and logged on an amplification curve that can be quantitatively related to the number of gene copies present in the sample (Wilhelm and Pingoud, 2003). The composition of an amplification curve requires three separate phases: 1) a lag phase where the level of fluorescence is too low to measure, 2) an exponential phase, where the level of fluorescence increases exponentially, and 3) a plateau phase, where the level of fluorescence is no longer increasing (Wilhelm and Pingoud, 2003). Within the exponential phase, the threshold intersects the signal curve, known as the threshold value (C_T), which is used to quantify the number of gene copies of interest present in the sample relative to a standard curve or reference gene (Wilhelm and Pingoud, 2003). While this tool is extremely useful for known ARG, the

amount of information gained from this technique is limited due to our incomplete knowledge of ARG sequences (Schmieder and Edwards, 2012).

Metagenomics is the analysis of all genetic information present in an environmental sample (Thomas et al., 2012). This information is analyzed using next-generation sequencing technology and provides a wealth of information about the microbial composition and diversity of a wide range of samples (Thomas et al., 2012). There are three categories of metagenomic analysis for ARG analysis: targeted (PCR-based), functional, and sequence-based (Penders et al., 2013). Targeted metagenomics uses highly developed and relatively inexpensive qPCR-based methods to quantify the presence and abundance of ARG; this requires a known target sequence (Penders et al., 2013). Functional metagenomics requires cloning of DNA into a vector (such as a plasmid) which is inserted into a host, such as *E. coli*. The hosts are grown on antibiotic-containing media, and resistant colonies are sequenced. This method is useful for determining previously unknown ARG, but relies on the gene of interest being expressed by the host (Penders et al., 2013). Finally, sequence-based metagenomics directly sequences DNA extracted from environmental samples; the resulting sequences are compared to a database of known sequences. Sequence-based metagenomics is expensive, but has become more affordable in recent years (Penders et al., 2013).

Next-Generation Sequencing Methods

Automated Sanger methods of sequencing, developed in the 1990s are considered ‘first generation’ technology. The Sanger methods of sequencing are still used frequently, as they are able to read sequences of > 700 bp in length and are the most error-free sequencing technologies available (Thomas et al., 2012). However, Sanger sequencing is labor intensive and biased, as its first step is cloning of the genome of interest. This is sufficient for low-diversity environments

but is not adequate for species-rich environments (Thomas et al., 2012). These shortcomings have led to the development of newer methods, known as next-generation sequencing (NGS) (Metzker, 2010). These NGS technologies include Roche 454 pyrosequencing and Illumina Solexa sequencing (Metzker, 2010). The NGS methods share similar general steps including template preparation, sequencing and imaging, and data analysis, but differ in their methods of clonal amplification (Metzker, 2010).

Sample preparation is critical to obtaining quality results when performing metagenomic sequencing. The template used should not only be high-quality, but also a realistic representation of the sample (Thomas et al., 2012). First, DNA is extracted from the environmental sample of interest. For this step, it is critical that the extraction protocol be designed to isolate as much of the target (e.g. bacteria, host) as possible while minimizing contaminants and DNA not of interest (Thomas et al., 2012; Wooley et al., 2010). The extracted and purified DNA is subjected to a library preparation step, where a sequencing library is compiled by shearing the extracted DNA into segments of < 1kb (Metzker, 2010; Schmieder and Edwards, 2012). This process includes several steps and is often the most time consuming part of obtaining metagenomic data (Schmieder and Edwards, 2012). After library preparation, metagenomic sequencing can occur.

Roche 454 pyrosequencing, which was first introduced commercially in 2004, clonally amplifies single stranded DNA fragments by emulsion polymerase chain reaction (ePCR) using primers, template DNA, deoxynucleoside triphosphates (dNTPs) and polymerase (Mardis, 2008; Metzker, 2010). Before PCR amplification can occur, the individual primer molecules must be attached to a solid support to which the fragmented template DNA will adhere (Metzker, 2010). PCR amplification results in thousands of copies of template DNA attached to each microscopic bead. The beads are then chemically adhered to a glass slide or placed into the wells of a

picotitre plate (Metzker, 2010; Thomas et al., 2012). Each individual bead, or polymerase colony (polony), is a cluster of PCR amplicons originating from a single molecule of DNA (Wooley et al., 2010). This "sequencing-by-synthesis" reaction sequentially adds dNTPs one at time. If the dNTP added is complementary the template DNA, it is incorporated by DNA polymerase to the single stranded template, resulting in the release of an amount of pyrophosphate (PPi) proportional to the nucleotide added (Thomas et al., 2012; Wooley et al., 2010). . The PPi is then converted to ATP by the sulfurase and the ATP reacts with luciferase to produce light (Wooley et al., 2010). The light from the > 1.2 million simultaneous reactions is detected, measured, and recorded as the DNA sequence of the template. Any excess nucleotides are degraded by apyrase before the process is repeated for the next dNTP (Thomas et al., 2012; Wooley et al., 2010). The Roche 454 method is ideal for sequencing samples with low levels of DNA, and is able to read sequences of ~250 base pairs in length in approximately 10 h, yielding ample sequence data for a relatively low cost (~$20,000/ gigabase) compared to other shotgun-sequencing methods. Additionally, the Roche 454 method is able to run up to 12 samples simultaneously through multiplexing (Thomas et al., 2012; Wooley et al., 2010).

The more recently introduced Illumina/Solexa Genome Analyzer (GAIIX) is similar to Roche 454 pyrosequencing, as it follows the same "sequencing-by-synthesis" approach (Wooley et al., 2010). Rather than utilizing ePCR, as with Roche 454 pyrosequencing, Illumina sequencing uses solid-phase amplification. This includes two basic steps: priming and extending the template molecule, and formation of clusters through bridge amplification of the single-stranded template DNA and primers (Metzker, 2010). Genomic DNA is prepared for the Illumina run by fragmenting the sample and ligating adaptors to both ends of the DNA fragments (Mardis, 2008). The prepared genomic DNA is randomly attached to the inside surface of a flow

cell, forming fragment clusters which are denatured, annealed with sequencing primer, and amplified (Mardis, 2008). During this solid-phase amplification about 100-200 million different template clusters are created.

Illumina utilizes four-color fluorescent cyclic reversible termination (CRT) to measure and record sequences of the template DNA (Metzker, 2010). CRT is comprised of five steps: 1) addition of all four nucleotides, each chemically and reversibly labeled with its own fluorophore on their 3'-OH end 2) addition of a single fluorescently labeled dNTP nucleotide which compliments the base present on the template by DNA polymerase bound to the template, 3) removal of unincorporated nucleotides by a washing step, 4) imaging to record the fluorescence of the incorporated nucleotide, 5) cleavage and washing of the inhibiting group and fluorescent dye (Mardis, 2008; Metzker, 2010). The cycle repeats. Compared to Roche 454 pyrosequencing, Illumina produces much larger volumes (~960 gigabase) of DNA sequencing per run at a cost of ~ $50/gigabase (Thomas et al., 2012). The Illumina HiSeq2000 is able to perform hundreds of millions of reads with a read length of ~150 bp and can obtain continuous sequence reads of 300 bp by performing paired-end sequencing reads, but has longer read times (4-9 d) than the Roche/454 method (Thomas et al., 2012). Illumina sequencing is currently the most widely used NGS method (Metzker, 2010).

Metagenomic Analysis

After NGS is completed, the data must be assembled and annotated using metagenomic databases including the SEED and Meta Genome Rapid Annotation using Subsystems Technology (MG-RAST) (Overbeek et al., 2004; Thomas et al., 2012). The SEED is a suite of open source tools that provide the ability to annotate, or predict the function of, new genomes by comparing sequences present in the NGS data to known sequences published in databases

(Overbeek et al., 2004; Thomas et al., 2012) The SEED is organized into subsystems, which are comprised of groups of genes with related functions, that allow the user to locate and visualize genes relevant to biologically related subsystems. Additionally, it provides the ability to identify gene similarities between organisms, examine genes related to metabolic functions, and identify fragments not previously annotated (Overbeek et al., 2004)

The SEED is comprised of four servers that provide access to SEED data and bioinformatics tools: the Sapling Server, the Annotation Support Server, the Rapid Annotations using Subsystems Technology (RAST) server, and the Metabolic Modeling and Flux Balance Analysis (FBA) server (Aziz et al., 2012). The Sapling Server accesses the SEED through the entity-relationship data model (ERDB) and allows for direct comparisons against databases (Aziz et al., 2012). The Annotation Support Server assigns function to protein or genomic DNA sequences that have not been previously identified and organize these sequences into subsystems (Aziz et al., 2012). The RAST Server is able to receive and annotate sequence input files via a graphical web interface (Aziz et al., 2012). Finally, the Metabolic Modeling and FBA server provides access to the Model SEED biochemistry and genome metabolic model database (Aziz et al., 2012). These servers provide a variety of ways to analyze and annotate NGS genomic and metagenomic data.

The MG-RAST server is a publicly available system that utilizes the SEED databases to allow for comparative genomic analysis. The server was designed with the ability to accommodate new methods and information – necessary due to the constantly evolving nature of NGS (Meyer et al., 2008). MG-RAST is able to analyze NGS data both phylogenetically and metabolically, and also contains a search function, simplifying management of the large NGS datasets (Meyer et al., 2008). To use the MG-RAST server, a new metagenome is uploaded and

subjected to quality control with user-defined phylogenetic analysis parameters (Meyer et al., 2008). Comparative metagenomic analysis is the core function of the MG-RAST platform. Users compare their NGS data to complete genomes located on the SEED and other metagenomes analyzed using MG-RAST (Meyer et al., 2008). Once a sample is analyzed using MG-RAST, its information can be made public and added to the SEED, providing more data for other researchers to reference (Overbeek et al., 2004).

While NGS metagenomic analysis using databases provides a wealth of information, it is important to note that these technologies have limitations. For example, for each sample submitted, many (10-98%) of the sequences may have no match in the database (Meyer et al., 2008). Additionally, the sequencing step is much faster than the data analysis step, and therefore more computational space is needed to reduce the bottleneck in metagenomic analysis (Meyer et al., 2008). Finally, the quality of the annotation is dependent on the quality of the data submitted, and therefore it is essential that all steps leading to data analysis be optimized for database analysis (Meyer et al., 2008).

ANTIBITOTIC RESISTANT BACTERIA IN PRE-WEANED CALVES

Considering the possible effect on the resistance profile of the gastrointestinal (GI) and fecal bacteria in calves exposed to antibiotics during the pre-weaning period is of concern. Early exposure to antibiotics can select for AR bacteria (ARB) in the calf, which can result in decreased antibiotic efficacy later in life (Khachatourians, 1998). Additionally, early exposure to antibiotics can lead to the establishment of a gut microbial community that is different from calves free from exposure, with unknown but potentially detrimental consequences (Xie et al., 2013). Finally, exposure to antibiotics can increase the fecal shedding of ARB, amplifying the environmental load of ARG and increasing the risk to human health (Duse et al., 2015).

Establishment of Gut Microbial Community and Antibiotic Resistant Bacteria

The gut microbiome is an important source of proliferation of infectious agents and its development is critical for the long term health of the calf (Storz et al., 1971). The digestive tract of calves, particularly the large intestine, is densely populated by a diverse population of bacteria such as *E. coli*, enterococci, and eactobacilli (Sørum and Sunde, 2001). The initial population present in the gut is dominated by a limited number of genera that become increasingly diverse as the calf ages (Klein-Jöbstl et al., 2014; Mayer et al., 2012; Oikonomou et al., 2013). This diverse population is acquired over time, as calves are considered to be born with a sterile gut (Mayer et al., 2012). The calf gut population is extremely dynamic during first 48 h of life but reaches a somewhat steady state from about 3 d of age until weaning. The diversity of gut microbial communities has been shown to be different in calves fed pasteurized and non-pasteurized waste milk, demonstrating that oral exposure to bacteria influences the colonization of the gut (Edrington et al., 2012). Also when calves fed bacitracin (a broad spectrum antibiotic) containing milk replacer, the bacitracin-fed calves had significantly different microbiome populations when compared to controls (Xie et al., 2013). Additionally, exposure to antibiotics has been shown to have a lasting effects on its bacterial population and the incidence of ARB in humans. It seems likely that these differences would be observed, and possibly amplified, in the naïve neonatal calf gut (Jakobsson et al., 2010).

Not only does antibiotic exposure influence the colonization of the gut, it can also increase the proliferation of ARB in the microbiome of the calf. Fecal *E. coli* isolates from pre-weaned calves treated with antibiotics tend to possess more AR phenotypes than isolates from calves not receiving antibiotics (Berge et al., 2005). Systemic exposure to antibiotics is associated with an increased number of AR isolates (Berge et al., 2005). Feeding non-saleable

(antibiotic-containing) milk has been shown to increase the incidence of fecal shedding of streptomycin and nalidxic acid resistant *E. coli* and increase the occurrence of CTX-M positive *E. coli*, but in other studies this practice has been shown to have no effect (Brunton et al., 2014; Duse et al., 2015; Wray et al., 1990). All of these studies draw their conclusions through the use of MIC assays rather than NGS technologies, presenting a critical gap in the data.

Antibiotic resistance has also been shown to develop in the absence of antibiotics. For example, fecal ARG were observed using qPCR in samples from calves never exposed to antibiotics (Thames et al., 2012). Also, there were no observed differences between the prevalence of AR *Campylobacter* isolates from the feces of organic and conventionally-raised calves (Sato et al., 2004). However, a study where differing levels of antibiotic were fed in raw milk found an increase in the proportion of multidrug resistant (MDR) *E. coli* in the feces of calves fed antibiotic containing milk when compared with calves fed raw milk without antibiotic. This MDR *E. coli* peaked at 2 weeks of age (Pereira et al., 2014). Several additional studies have observed a peak in AR *E. coli* isolates between the ages of 2-4 weeks regardless of antibiotic exposure, indicating that the incidence of AR is not only dependent on antibiotic administration but also on age (Berge et al., 2005; Edrington et al., 2012). This is a critical area for research to overcome conflicting results and the lack of conclusive data.

Fecal ARB and their Environmental Effects

The incidence of ARB in the calf gut has the potential to be harmful to the animal, but the more pressing concern is the imminent threat to human health through environmental effects of fecally-shed ARB. Manure from animals raised in agricultural settings is stored, composted, or directly spread onto pasture or cropland (McEwen and Fedorka-Cray, 2002). This waste may contaminate the environment, groundwater, streams, waterways, and eventually our food supply,

with ARB or residual ARG (Khachatourians, 1998; McEwen and Fedorka-Cray, 2002). Water contamination is of primary concern and waterways close to agricultural areas tend to contain higher levels of ARG than other areas. Additionally, drinking water is a known source of ARB and ARG, as current methods of wastewater treatment are unable to effectively remove, and in some cases even select for, these contaminants (Shi et al., 2013).

The use of antibiotic- or ARG-containing manure as fertilizer increases the incidence of ARG in crops exposed to the manure. Additionally, crops such as green onions and cabbage are able to uptake antibiotics present in the soil, which can cause allergic reactions and increased incidence of AR in humans consuming the crop (Kumar et al., 2005). ARG have been detected using qPCR on tomatoes, peppers, radishes, and carrots fertilized with dairy manure, demonstrating that areas that come into contact with contaminated dairy manure are of risk to humans and human health (Marti et al., 2013).

CONCLUSIONS AND RESEARCH OBJECTIVES

In the dairy calf, reports of the effect of antibiotic exposure through antibiotic-containing milk on the incidence of ARG are mixed. The limited number of studies on AR as a result of feeding of non-saleable milk to calves have primarily used culture-based, rather than molecular, approaches to determine AR (Aust et al., 2012). Molecular-based research on the effect of feeding non-saleable milk containing antibiotic residue is needed, as traditional culture-based methods of testing for AR consider only a small fraction of the microorganisms involved in this resistance. Only 1% of bacteria are cultureable so culture-based methods of detecting antibiotic resistance show just a small piece of the picture (Pace, 1995). Molecular methods like qPCR and NGS analyze all present microorganisms for AR, providing a much more complete picture. Additionally, acquired resistance occurs through HGT between bacteria, so employing a

quantitative measure like qPCR can give a reasonable indication of the amount of AR being excreted by the calves. Assessment of functional gene composition is attainable by utilizing metagenomic analysis methods like Illumina HiSeq, providing direction for which ARG to quantify with qPCR (Thomas et al., 2012).

The objective of this research was to utilize NGS and metagenomic technologies to assess the effect of feeding pirlimycin residue-containing milk on the abundance and relative abundance of ARG in the feces of the calf, and to capture temporal variation in ARG coding sequences excreted in the fecal metagenome in calves fed milk spiked with pirlimycin. Ultimately, the goal of this research is to provide a better understanding of the triggers for ARG proliferation, resulting in the potential for improved management techniques to reduce the dissemination of ARG into the environment.

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CHAPTER 3: ANTIBIOTIC RESISTANCE GENE ABUNDANCE IN FECES OF CALVES FED PIRLIMYCIN-DOSED WHOLE MILK

ABSTRACT

Exposure to antibiotics has the potential to increase the incidence and proliferation of antibiotic resistance genes (ARG) in the gut and fecal microbiome. Non-saleable, antibiotic-containing milk from cows treated with antibiotics (waste milk) is commonly fed to dairy calves but the effects of ingestion of antibiotics at an early age on the gut microbiome and the development of ARG in the naïve gut are not well understood. Pirlimycin, a lincosamide antibiotic acting against Gram positive bacteria through inhibiting protein synthesis by binding to the 50S ribosome, is commonly used as mastitis therapy. Lincosamides are also considered highly important in human medicine, often used against *Staphylococcus aureus* and *Clostridium difficile* infections. Emerging microbial resistance to pirlimycin is of concern for both animal and human health and the World Health Organization has classified pirlimycin as a "highly important" antibiotic (2014). The objective of this study was to determine the effect of early lincosamide antibiotic exposure on the abundance of ARG in feces of milk-fed calves. Eight female Holstein calves were blocked by age, paired by block, and randomly assigned to pasteurized whole milk (control; n = 4) or pasteurized whole milk containing 0.2 mg/L of pirlimycin (treatment; n = 4). Calves were enrolled after receiving two colostrum feedings and were fed 5.68 L of pasteurized whole milk, treatment, or control, divided into two daily feedings, from d 1 to d 50 of age. After weaning calves were fed non-medicated starter grain *ad libitum*. Fecal samples were collected weekly until 85 d of age and freeze-dried. DNA was extracted using QiaAmp® Fast DNA Stool Mini Kit and qPCR was used to quantify the absolute abundance (gene copies/g of wet feces) and relative abundance (gene copies/copies of 16S rRNA

genes) of *erm*(B), *tet*(O), *tet*(W) and 16S rRNA genes. Data was analyzed using PROC GLIMMIX in SAS. Abundance of 16S rRNA genes, *tet*(O) and *tet*(W) were not different between control and pirlimycin-fed calves nor were the relative abundance of *tet*(O) (mean = 0.05 *tet*(O) copies/16S rRNA genes) or *tet*(W) (0.56 *tet*(W) copies/16S rRNA genes). While abundance of *erm*(B) was higher in pirlimycin-fed calves compared to control calves (6.46 and 6.04 log gene copies/g wet feces; $P = 0.04$) the relative abundance of *erm*(B) (0.27 gene copies/16S rRNA genes) in feces of calves was not influenced by treatment. There was an effect of day ($P < 0.10$) for absolute abundance of *tet*(O), *tet*(W), and *erm*(B) indicating that the levels change with time as the fecal microbiome develops. This study suggests that feeding pirlimycin-containing non-saleable milk to growing calves may increase environmental loading of e*rm*(B), which codes for resistance to highly important macrolide and lincosamide antibiotics. Additional research is needed on effects of feeding waste milk to calves on other fecal ARG and on the post-excretion and post-application fate of these genes.

INTRODUCTION

Non-saleable (waste) milk is an otherwise unusable byproduct of dairy farms comprised of colostrum, mastitic milk, and milk that cannot be sold due to its high somatic cell count, residual antibiotic, or other residual medication (Aust et al., 2012; Randall et al., 2014). In the dairy industry, feeding non-saleable milk is a common practice, occurring on approximately 28% of United States dairy farms (USDA, 2011). Non-saleable milk accounts for approximately 1-4% of milk production in Germany, but estimates for the United States are not available (Aust et al., 2012). Non-saleable milk can be fed pasteurized or unpasteurized (raw); the former is recommended to reduce the risk of proliferation of infectious disease (Godden and Eastridge, 2011). Feeding this milk in place of milk replacer reduces the cost of raising heifers by $34 per calf from birth to weaning (Godden et al., 2005). Feeding pasteurized non-saleable milk also results in higher growth rates and lower morbidity and mortality rates compared to feeding milk replacer (Aust et al., 2012; Brunton et al., 2012; Godden et al., 2005).

While there are benefits to feeding non-saleable milk, there are associated risks including exposure of the naïve calf gut to functional antibiotics and pathogens, even if it is pasteurized. Bacterial counts are higher in raw non-saleable milk (6.6×10^4 cfu/mL) compared to milk replacer (3.9×10^3 cfu/mL) and pasteurized milk (2.3×10^3 cfu/mL) (Aust et al., 2012; Elizondo-Salazar et al., 2010). Even after pasteurization following guidelines for non-saleable milk, pathogens in the milk may still be viable, especially if the cfu in raw milk is high (Aust et al., 2012; Edrington et al., 2012; Godden and Eastridge, 2011). For example, a study examining the use of ultraviolet (UV) light for bacterial inactivation found that the bacterial levels present in many of their samples were not adequately reduced by UV treatment (Gelsinger et al., 2014). Additionally, standard pasteurization treatments of raw milk can result in as little as 0.01%

degradation of antibiotics in milk (Zorraquino et al., 2008). For example, in a study by Randall et al. (2014) residual cefquinome antibiotic was detected in 64.1% of waste milk samples collected from 103 different farms. Another study demonstrated ultra-high pasteurization of cefquinome containing milk did not successfully degrade the antibiotic present (Horton et al., 2015). Certain temperature treatments such as batch pasteurization (30 minutes at 63ºC) and high temperature short time pasteurization (15 seconds at 72ºC followed by rapid cooling), are effective to reduce bacterial cfu, they do not adequately reduce antibiotic content (Godden and Eastridge, 2011). Other methods of waste milk management such as fermentation at high temperatures (Horton et al., 2015), addition of antibiotic degrading enzymes (Li et al., 2014), pH adjustment (Keith et al., 1983), and electrochemical oxidization (Kitazono et al., 2012) have been found to be somewhat effective at degrading antibiotics, however, no treatment is able to completely remove the antibiotic residue (Horton et al., 2015; Zorraquino et al., 2008). Pirlimycin antibiotic was selected for this study because a large proportion, more than half of the intramammary dose, is excreted as the whole and functional compound into the milk, feces, and urine, making this a common antibiotic contaminant in non-saleable milk (Moulin and Fougčres). Relevant to the current study, pasteurization has been demonstrated to be ineffective at degrading lincosamides, which are highly stable and loses minimal antimicrobial activity when pasteurized (Zorraquino et al., 2011). Therefore when calves are fed pasteurized non-saleable milk, they and their gut microbiome are exposed to whole, functional antibiotic.

The development of antibiotics changed the face of medicine by allowing doctors to combat previously untreatable diseases. However, resistance to antibiotics developed almost as soon as antibiotics were discovered (Abraham and Chain, 1940). Antibiotic resistance is becoming increasingly common in the United States, costing the US health system $21-$34

billion annually (World Health Organization, 2014). Commonly acquired infections, such as *Staphylococcus aureus* and *Escherichia coli* are becoming increasingly resistant to even the most potent antibiotics, making antibiotic resistance an issue of critical concern. The possibility of a post-antibiotic era, where antibiotics are no longer able to treat even minor infections, is becoming increasingly realistic (World Health Organization, 2014). Consequently, this rise in antibiotic resistance must be addressed.

The World Health Organization has classified animal agriculture as one of the main human actions contributing to the acceleration of the emergence of antibiotic resistance, as more than half of antibiotic use in the US is agricultural (World Health Organization, 2012). In 2010, approximately 13.3 million kg of antimicrobials were sold in the United States for use in food-producing animals, while 3.28 million kg of antibacterial drugs were sold for human use. Thus, livestock production's portion of antibiotic usage is close to 80% of total domestic use (FDA, 2010; 2012). This administration of antibiotics can lead to antibiotic resistance in the microbial communities of the animal as well as those in the soil and water, due to the dissemination of residual antibiotic, antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARG) in livestock feces (McEwen and Fedorka-Cray, 2002).

The effect of antibiotic exposure on the fecal bacteria of dairy calves has been studied for many years, but the results yielded are inconclusive. The digestive tract of calves is inhabited by an initially homogeneous population of bacteria that increases in diversity as the calf ages (Mayer et al., 2012; Oikonomou et al., 2013). When the naïve calf gut is exposed to antibiotics, the bacterial population becomes different from the population present in calves with no antibiotic exposure (Langford et al., 2003; Thames et al., 2012). While it is known that exposure to antibiotics leads to the proliferation of ARG, studies of fecal ARG dissemination in dairy

calves with oral exposure to antibiotics yield conflicting results, possibly due to the limitations of the methods employed (Aust et al., 2012; Wray et al., 1990). However, it is known that waterways and cropland located proximally to agricultural areas tend to have higher incidences of ARB and ARG than other areas, and this higher incidence of ARG can contaminate crops (Marti et al., 2013; Shi et al., 2013). Further research is needed to understand the role of early antibiotic exposure on ARG proliferation in the naïve gut microbiome of the dairy calf.

The objective of this research was to utilize quantitative technologies to assess the effect of feeding pirlimycin containing milk on the abundance and relative abundance of ARG in the feces of the calf and to capture temporal variation in ARG coding sequences excreted in the fecal metagenome. Ultimately, the goal of this research is to provide a better understanding of the triggers for ARG proliferation, resulting in the potential for improved management techniques to reduce the dissemination of ARG into the environment.

MATERIALS AND METHODS

This research was conducted under the review and approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (protocol number 14-010-DASC).

Experimental Treatments

Four lactating cows receiving intramammary pirlimycin were sampled at nine consecutive milking times to establish antibiotic concentrations in their milk (mean = 413 ng/mL, range = 45 – 2752). A value representing the antibiotic concentration in the lower half (median = 243 ng/mL) of treated cows sampled was used to determine the dose to administer to calves. Eight female Holstein calves were selected at birth from the Virginia Tech Dairy Center (Blacksburg, VA). Calves were assigned to pasteurized whole milk (control; n = 4) or milk

containing 0.2 mg/L of pirlimycin (Pirsue®, Zoetis, Madison, NJ; treatment; n = 4) and were blocked by age and paired by block. Calves were fed 5.68 L of pasteurized whole milk, treatment or control, divided into two daily feedings, from d 1 to d 43 of age and one 2.84 L feeding from d 44 to d 50 of age. After weaning at the age of 50 d, calves were fed *ad libitum* non-medicated starter grain until the completion of the study, at 85 d of age.

Newborn Processing

Dams of enrolled calves received ToMorrow® cephaprine benzathine upon dry-off. After birth, calves were separated from their dams and received two 1.89 L feedings of thawed colostrum approximately 8 h apart, the first feeding occurring within two hours after birth. At birth, calf navels were dipped in a 7% solution of iodine and vaccinations were administered according to current standard operating procedures at the Virginia Tech Dairy Center. Vaccines were for bovine rhinotracheitis and parainfluenza 3 (2 ml intranasally, TSV-2, Pfizer Animal Health, Exton, PA) rota- and corona virus (2 ml orally, Calf Guard, Pfizer Animal Health, Exton, PA), as well as a vaccine for five clostridial diseases with a toxoid (2 ml of Vision Seven, Bayer Corp.). Calves also received 1 ml of BoSe subcutaneously [0.5 mg of selenium, 25 mg (34 IU) of vitamin E; Schering-Plough Animal Health Corp., Union, NJ), and Vitamin A and D (250,000 IV vitamin A, 37,500 IU vitamin D; Phoenix Pharmaceutical, Inc., St. Joseph, MO)]. At 7 and 21 days of age, calves were vaccinated for bovine rhinotracheitis and parainfluenza 3 (2 ml intranasally, TSV-2, Pfizer Animal Health, Exton, PA).

Feeding and Housing

This study was conducted from 1 d to 85 d of age. Animals were enrolled and treatments were initiated at 1 d of age. Calves were fed grade A pasteurized milk (James River Work Center, State Farm, VA) containing pirlimycin if applicable. Milk was delivered weekly and was

stored in a 4ºC walk in cooler until feeding. Pirlimycin was added to 18.9 liter- bags of milk, thoroughly mixed, assigned to each treatment calf, and kept refrigerated until feeding. Each bag lasted 6 feedings. Milk was heated to 38°C twice daily at 0600 and 1800 h and calves were fed using bottles specifically assigned to the individual calf to avoid cross-contamination. Calves were supervised during feeding and were allowed as much time as needed to consume the milk provided. Equipment was thoroughly cleaned using soap and warm water following each feeding. Calves were fed *ad libitum* non-medicated starter grain (Fast Start TCR – 18% CP, Southern States Cooperative, Richmond, VA) and were allowed *ad libitum* access to water beginning at 1 d of age.

Calves were housed at the Virginia Tech Dairy Center in individual sawdust bedded fiberglass hutches (1.83 m × 1.37 m) with metal hog panels providing a 2.5 m^2 fenced area. Heifers were placed in hutches prior to their initial colostrum feeding, and remained there until weaning. Weaning was initiated at 43 d of age by reducing milk offered by 50%, feeding once daily at 0600 h. Calves were weaned when starter grain consumption reached 1.8 kg daily, no sooner than 50 d of age. This target was attained by all calves. At weaning, calves remained in their hutches for 3 to 5 days and were then moved to in a 3-sided barn with access to a dry lot in groups of four to six. Weaned calves were group-fed *ad libitum* alfalfa hay and the starter grain previously described.

Calf Health

Prior to the 0600 h feeding, body temperatures and fecal (0 to 3), respiratory (0 to 3), ear (0 to 3), and eye scores (0 to 3) were recorded using a Calf Health Scoring Criteria publication from the University of Wisconsin School of Veterinary Medicine (Larson et al., 1977). All

animals remained healthy throughout the study and no calves received any antibiotic other than their assigned treatment.

Sample Collection

Fecal samples were collected rectally using a clean glove and sterile lubricant for each collection from all calves on d 1 and then once weekly until d 85. Fecal samples were collected n. Approximately 100 g of wet fecal sample was collected into a plastic sterile snap cap vial and immediately placed into a -20°C freezer.

Sample Preparation and DNA Extraction

Fecal samples were thawed and 10 g of wet feces/sample was weighed into a sterile 50 mL conical, covered with aluminum foil, and frozen at -80°C for one hour. Samples were then freeze-dried (LABCONO, Kansas City, MO) for 48 hours.

Freeze-dried samples were mixed using a sterilized disposable spatula and 0.10XX g of freeze-dried feces was weighed into a sterile 2 mL microcentrifuge tube. Samples were extracted using the QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Germantown, MD) following the included pathogen detection protocol with modifications. The Inhibitex buffer was warmed (40-50°C) prior to its addition to reduce the incidence of precipitate. In the lysis step (step 3), samples were heated to 90°C using a dry bath for 5 minutes to improve lysis of gram-negative bacteria. Prior to step 5 an additional centrifguation steps were added to reduce the chance of residual fecal matrix contaminants. Also prior to step 5, 4 µL of RNase A (conc. 5µg/µL; Eipcentre®, Madison, WI) was added to reduce RNA contamination of the sample. In the elution step (step 14), 50 µL of buffer EB (10mM Tris-Cl) warmed to 50°C was utilized, as it does not contain EDTA, which can inhibit the library preparation step for Illumina analysis. 30 µL of the

eluted 50 µL was reapplied to the column and eluted, to ensure maximum DNA elution. All centrifugation steps were executed at 16,200 × *g*. Eluted DNA was stored at -20°C.

Real-Time Quantitative Polymerase Chain Reaction (qPCR) Analysis

DNA from fecal samples collected at all time points were extracted as previously described, and were analyzed with qPCR for 16S rRNA genes(Suzuki et al., 2000), *erm*(B) (Jacob et al., 2008), *tet*(O) (Aminov et al., 2001), and *tet*(W) (Ng et al., 2001) genes using previously reported qPCR protocols. Primer sequences are included in the appendix.

A CFX96^{TM} Real-Time System (Bio-Rad, Hercules, CA) was used to analyzed qPCR quantification in triplicate. A 10 µL reaction mixture including 5µL SsoFast EvaGreen (Bio-Rad, Hercules, CA), 0.8 µL (5M) of forward and reverse primer, 2.4 µL of molecular grade water, and 1 µL of diluted sample DNA (1:50, 16S rRNA; 1:80 *erm*(B); 1:80, *tet*(W); 1:80 *tet*(O)) was prepared for each well. Reaction temperatures were first 98°C for 2 minutes (temperature stabilization), followed by 39 cycles of 98°C (DNA denaturation), and subsequent annealing temperatures of 60.0°C (16S rRNA genes and *tet*(W)), 50.3°C (*tet*(O)), and 63°C (*erm*(B)), and finally, 45 cycles of 98°C to allow for DNA extension. Dry matter content was measured and data were back-calculated and presented as g of wet feces. Results are presented as both absolute gene abundance (copies/g of wet feces) and relative abundance to the overall bacterial population (copies of gene of interest/copies of 16S rRNA genes).

Statistical Analysis

Quantitative PCR data was analyzed using PROC GLIMMIX in SAS 9.4 (SAS Institute Inc., Cary, NC). Prior to analysis, non-normal data were logarithmically transformed to achieve data normality. Tukey pair-wise comparisons were used to determine treatment differences for

day and treatment. Results were considered significant at $P \leq 0.10$ and a $P \leq 0.15$ was considered a trend.

RESULTS

qPCR Results

16S rRNA genes

There was no effect of treatment ($P > 0.10$, **Error! Reference source not found.**) on the ecal abundance of 16S rRNA genes (mean = 9.80 log gene copies/g wet feces). There was an effect of day ($P < 0.02$, **Error! Reference source not found.**) but no differences between time oints. Treatment × day interaction was not significant.

Tetracycline resistance genes

There was no effect of treatment ($P > 0.10$, **Error! Reference source not found.**) on the bsolute fecal abundance of *tet*(W) (mean = 9.77 log gene copies/g wet feces). There was an effect of day on absolute abundance for *tet*(W) ($P < 0.04$; **Error! Reference source not found.**) ut no differences were observed between time points (Figure 2A). Treatment × day interaction for *tet*(W) absolute abundance was not significant. There was no effect of treatment ($P > 0.10$, Table 2) on the relative fecal abundance (gene copies/copies16S rRNA) of *tet*(W) (mean = 0.56 log gene copies/copies 16S rRNA genes). There was a trend for an effect of day on relative abundance for *tet*(W) ($P = 0.15$; Table 2) but no differences were observed between time points (Figure 2B). Treatment × day interaction for *tet*(W) relative abundance was not significant.

There was no effect of treatment ($P > 0.10$, **Error! Reference source not found.**) on the bsolute fecal abundance of *tet*(O) (mean = 7.77 log gene copies/g wet feces). There was an effect of day on absolute abundance for *tet*(O) ($P < 0.02$; **Error! Reference source not found.**) but no differences were observed between time points (Figure 3A). Treatment × day interaction for

tet(O) absolute abundance was not significant. There was no effect of treatment or day ($P > 0.10$, Table 2) on the relative fecal abundance (gene copies/copies16S rRNA genes) of *tet*(O) (mean = 0.08 gene copies/copies 16S rRNA genes). Treatment × day interaction for *tet*(O) relative abundance was not significant.

Macrolide resistance genes

There was an effect of treatment ($P < 0.04$, **Error! Reference source not found.**) on the ecal absolute abundance of *erm*(B) (control = 6.04 log gene copies/g wet feces; treatment = 6.46 log gene copies/g wet feces). There was an effect of day for log copies of *erm*(B) per gram of feces ($P < 0.0001$, **Error! Reference source not found.**) but no differences were observed etween time points (Figure 4A). Treatment × day interaction for absolute abundance was not significant. There was no effect of treatment ($P > 0.10$, Table 2) on the relative fecal abundance (gene copies/copies16S rRNA genes) of *erm*(B) (mean = 0.28 log gene copies/g wet feces). There was no effect of day on relative abundance for *erm*(B) ($P > 0.10$; Table 2) and no differences were observed between time points (Figure 4). Treatment × day interaction for *erm*(B) absolute abundance was not significant.

DISCUSSION

The bacterial genes analyzed for qPCR were 1) 16S rRNA genes, which was used to quantify bacterial abundance as well as to normalize the abundance of the ARG analyzed; 2) *tet*(W) and *tet*(O), both of which code for resistance to tetracyclines; and 3) *erm*(B), which codes for macrolide and lincosamide resistance. Each of the three resistance genes (*tet*(W), *tet*(O) and *erm*(B)) were found in the vast majority of the samples, including samples from control calves not exposed to antibiotics as well as samples collected at the initiation of the study. For example, *tet*(W) was detected in all day 1 samples, *tet*(O)was detected in 75% of day 1 samples, and

erm(B) was detected in 87% of day 1 samples. Until recently, the GI tract of calves was considered to be sterile, rapidly acquiring a diverse population after birth (Mayer et al., 2012). However, it has recently been demonstrated that meconium from calves contains bacteria (Mayer et al., 2012)The first sample in this study was collected at 24 hours of age, and therefore this data cannot speak to the sterility of the newborn calf gut. However, the occurrence of all three ARG in the initial fecal sample of the majority of the population sampled indicates that ARG incidence is regardless of antibiotic exposure status. Similar results were observed by Thames et al. (2012) where tetracycline, sulfonamide, and macrolide-lincosamide resistance genes were detected using qPCR in the feces of 6 week old calves never exposed to antibiotics. Additionally, studies examining the abundance of AR bacterial isolates in feces of calves raised with or without antibiotic exposure have observed no differences in the prevalence of AR fecal bacterial isolates (Sato et al., 2004; Wray et al., 1990).

While the calf gut population is initially dynamic, it has been shown to reach a stable state by about 3 d of age, plateauing until weaning (Edrington et al., 2012). The 16S rRNA gene copies/g wet feces measured in this study align with this general observation, remaining fairly steady throughout the study, with no significant differences between consecutive time points. While the qPCR data obtained in this study cannot speak to the diversity of the bacterial population present, the log copies of 16S rRNA genes graphed in Figure 1 remain fairly constant throughout the duration of the study, although there is an effect of day (P <0.02) on the number of log gene copies. However, there is a numerical (but not statistical) decrease in the number of copies of 16S rRNA genes in both control and treatment calves around weaning – at d 43, prior to milk offered being reduced to 2.93 L/d, and at d 57, one week after the cessation of milk feeding. This decrease could be attributed to the nutritional shift, from a in the primarily milk-

based diet to one based on grain, as bacterial population is highly influenced by diet (Callaway et al., 2010).

That lack of treatment and treatment × day interaction on the number of copies of 16S rRNA genes in fecal bacteria suggests a lack of effect on the overall bacterial population due to pirlimycin exposure. It is important to note that the qPCR measurement of 16S rRNA genes only describes total bacterial population. While the bacterial load may have remained stable it is possible that the microbial profiles may differ between groups. For example, Edrington et al. (2012) observed a greater number of bacterial species in the feces of calves fed pasteurized waste milk compared with those fed raw waste milk. These findings did not consider the influence of antibiotic present in the milk on bacterial diversity. Instead, the study only considered the effect of the bacterial load (or load reduction due to pasteurization) of the liquid feed. Another study examining microbial diversity in calf feces found that the Chao1 index, an indicator of bacterial diversity, was lower in calves treated with broad-spectrum antibiotics for pneumonia (Oikonomou et al., 2013). This decrease in bacterial diversity persisted for three weeks post-antibiotic treatment. In the current study all calves were fed only pasteurized milk, but treatment calves received doses of antibiotic in their milk throughout the first seven weeks of life. The results from the previous studies suggest that it is possible that the treatment employed did influence the diversity of the fecal bacterial communities of the calves in the current study. Additional exploration using metagenomic techniques would confirm or negate this hypothesis.

The effect of antibiotic exposure on the two tetracycline resistance genes measured, *tet*(O) and *tet*(W), was minor. There was an effect of day on fecal absolute abundance of both *tet*(O) ($P < 0.02$) and *tet*(W) ($P < 0.04$) (**Error! Reference source not found.**) but there was no ffect of treatment, day, or treatment × day on the relative abundance of *tet*(O), and there was only

a trend ($P < 0.15$) of day for *tet*(W). Thus while the number of copies of these resistance genes increased as the calf aged, the relative abundance (number of copies/number of bacteria) of these ARG did not change. While no tetracycline antibiotics were administered throughout the course of this study, tetracycline resistance genes are of interest as they are found in many different bacteria isolated from a variety of sources, including humans, animals, soil, and water. T*et*(O)is thought to be associated primarily with gram-positive bacteria, while *tet*(W)is more commonly associated with gram-negative bacteria, although neither classification is absolute (Chopra and Roberts, 2001). Additionally, certain tetracycline resistance genes are known to reside on the same transposon as the macrolide-lincosamide resistance gene *erm*(F) (Chopra and Roberts, 2001). This potential conjugal transfer of resistance genes related to macrolide-lincosamide exposure could be of interest in this study, as the antibiotic fed was a lincosamide.

A previous study measuring antibiotic resistance genes in antibiotic-exposed and antibiotic naïve calves identified tetracycline resistance genes in both groups (Thames et al., 2012). Similar to the results of the current study, feeding antibiotic-containing milk replacer did not have an effect on absolute abundance of the measured tetracycline genes. Additionally, another study observed no differences in tetracycline resistance in fecal *E. coli* from calves fed diets including tetracycline antibiotic compared with those fed a diet free of antibiotic (Aust et al., 2012). However, a study by Khachatryan et al. (2004) found fecal *E. coli* isolates from calves < 3 months in age were more likely to be resistant to tetracycline in calves fed diets containing oxytetracycline compared to calves fed a diet free of antibiotic. Therefore, it is possible that the reason we did not observe differences in tetracycline ARG between groups could be the mechanism of action of the antibiotic used in this study. Additionally, the relatively low

biological cost of attaining and maintaining *tet* resistance genes may explain the lack of differences between the two groups (Khachatryan et al., 2006).

The effect of day observed in this study has also been reported previously, but over a longer and less frequently sampled dataset (Khachatryan et al., 2004). These authors identified 79.2% of fecal *E. coli* isolates from preweaned calves as resistant to tetracyclines while only 35.7% of isolates from 3-6 month old heifers were resistant. These previously reported results align with our observation of an effect of age on the fecal shedding of *tet*(W) and *tet*(O)but no effect on the proportion of bacteria carrying this resistance gene. It is worth noting that samples from earlier in the study (d 8, d 15) had numerically (not significantly) higher proportions of *tet*(W)than samples collected at later dates (Figure 2B). While this observation is not significant, it is interesting, as it has been previously observed that younger animals tend to have higher amounts of ARB because younger animals are more commonly exposed to antibiotics than older ones (Khachatryan et al., 2004).

The final and most relevant ARG of interest analyzed in this study, *erm*(B), codes for resistance to lincosamides such as pirlimycin. The increased absolute abundance of *erm*(B) observed in treatment calves aligns with our hypothesis. While there was no significant treatment × day interaction, there is a time point that merits comment. There is a numerical, if not significant, decrease in the absolute of *erm*(B)at d 57 of age for both control (mean = 4.67 log copies/g feces) and treatment (mean = 5.84 log copies/g feces) groups (Figure 4A). This time point is important as it is the first sample collected after cessation of antibiotic exposure (d 50). However, while the number of copies of *erm*(B) decreased, data normalized to compensate for bacterial population (copies/16S rRNA genes) showed no differences. Therefore, it is possible that this observed decrease in the number of *erm*(B) copies could be attributed to the transient

nature of the young calf gut, where the population was experiencing a shift due to weaning rather than the removal of the antibiotic.

A second data point of interest was on d 71 where a non-significant spike was observed as an increase in the relative abundance of *erm*(B)in control calves (mean = 0.01 *erm*(B) copies/16S rRNA genes) compared with treatment calves (mean = 1.78 *erm*(B) copies/16S rRNA genes) (Figure 4B). This spike occurred two weeks after the cessation of feeding milk which was implemented at d 50, and therefore cannot be attributed to a change in diet, although calves were likely consuming more grain than in previous weeks. These results support our hypothesis that calves fed antibiotic containing milk will have more bacteria that have the capacity for macrolide-lincosamide resistance. It is possible that this spike in *erm*(B) relative abundance was due to stress on the bacterial community caused by combating a low-level infection, as ARG can be transferred along with other proximal genes, but that does not explain the difference seen between the two groups (Chopra and Roberts, 2001). This spike occurred approximately 14 days after calves were moved from individual hutches into group pens. Therefore, it is possible that what is being observed is the effects of the combined stresses of weaning, diet change, and increased pathogen exposure due to movement into group housing (Yun et al., 2014). It is also possible that the change observed was due to a shift in the bacterial population caused by the transition to a purely grain based diet, however, bacterial populations are known to stabilize around one month of age (Rey et al., 2014). Each of these observed differences in *erm*(B) prevalence was transient, and populations returned back to their baseline levels by the next sampling.

Environmental Concerns

The results from this study primarily show differences in the absolute abundance of resistance genes rather than their relative abundance. Therefore, these effect of these differences on the calf gut is likely less of a concern than the environmental effects of increased ARG shedding and dissemination. Fecal shedding of *tet*(O) and *tet*(W) did not differ between treatments, but shedding of *erm*(B) was increased ($P > 0.04$) in treatment calves. One of the biggest problems posed by fecal ARG shedding is water contamination, as water in close proximity to agricultural activity tends to contain higher levels of ARG than more distant waters, likely due to runoff from farms (Pruden et al., 2006; Santamaria et al., 2011). Additionally, drinking water is a known source of ARB and ARG, as current methods of wastewater treatment are unable to effectively remove - and in some cases even select for - these contaminants (Shi et al., 2013). Human contact with ARB-containing water proximal to agricultural sources is a direct point of entry for ARG and ARB to the human microbial community, and these results show the potential influence antibiotic exposure can have on ARG dissemination into the environment.

Study Limitations

There are several possible reasons few differences were observed in this study, the first of which is the mechanism of action of the antibiotic applied. Pirsue®, the form of pirlimycin dosed into the milk fed, is specifically designed to penetrate into mammary tissue to combat mastitic infections. This antibiotic was selected for this study because a large proportion, more than half of the intramammary dose, is excreted as the whole and functional compound into the milk, feces, and urine, making this a common antibiotic contaminant in non-saleable milk (Moulin and Fougčres). Pirlimycin is also a relevant antibiotic, as nearly all pirlimycin residue in milk is in the parental form. However, while it is well known that pirlimycin is secreted into the milk of Pirsue®-treated cows, the pharmacokinetics of the calf digestive tract on the compound is

unknown, as macrolides and lincosamides are not labeled for oral administration. While data on the digestive metabolism of macrolides and lincosamides is available, tissue deposition studies of macrolides administered subcutaneously have identified deactivated metabolites present in liver tissue samples, demonstrating a level of metabolic inactivation (Linhares et al., 1998). Additionally, ML are designed to penetrate into the cell to bind to the ribosome. The efficacy of antibiotics against bacterial diseases can be reduced in the presence of extracellular fluid at low pH, so if the pirlimycin compound was inactivated by the acidic conditions of the stomach prior to internalization by a cell, it would not exert its effect. (Linhares et al., 1998).

Another possible reason for the lack of treatment effect is the high plane of nutrition in the course of the study. There is a physiological cost, or fitness cost, to bacteria to express resistance genes, and therefore these genes are more likely to be expressed during times of stress (Pereira et al., 2011). There is a known, but not well understood, relationship between immune system activation and nutrition. Several studies have demonstrated that calves receiving more nutrition have lower mortality rates and better immune responses than calves fed a lower plane of nutrition (Godden et al., 2005). The calves in this study were fed more than most calves on a commercial dairy, and therefore had more energy to devote to immune system support. It is reasonable to posit that the extra nutritional resources available to the support immune system would prevent the occurrence of conditions that favor the proliferation of ARG in the gut, explaining the results observed in this study.

Finally, this was a pilot study, and the number of animals utilized was small. It is possible that applying increased power to this experiment would result in greater treatment effects, especially in regard to *erm*(B) absolute abundance, where potential differences were identified. Additionally, the method employed, qPCR, while quantitative, has large amounts of variability,

and therefore increasing the number of animals used could reduce the variability observed. Importantly, it is possible that minimal differences were observed because the exposure to pirlimycin antibiotic did not cause an increase in fecal dissemination of the resistant genes quantified in this study.

CONCLUSIONS

The techniques applied in this study provide a quantitative measure of the effects of oral pirlimycin exposure on the ARG proliferation in the calf gut. The antibiotic administered largely did not affect the proliferation and excretion of the ARG measured. However, the absolute abundance of *erm*(B) was greater in calves exposed to pirlimycin than in control calves and there was a transient spike of *erm*(B) observed on day 71 in treatment calves. Calf age played a significant role in the fecal shedding of ARG, and future researchers can use this information to identify critical days for management of feces excreted. Future research can also focus on using deep sequencing techniques to identify the effects of antibiotic on the full fecal microbiome in calves. Additionally, future research needs to be conducted testing the effects on calf fecal ARG by antibiotics other than pirlimycin commonly present in waste milk.

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Table 1. qPCR analysis: Effect of fed pirlimycin on antibiotic resistance gene absolute abundance in dairy calf feces (n = 4/trt)

Gene copies (log10) per gram of feces							
	Antibiotic[1]		Control[2]		*P* = [3]		
Gene	LSM[4]	SEM[5]	LSM	SEM	Treatment	Day	Interaction[6]
16S rRNA genes	9.96	0.16	9.64	0.17	NS	< 0.02	NS
tet(O)	7.74	0.10	7.80	0.13	NS	<0.02	NS
tet(W)	9.58	0.07	9.50	0.08	NS	< 0.04	NS
erm(B)	6.46	0.10	6.04	0.11	<0.04	<0.0001	NS

[1] n = 4 orally dosed antibiotic in milk fed, 0.2 mg/L

[2] n = 4 no antibiotic fed

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

[4] Least square means for treatment

[5] Standard error for treatment LSM

[6] Two way interaction of treatment × day

Table 2. qPCR analysis: Effect of fed pirlimycin on antibiotic resistance gene relative abundance in dairy calf feces (n = 4/trt)

Gene copies per 16S rRNA genes							
	Antibiotic[1]		Control[2]		*P* = [3]		
Gene	LSM[4]	SEM[5]	LSM	SEM	Treatment	Day	Interaction[6]
tet(O)	0.09	0.06	0.01	0.08	NS	NS	NS
tet(W)	0.55	0.11	0.58	0.12	NS	< 0.15	NS
erm(B)	0.16	0.12	0.03	0.14	NS	NS	NS

[1] n = 4 orally dosed antibiotic in milk fed, 0.2 mg/L

[2] n = 4 no antibiotic fed

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

[4] Least square means for treatment

[5] Standard error for treatment LSM

[6] Two way interaction of treatment × day

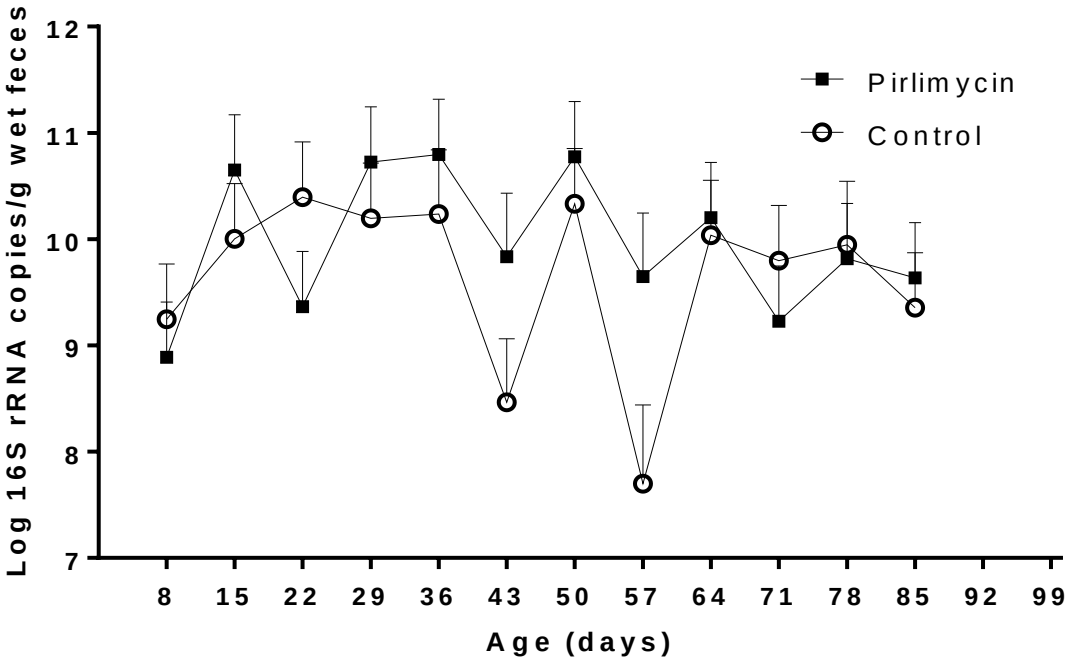

Figure 1. qPCR analysis: Abundance of 16S rRNA genes (log10 copies per 1.0 g wet feces) in fecal samples

Samples were collected from control (n=4) and pirlimycin hydrochloride treated (n=4) calves. Day 1 (pre-treatment) samples were used as a covariate.

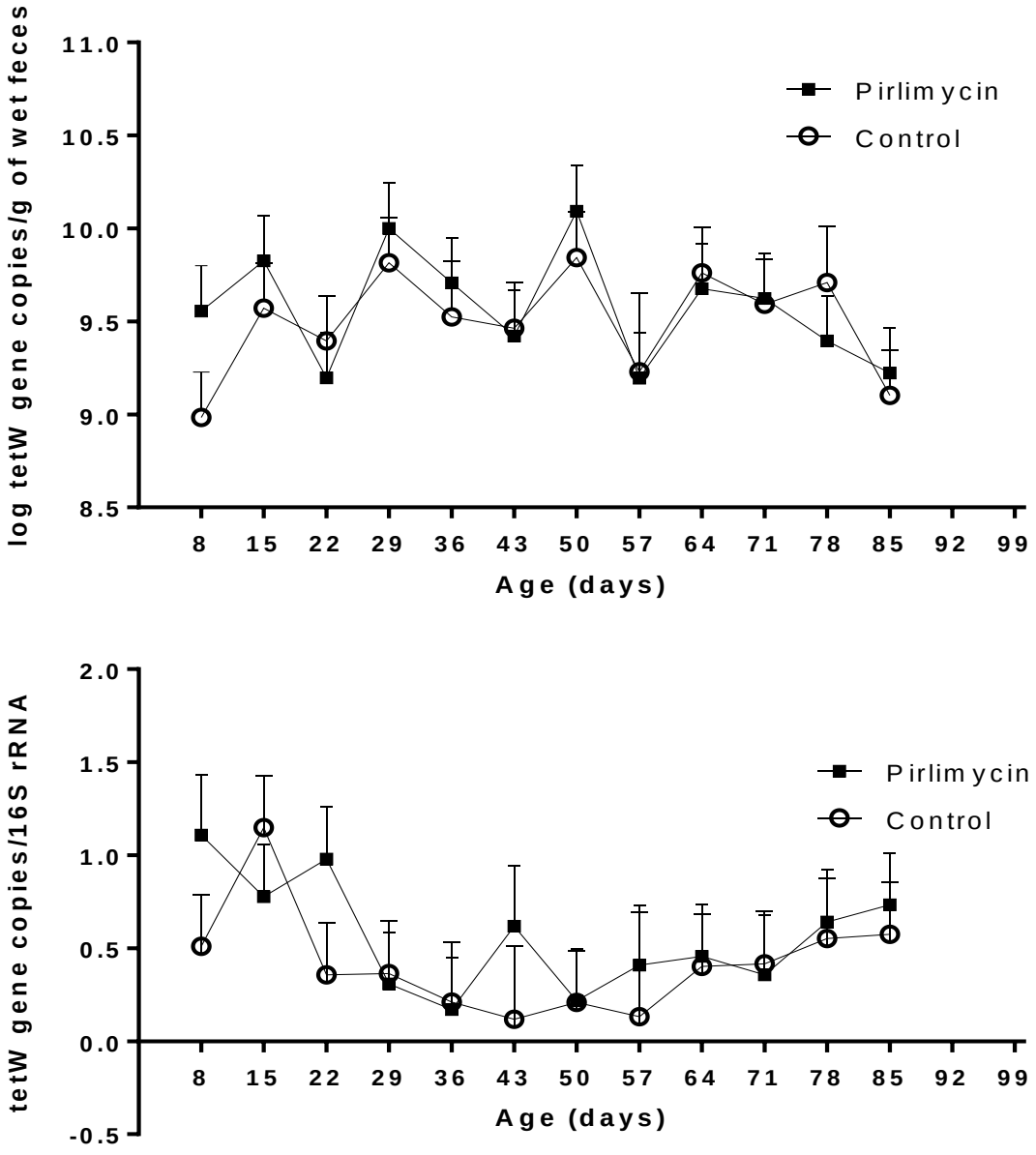

Figure 2. qPCR analysis: (A) Abundance of *tet*(W) (log10 copies per 1.0 g wet feces) in fecal samples. (B) Relative abundance of *tet*(W) (gene copies/16S rRNA genes) in fecal samples Samples were collected from control (n=4) and pirlimycin hydrochloride treated (n=4) calves. Day 1 (pre-treatment) samples were used as a covariate.

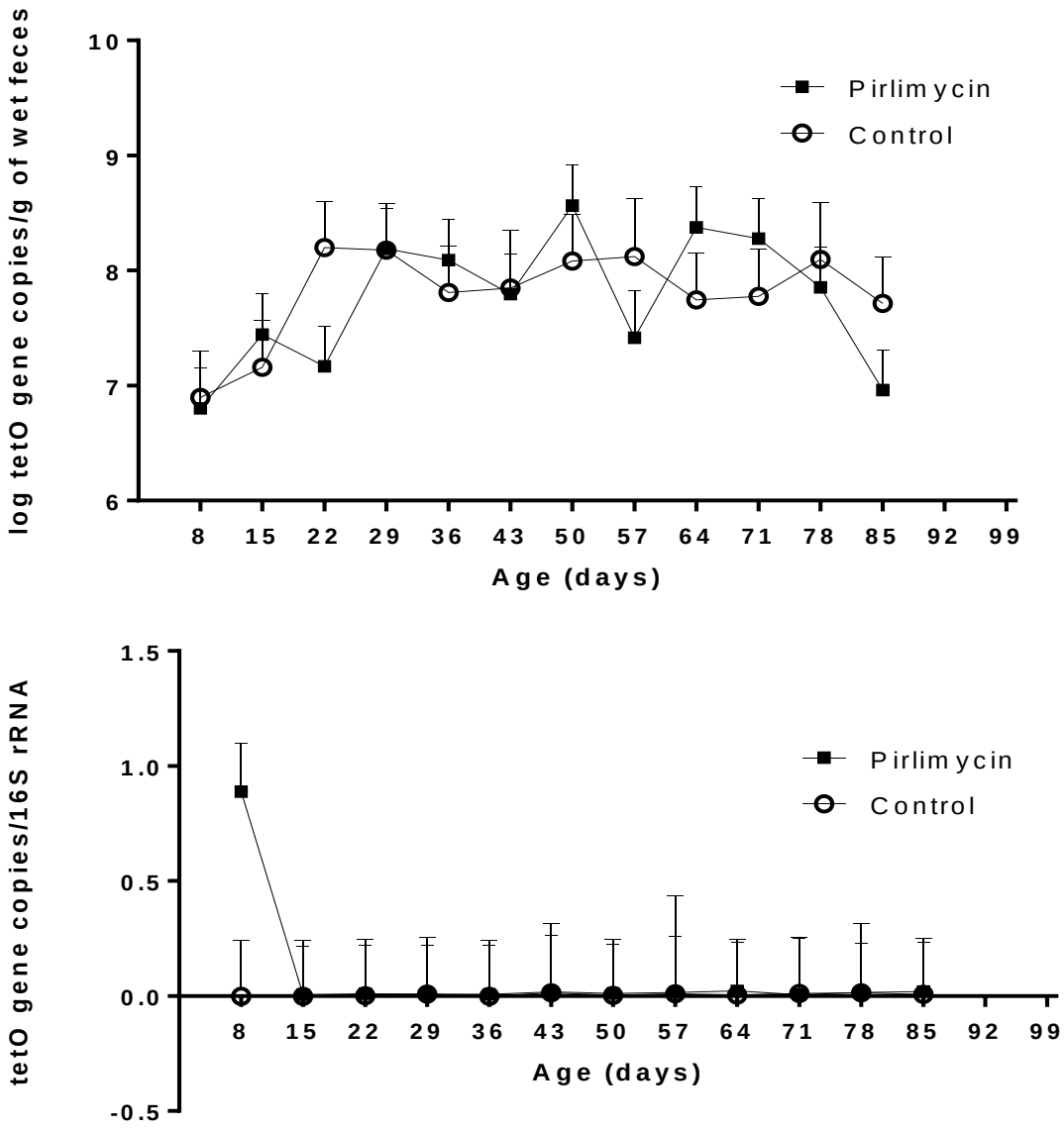

Figure 3. qPCR analysis: (A) Abundance of *tet*(O) (log10 copies per 1.0 g wet feces) in fecal samples. (B) Relative abundance of *tet*(O) (gene copies/16S rRNA genes) in fecal samples. Samples were collected from control (n=4) and pirlimycin hydrochloride treated (n=4) calves. Day 1 (pre-treatment) samples were used as a covariate.

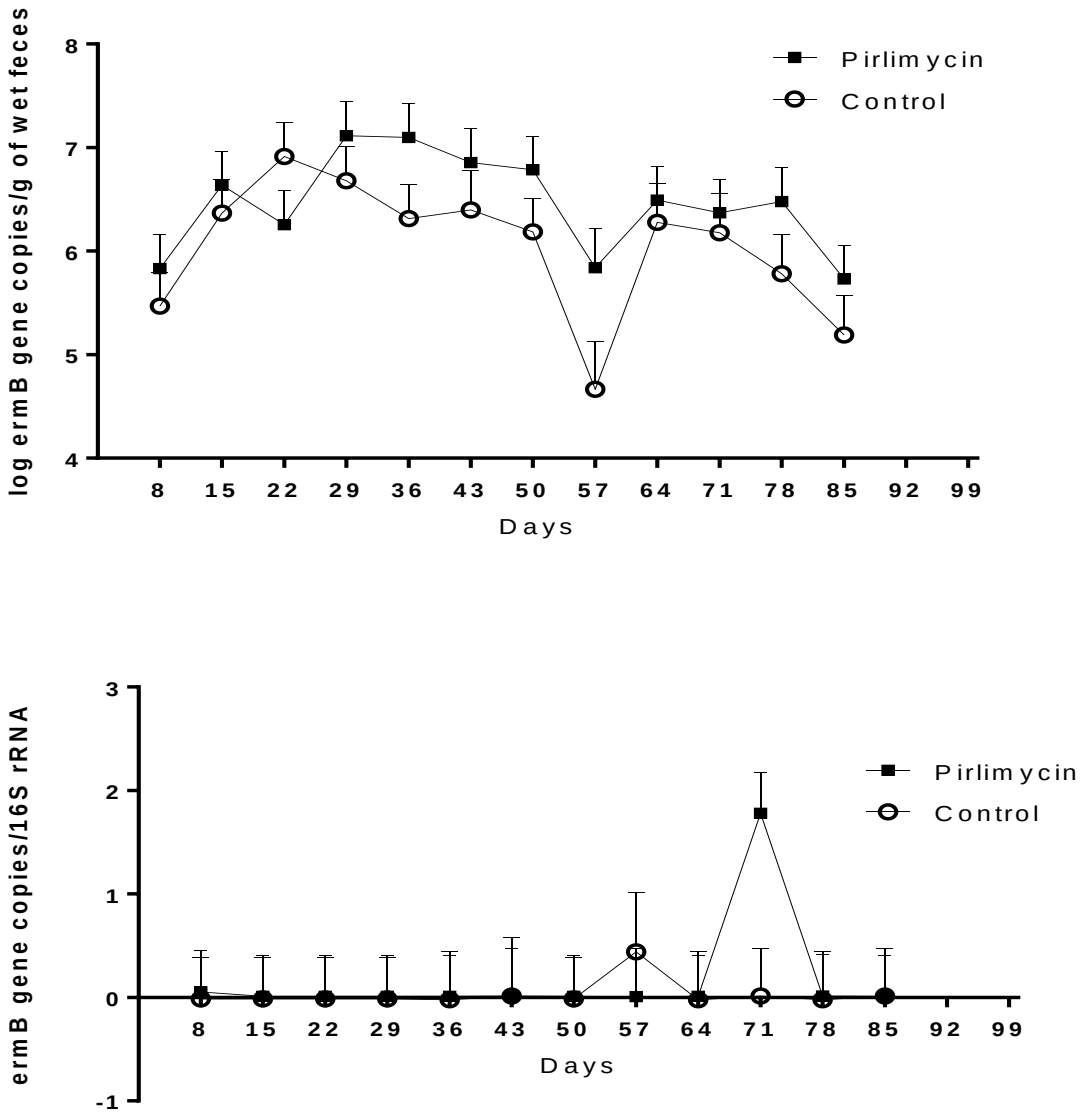

Figure 4. qPCR analysis: (A) Abundance of *erm*(B) (log10 copies per 1.0 g wet feces) in fecal samples. (B) Relative abundance of *erm*(B) (gene copies/16S rRNA genes) in fecal samples. Samples were collected from control (n=4) and pirlimycin hydrochloride treated (n=4) calves. Day 1 (pre-treatment) samples were used as a covariate.

APPENDIX A

Forward and reverse primer sequences for qPCR analysis

Primer	Target gene	Primer sequence 5'-3'	Reference
1369F	16S rRNA	CGGTGAATACGTTCYCGG	(Suzuki et al., 2000)
1492R	16S rRNA	GGWTACCTTGTTACGACTT	
tet(O)-Fwd	*tet*(O)	ACGGARAGTTTATTGTATACC	(Aminov et al., 2001)
tet(O)-Rev	*tet*(O)	TGGCGTATCTATAATGTTGAC	
tet(W)-Fwd	*tet*(W)	GAGAGCCTGCTATATGCCAGC	(Aminov et al., 2001)
tet(W)-Rev	*tet*(W)	GGGCGTATCCACAATGTTAAC	
erm(B)-Fwd	*erm*(B)	GAATCCTTCTTCAACAATCA	(Jacob et al., 2008)
erm(B)-Rev	*erm*(B)	ACTGAACATTCGTGTCACTT	