Excretion of antibiotic resistance genes by dairy calves

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ABSTRACT

Twenty-eight Holstein and crossbred calves of both genders were used to evaluate the effect of milk replacer antibiotics on abundance of selected antibiotic resistance genes (ARG) in the feces. Calves were blocked by breed, gender, and birth order, and assigned to one of three treatments at birth. Treatments were control (containing no antibiotics in the milk replacer), subtherapeutic (neomycin sulfate and oxytetracycline hydrochloride each fed at 10 mg/calf/d), and therapeutic (no antibiotics in the milk replacer until d 36, then neomycin sulfate and oxytetracycline hydrochloride each fed at 1000 mg/calf/d for 14 d). Calves were fed milk replacer twice daily at 0600 h and 1800 h. Fecal and respiratory scores and rectal temperatures were recorded daily. Calves were weighed at birth and weaning to calculate average daily gain. Beginning at six weeks of age fecal grab samples were collected from heifers at 0600 h, 1400 h, 2000 h, and 2400 h for 7 d, while bull calves were placed in metabolism crates for collection of all feces and urine. DNA was extracted from feces, and ARG corresponding to the tetracyclines (*tet*C, *tet*G, *tet*O, *tet*W, and *tet*X), macrolides (*erm*B, *erm*F), and sulfonamides (*sul*1, *sul*2) classes of antibiotics along with the class I integron gene, *int*I1, were measured by quantitative

polymerase chain reaction (qPCR). No *tet*C or *int*I was detected. There was no significant effect of antibiotic treatment on the absolute abundance (gene copies/ g wet manure) of any of the ARG except *erm*F, which was lower in the antibiotic-treated calf manure probably because host bacterial cells carrying *erm*F were not resistant to tetracycline or neomycin. All ARG except *tet*C and *int*I were detectable in feces from 6 weeks onwards, and *tet*W and *tet*G significantly increased with time ($P < 0.10$), even in control calves. Overall, the majority of ARG analyzed for were present in the feces of the calves regardless of exposure to dietary antibiotic. Feed antibiotics had little effect on the ARG monitored; other methods for reducing the ARG pool should also be investigated.

Key words: antibiotic, antibiotic resistance gene, dairy calves

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ABBREVIATION KEY

ADG: Average daily gain

ARG: Antibiotic resistance gene(s)

BW: Body weight

CP: Crude protein

DM: Dry matter

qPCR: Quantitative polymerase chain reaction

Chapter 1: Review of Literature

Introduction

Antibiotics are fed to livestock at subtherapeutic levels for both growth promotion and disease prevention, because their use reduces morbidity and mortality. The extensive use of antibiotics in animal agriculture and the development of antibiotic resistant bacteria have created increasing concern. Livestock are often cited as a potential reservoir for resistant bacteria and antibiotic resistance genes (ARG). The unintentional selection of bacteria that are resistant to powerful antibiotics could have devastating human health consequences. However, there are animal welfare implications when antibiotic use is limited. Sustainability of the dairy industry depends on a balance between concerns for animal welfare, economical animal production practices, and human health.

Subtherapeutic Use of Antibiotics in Dairy Calves

Antibiotics are used in the agricultural industry for the prevention and treatment of disease and for growth promotion (Chee-Sanford et al., 2009). Dairy calves are susceptible to a multitude of pathogens prior to weaning, so antibiotics are commonly added to milk replacers to prevent or treat disease (Berge et al., 2005). Of the 85.9% of US dairies feeding milk replacers in a 2011 survey, two thirds were feeding medicated milk replacers (USDA, 2012).The two

approved antibiotics most commonly used in milk replacers in the US are neomycin and oxytetracycline. Neomycin prevents and treats scours, because it is not absorbed by the body of the animal. Instead, it remains in the digestive tract of the calf, where it controls growth of pathogenic organisms. Oxytetracycline, in contrast, is absorbed by the body of the calf, and is responsible for prevention and treatment of respiratory illnesses.

Prophylactic use of antibiotics in milk replacer increases average daily gains (ADG; (Lassiter et al., 1963), improves feed consumption (Donovan et al., 2002), and increases feed efficiency (Quigley and Drew, 2000).Inclusion of neomycin and oxytetracycline in milk replacer increased the probability of normal fecal scores (Heinrichs et al., 2003) and decreased incidences of scours and number of days scouring (Quigley et al., 1997). Calves treated with long-acting antibiotics such as tulathromycin were 50% less likely to be treated for bovine respiratory disease than calves treated with a short-acting antibiotics (Stanton et al., 2010).

Growth promotion with antibiotics is partly attributed to prevention of enteric infections, which causes increased intake, and in turn, increased growth. Broad-spectrum antibiotics can decrease incidences of diarrhea and enteric diseases by destroying disadvantageous bacteria that inhabit the digestive tract (Casewell et al., 2003). However, it is important to note that antibiotics are only effective against bacterial sources of calf scours, not against protozoan and viral infections that are common in young calves (Lorenz, 2009). Also, use of antibiotics in a

subtherapeutic or therapeutic manner is not a substitute for passive transfer of immunity through colostrum (Berge et al., 2005), however. Calves that do not receive adequate amounts of high quality colostrum will suffer more ill health episodes than calves that do receive adequate colostrum, regardless of antibiotic use (Berge et al., 2005).

Another feeding practice that supplies antibiotics to calves is the feeding of milk collected from antibiotic-treated cows. This "waste milk" is fed to calves on one third of US dairy farms (USDA, 2012). The amount of antibiotics in waste milk is highly variable, changing with the number of cows being treated for mastitis or other diseases, and the number of cows that are in the mandatory withdrawal period following treatment with antibiotics (Langford et al., 2003).

There is some evidence that subtherapeutic use of antibiotics may be detrimental to the health of calves (Berge et al., 2009). The ratios of natural flora found in the digestive tract of the calf may be negatively affected with antibiotic use in the milk replacer. This may result in increased incidence of disease, decreased weight gain, and decreased starter grain consumption. Berge et al. (2009) found the calves fed milk containing antibiotics had 31% more days of scouring than calves fed milk not containing antibiotics, probably due to antibiotic-associated diarrhea, in which antibiotics make the normal gut microflora vulnerable to colonization by

pathogens. Also, antibiotics are not effective against illnesses caused by protozoa or viruses, common in young calves (Donovan et al., 2002).

Antibiotic Resistance

The gut micro flora may gradually become resistant to the antibiotics to which they are constantly exposed. This may reduce the effectiveness of commonly used antibiotics and may also contribute to multi-drug resistance, reducing treatment options in cases of infection. The microbial genes that code for resistance to antibiotics (antibiotic resistance genes, ARG) are considered environmental contaminants because they can persist in water and soil beyond the lifespan of the host microbe (Pruden et al., 2006). The intestinal tract of livestock and the manure storage systems serve as reservoirs for ARG. Because some ARG exist naturally in the background, they are found associated with both conventional and organic agriculture (Pruden et al., 2010).

Transfer of antibiotic resistance genes among organisms

ARG may be transferred among organisms in a variety of ways. These genes are sometimes transferred through plasmids, either by horizontal gene transfer or conjugation (Manson et al., 2010). Also, ARG may be transferred between pathogenic and benign strains of the same genus of organisms, troubling from a food safety standpoint (Zago et al., 2010). Recombination between sections of the chromosomes of bacteria and plasmids from

environmental reservoirs can increase the prevalence of ARG (Manson et al., 2010). More research on this topic is needed to understand the amplification and attenuation of ARG in various reservoirs (Pruden et al., 2010).

A variety of tetracycline ARG were observed in waters that received effluent from an oxytetracycline production plant in China. The genes identified included *tet*A, *tet*W, *tet*C, *tet*J, *tet*L, *tet*D, *tet*Y, and *tet*K. Class I integrons, mobile genetic elements with the capability of being passed among organisms, were detected in the majority of wastewater and river water from and around the production plant, but the integrons were not associated with any of the *tet* genes, which likely means that the *tet* genes were not being transferred among organisms (Abbassi-Ghozzi et al., 2011).

Antibiotic resistance in gut and fecal bacteria

With its warm, moist environment and high level of available nutrients the gastrointestinal tract acts as a reservoir for antibiotic resistance (Unno et al., 2010) and it is an ideal environment for the transmission of ARG among the gut micro flora (Jernberg et al., 2010). In addition to selecting for ARG, the introduction of antibiotics in to the gut environment may increase the number of gene transfer events that occur (Jernberg et al., 2010).

In calves fed waste milk, antibiotic-resistance of fecal bacteria increases in relation to the amount of antibiotics present in the milk (Langford et al., 2003). The variability of the antibiotic

content of waste milk has consequences with respect to antibiotic resistance, because constant exposure to low levels of antibiotics is considered the major route for development of antibiotic resistance (Langford et al., 2003).

Antibiotic resistance tends to be higher on dairy farms that have issues with calf scours, likely due to attempts to treat scours with antibiotics (Langford et al., 2003). Fecal isolates collected from dairy calves are more likely to be resistant to antibiotics than isolates from other types of cattle. In a survey of 2,255 cattle at slaughterhouses from nine regions in France, multiple resistance was found in one isolate from culled cows, one isolate from young beef cows, and 43 isolates from dairy calves (Chatre, 2010). This higher incidence of multi-resistance found in the young calves may be due to the high frequency with which dairy calves are treated for diseases related to their immature immune system.

In South Korea, antibiotic use is approximately 1.5 times greater than neighboring countries, and antibiotic resistant bacteria are isolated in South Korea more frequently than in any other industrialized country (Unno et al., 2010). However isolates collected from humans were less resistant than isolates collected from pigs or chickens (Unno et al., 2010).

Resistance is becoming more common among pathogenic bacteria associated with food animal products. Non-typhoidal salmonellosis causes infections in humans, is commonly acquired by consumption of contaminated food animal products, and is becoming increasingly

difficult to treat (Abbassi-Ghozzi et al., 2011). Nearly 80% of salmonella isolates recovered from stool samples of infected patients in Tunisia were resistant to at least one antibiotic (Abbassi-Ghozzi et al., 2011).

In South Africa, ARG previously found only in clinical isolates were isolated from human wastewater treatment plant effluents, suggesting that genetic exchange is occurring between clinical and environmental bacteria (Okoh and Igbinosa, 2010). ARG found in wastewater treatment plant effluents become widely dispersed in the environment (Okoh and Igbinosa, 2010) and many scientists fear that the major water pollution issues in South Africa due to mismanagement of domestic sewage and industrial wastewater will exacerbate with the spread of ARG (Okoh and Igbinosa, 2010).

Antibiotic resistance genes in pristine populations

Examination of "pristine" populations allows greater understanding of the contribution of antibiotic use to the problem of antibiotic resistance. In fecal isolates of free-ranging red foxes in Portugal, obviously never treated with vancomycin, 13.5% of isolates contained the *van*A gene (Radhouani et al., 2011).The excreta of wild raptors (no history of antimicrobial treatment) housed in a Spanish wildlife rehabilitation center contained multi-drug resistant *Salmonella* and quinolone-resistant *Campylobacter* isolates (Molina-Lopez et al., 2011). Similarly, 67% of

human subjects in a remote section of Bolivia were found to carry fecal E. coli resistant to one or more antibiotics (Bartoloni et al., 2004).

The fact that resistance has been documented in animal populations with little to no human contact is troubling. However, these results also suggest that even a complete halt in the use of antibiotics will not eliminate antibiotic resistance. After all, most antibiotics are produced by fungi and bacteria so are, to some extent, produced naturally, and antibiotic resistance has been documented in bacterial samples collected in years prior to the first industrially produced antibiotics (Allen et al., 2010). Also, even remote populations considered pristine may be exposed to ARG transported by migratory birds.

Effects of Antibiotics in the Environment

Antibiotics are excreted largely intact (Liu et al., 2013)and may have damaging effects on natural ecosystems when land applied in manure (Dolliver and Gupta, 2008). The half-life of antibiotics following land application is affected by soil chemistry, exposure to light and heat, and land management (Storteboom et al., 2007). The half-life of oxytetracycline in land-applied manure was 30 days, with the parent compound still detectable in the manure after five months of maturation (Liguoro et al., 2003). In contrast, tylosin degrades rapidly and was no longer detectable in manure 45 days after treatment of experimental animals ceased.

Runoff from agricultural land can deliver antibiotics to surface water but the extent of agricultural contribution is relatively unstudied. In Northern Germany, ß-lactams, macrolides, sulfonamides, trimethoprim, fluorochinolones, and tetracyclines were not detected in dairy manure or leachate from dairy farms, but water samples from a river surrounded by livestock farms contained up to 68 µg/l of oxytetracycline (Matsui et al., 2008). Antibiotics in surface water become associated with the sediment in the aquatic environment (Arikan et al., 2008). Antibiotic concentrations in the soil of the riverbed was highly variable throughout the year due to weather patterns and numbers of animals receiving treatment (Matsui et al., 2008).

Within the soil, antibiotics affect microbial populations. Antibiotics have differential effects on gram-negative or gram-positive bacteria, and altering these ratios affects populations of fungi, other soil microorganisms, soil biomass, and nutrient transfer among organisms within the community (Berge et al., 2010). Tyrosine, for example, reduces soil respiration rate and fungal biomass production (Berge et al., 2010). Ionophores, used for growth promotion and prevention of coccidiosis in cattle, are generally considered toxic to microbes in the soil and aquatic environments, but the toxicity of ionophores varies with the amount of runoff from agricultural land, and the degree to which bioaccumulation occurs (McGregor et al., 2007). The effect of manure management on prevalence of ARG in manure applied to agricultural land has not been fully evaluated, but there is concern that this practice distributes ARG to other

environments. Some manure handling systems have been shown to affect the amount of ARG found in manure (Storteboom et al., 2007). For example in composted dairy manure a positive correlation was observed between concentration of *tet*W in the dairy manure and total concentration of tetracyclines. The half-life of oxytetracycline was reduced from 7-31 days in control manure (not composted) to 6-15 days in composted manure (Storteboom et al., 2007). The half-life of antibiotics is important because the longer the organisms are exposed to an antibiotic, the greater the potential for selection for resistant organisms to occur.

Regulations Limiting Antibiotic Use

Because of concerns about growing microbial resistance to medically important antibiotics, some of these compounds (e.g., chloramphenicol and nitrofurans) have been banned for use in food animals (Vaz et al., 2010). Also, the European Union countries have banned subtherapeutic antibiotic use in livestock (Boerlin et al., 2001), beginning with a partial ban in Switzerland in 1972. Not included in the 1972 ban were avoparcin, a glycopeptide, and tylosin, a macrolide, and use of these was associated with a rise of intestinal bacteria resistant to these medically important drugs. By 1999 all antimicrobial growth promoters were banned in Switzerland (Boerlin et al., 2001). Isolates collected from pigs before and after this complete ban suggest that resistance to these antibiotics rapidly declined following the removal of selection pressure, a positive result from a human health standpoint (Boerlin et al., 2001). Effects on

bacteria in poultry excreta were not as consistent. Prior to the ban of antibiotic growth promoters in Switzerland, isolates of *E. faecalis*, *E. faecium,* and *lactobacilli* collected from poultry showed high levels of resistance to the antibiotics ampicillin, ciprofloxacin, chloramphenicol, gentamycin, streptomycin, tetracycline, erythromycin, vancomycin, and bacitracin (Frei et al., 2001). Following the ban of antibiotic growth promoters, the prevalence of antibiotic resistance in *S. typhimurium* was notably lower but resistance in indicator organisms such as *E. coli* and *E. faecium* was unchanged (Bengtsson and Wierup, 2006).

The EU banned avoparcin in 1997 and bacitracin, spiramycin, tylosin and virginiamycin in 1999. Immediately following the EU ban, a slight decrease in antibiotic resistance was observed via rectal swabs collected from food animals at slaughter, but incidences of food-borne diseases increased (Casewell et al., 2003). Also, therapeutic use of antibiotics in livestock increased following the ban (Casewell et al., 2003).

Co-selection of ARG is also a problem and may lead to persistence of resistance to a banned antibiotic. ARG present on the same mobile genetic element may be co-selected and or be passed among different organisms. For instance, following a ban on use of avoparcin in chickens in Taiwan, the incidence of vancomycin-resistant *Enterococci* was decreased (Lauderdale et al. 2007). However, vancomycin resistance did persist following the ban and resistance to tetracyclines and macrolides stayed the same or increased. The perpetuation of

vancomycin resistance may be due to co-selection; in bacteria isolated from swine feces, vancomycin and macrolide ARGs were located on the same genetic element (Aarestrup, 2000).

The mixed results of antibiotic bans on antibiotic resistance reflect the reality that antibiotic resistance takes time to decline in the intestinal microflora of the food animal population. For instance, although the most effective way to rid flocks of fowl typhoid (caused by *Salmonella enterica*) is to remove chickens that carry it, in South Korea antibiotics such as quinolones, aminoglycosides, and fluoroquinolones are frequently the first method of treatment when an outbreak occurs. Outbreaks are now rare in Korea but infections due to antibiotic resistant *S. enterica* persists and reduced susceptibility to fluoroquinolones is observed (Kang, 2010). Over time it is expected that restrictions on antibiotic use will lead to sustained reductions in resistance; several global organizations are monitoring the situation (Bengtsson and Wierup, 2006).

The European laws banning antibiotic growth promoters had some unintended negative effects for livestock (Casewell et al., 2003). Diseases that had been suppressed by the growth promoters, such as clostridial necrotic enteritis in poultry, became major health concerns in these countries following the bans (Casewell et al., 2003). Also, there is speculation that in some ways human health may be adversely affected due to the ban on antibiotic growth promoters (Phillips, 2007). One potential human health issue lies in increased variation in carcass size associated

with cessation of use of growth promoting antibiotics (Phillips, 2007). Greater variability in carcass size increases the risk of digestive tracts of food animals being broken open by machinery used to process carcasses, spilling bacteria on to the meat destined for human consumption (Phillips, 2007). However the actual impact of this variation in carcass size on contamination of meat has not been fully evaluated (Hammerum et al., 2007).

In August of 2009, the U.S. FDA announced new regulations for the amounts of antibiotics allowed in milk replacer (21CFR § 520.1484; 21CFR § 520.1660d). The previous regulation allowed neomycin and oxytetracycline to be fed continuously at a rate of 400 and 200 g/ton, respectively. The new regulations allow two different doses of antibiotics to be fed, one low for disease prevention (10-20 g/ton of neomycin and oxytetracycline, or ~10 mg/d of each for a 45.4 kg calf), and one higher for therapeutic use (1600 g/ton of neomycin and oxytetracycline supplying ~1000 mg/d). Long term effects of this change on animal health and antibiotic resistance of gut and fecal bacteria remain to be determined.

With growing concerns regarding antibiotic resistance, other options for disease control and growth promotion in livestock should be evaluated. Examples include prebiotics, more nutritionally dense milk replacers, and plasma-derived products (Quigley and Drew, 2000; Heinrichs et al., 2005).

Methods of Characterizing Antibiotic Resistance

Early research in bacterial resistance to antibiotics utilized classic bacterial isolation and identification techniques. These are useful for identifying known resistant organisms such as MRSA, but do not yield much information about relative resistances or the genetic basis for the resistance. A later development was the disc diffusion assay, in which an agar plate containing a small disc impregnated with the antibiotic of interest is used to determine an organism's 'zone of inhibition'. Organisms that were capable of growing closer to the impregnated disc were considered more resistant to the antibiotic than organisms that grew only farther away from the antibiotic disc. These methods provide insight on relative resistances of isolated organisms, but they are culture dependent, and give no information on the genetic basis for the resistance.

The analysis of ARG with quantitative polymerase chain reaction (qPCR) offers multiple benefits compared to earlier phenotypic approaches. First, with qPCR the genetic basis of the resistance can be easily determined, as can the relative abundance of the genes. This is important because multiple genes can encode for resistance to the same antibiotics. Also, ARG are often physically linked on mobile genetic elements, and be picked up by other organisms. Analysis with qPCR allows determination of these links. Perhaps most important, molecular techniques like qPCR are culture independent.

What Are These Genes?

The genes analyzed in the present experiment were chosen to examine the effects of feeding medicated milk replacer to calves on ARG related to antibiotics that were fed (*tet* genes), genes related to antibiotics that were not fed (*sul* and *erm* genes), and genes associated with transfer of ARG between organisms (class 1 integrons, *int*I).

Examples of tetracyclines include oxytetracycline, fed to the calves in the current experiment, tetracycline commonly used to treat acne, and doxycycline used to treat a variety of bacterial sexually transmitted diseases. The tetracycline genes evaluated make the bacteria resistant to the antibiotic through different mechanisms. For instance, *tet*C and *tet*G allow for antibiotic resistance through tetracycline efflux pumps, allowing the bacteria to pump the antibiotic out of the cell. In contrast *tet*O and *tet*W give the organism ribosomal protection, protecting the ribosome from disruption of translation caused by tetracyclines. Finally, *tet*X codes for a NADP-requiring oxidoreductase enzyme, which modifies tetracycline antibiotics so that they are no longer toxic to the cell. The precise mechanism of this modification is currently unknown.

Erythromycin is a macrolide antibiotic similar to penicillin that is important to human health. It is commonly used for treating respiratory infections, but is not used in animal agriculture. While the calves were never exposed to erythromycin, two genes (*erm*B and *erm*F)

were found in feces samples; they confer resistance to erythromycin by methylating adenine at a specific point in the bacterial genome.

The genes *sul*I and *sul*II confer resistance to sulfonamides (a.k.a. sulfa drugs). Sulfadimethoxine is a sulfonamide commonly used in veterinary medicine to treat respiratory and soft tissue infections, and to control coccidiosis in some species. These genes give the organism a sulfonamide-resistant dihydropteroate synthase. The dihydropteroate synthase is naturally occurring in microorganisms, and is involved in folate synthesis. When a sulfonamide antibiotic is applied it inhibits this enzyme, "starving" the organism of folate, causing it to die. Bacteria expressing the *sul*I and *sul*II genes can resist this effect, allowing normal enzyme function. *Sul*I is also typically associated with class 1 integrons.

Finally, *int*I is not an ARG but is a gene that codes for class 1 integrons. These are mobile genetic elements that can be passed among organisms, allowing them to gain resistance to antibiotics.

Summary and Objectives

The concerns over antibiotic use in livestock and potential antibiotic resistance created and maintained in the gastrointestinal tracts of food animals are increasing. Antibiotics can cause damaging environmental effects, but they are important to the livestock industry because they treat and prevent common diseases, and increase rate and efficiency of growth. While it is widely

accepted that medically important antibiotics should not be used subtherapeutically for livestock production, co-selection of ARG may perpetuate resistance to banned medically important antibiotics with use of other, non-medically important, antibiotics. To evaluate the effect of subtherapeutic use of antibiotics on genetic resistance to those and other antibiotics, more research is needed quantifying the excretion of ARG. Therefore, the objectives of this research were to

1. determine the effect of newly mandated doses of antibiotics in medicated milk replacers on calf growth and health, and abundance of ARG in feces from calves fed milk replacer, and
2. determine changes in abundance of ARG in feces from calves fed milk replacer over time as they transition to weaning.

Chapter 2: Excretion of Antibiotic Resistance Genes by Dairy Calves

Abstract

Twenty-eight Holstein and crossbred heifer and bull calves were used to evaluate the effect of milk replacer antibiotics on abundance of selected antibiotic resistance genes (ARG) in the feces. Calves were blocked by breed, gender, and birth order, and assigned to one of three treatments at birth. Treatments were control (containing no antibiotics in the milk replacer), subtherapeutic (neomycin sulfate and oxytetracycline hydrochloride each fed at 10 mg/calf/d), and therapeutic (no antibiotics in the milk replacer until d 36, then neomycin sulfate and oxytetracycline hydrochloride each fed at 1000 mg/calf/d for 14 d). Calves were fed milk replacer twice daily at 0600 h and 1800 h. Fecal and respiratory scores and rectal temperatures were recorded daily. Calves were weighed at birth and weaning to calculate average daily gain. Beginning at six weeks of age fecal grab samples were collected from heifers at 0600 h, 1400 h, 2000 h, and 2400 h for 7 d, while bull calves were placed in metabolism crates for collection of all feces and urine. DNA was extracted from feces, and ARG corresponding to the tetracyclines (*tet*C, *tet*G, *tet*O, *tet*W, and *tet*X), macrolides (*erm*B, *erm*F), and sulfonamides (*sul*1, *sul*2) classes of antibiotics along with the class I integron gene, *int*I1, were measured by quantitative polymerase chain reaction (qPCR). No *tet*C or *int*I was detected. There was no significant effect

of antibiotic treatment on the absolute abundance (gene copies/ g wet manure) of any of the ARG except *erm*F, which was lower in the antibiotic-treated calf manure probably because host bacterial cells carrying *erm*F were not resistant to tetracycline or neomycin. All ARG except *tet*C and *int*I were detectable in feces from 6 weeks onwards, and *tet*W and *tet*G significantly increased with time ($P < 0.10$), even in control calves. Overall, the majority of ARG analyzed for were present in the feces of the calves regardless of exposure to dietary antibiotic. Feed antibiotics had little effect on the ARG monitored; other methods for reducing the global ARG pool should also be investigated.

Key words: antibiotic, antibiotic resistance gene, dairy calves

Introduction

Antibiotics are fed to livestock at subtherapeutic levels for both growth promotion and disease prevention because their use reduces morbidity and mortality, but livestock are often cited as a potential reservoir for antibiotic resistant bacteria and ARG. The unintentional selection of bacteria resistant to powerful antibiotics could have devastating human health consequences. However, animal welfare considerations must also be included in the discussion of appropriate antibiotic use for livestock. Sub-therapeutic use of antibiotics was banned in the European Union in the late 1990s and decreased antibiotic resistance in some bacteria was immediately observed (Casewell et al., 2003) but negative impacts on animal health and survival were also reported.

In the dairy industry, the most common sub-therapeutic use of antibiotics is neomycin and oxytetracycline in milk replacer for young calves. Neomycin prevents and treats scours, because it is not absorbed by the body of the animal. Instead, it remains in the digestive tract of the calf, where it controls growth of pathogenic organisms. Oxytetracycline, in contrast, is absorbed by the body of the calf, and is responsible for prevention and treatment of respiratory illnesses. Based on concerns regarding human health effects, the FDA issued new rules limiting antibiotic use in milk replacers for calves in 2009 (21CFR § 520.1484; 21CFR § 520.1660d). The objectives of this research were to determine the effect of newly mandated doses of

antibiotics in medicated milk replacers on calf growth and health and abundance of ARG in feces from calves fed milk replacer, and to determine changes in abundance of ARG in feces from calves fed milk replacer over time as they transition to weaning.

Materials and Methods

Experimental Treatments

Forty-one newborn calves were blocked according to breed (Holstein or crossbred), birth order, and gender. Crossbred calves were either Holstein- or Jersey-sired and out of Jersey x Holstein, Jersey x Holstein x Brown Swiss, or Jersey x Holstein x Swedish Red dams. Within block, calves were randomly assigned to treatments at birth. Treatments were control (containing no antibiotics in the milk replacer), subtherapeutic (neomycin sulfate and oxytetracycline hydrochloride each fed at 10 mg/calf/d provided from d 1 until weaning), and therapeutic (no antibiotics in the milk replacer until d 36, then neomycin sulfate and oxytetracycline hydrochloride each fed at 1000 mg/calf/d for 14 d).

Newborn Processing

Within 2 h after birth, calves were moved from the calving pen to individual stalls in an enclosed barn. Calf navels were dipped in a 7% tincture of iodine, and calves were vaccinated intranasally with Nasalgen (Merck Animal Health, Millsboro, DE). Birth date, BW, and identity of the dam were recorded. Calves received 1.89 L of thawed colostrum as soon as possible after

birth, usually within 2 h. Colostrum was frozen in 1.89 L bottles and thawed before feeding to the calves. When collected, colostrum was scored with a colostrometer, and all calves received colostrum scored green. A second feeding of 1.89 L of colostrum was administered 6 h after the first feeding and calf navels were dipped a second time.

Feeding and Housing

Calves were enrolled and treatments were imposed at 1 d of age. Calves were fed milk replacer (Cow's Match, Land O'Lakes Animal Products Co., Arden Hills, MN) twice daily individually via nipple buckets, at 0600 and 1800 h. Milk replacer contained 28% CP and 20% fat, with whey protein as the protein source and human grade edible lard as the fat source. Milk replacer was mixed in 19 L batches of a 17.6% DM solution with powder measured into the mixing bucket and water added to bring the milk replacer up to volume. The mixture was thoroughly combined by hand using a large whisk. Amount of liquid fed provided 0.68 to 1.1 kg of DM per calf, depending upon birth weight, resulting in feeding 2.1 to 3.4 times more protein and 1.5 to 2.4 times more fat than traditional feeding programs. This feeding rate has been termed "intensive feeding" and is higher than conventional calf feeding programs, which deliver 0.454 kg of milk replacer DM containing 20% fat and 20% protein. The intensive feeding program approaches amounts calves would consume if allowed free access to liquid diets.

Antibiotic treatments were added directly to the nipple buckets, which were used exclusively for a specific treatment to avoid cross-contamination. Calves were supervised during feeding, and 15 min was allowed for consumption. Any refusals were weighed and recorded. After feeding, all equipment was thoroughly cleaned. Calves were fed starter grain (Intensity - 22% CP, 10.13 mg/kg of monensin, Southern States Cooperative, Richmond, VA) beginning at 1 d of age. Calves were initially fed 0.22 kg of starter grain, and the amount offered increased in 0.22 kg increments as consumption increased.

Calves were housed at the Virginia Tech Dairy Cattle Center in individual fiberglass or plastic hutches (1.83 m x 1.37 m) with metal hog panels to create a fenced area (~2.5 m^2). At 39 d of age bull calves were moved in to metabolism crates to facilitate total feces and urine collection (described below). After the total collection period was complete, bull calves were moved back to their original hutches to be weaned. Heifers remained in hutches throughout the study.

Weaning was initiated at 50 d by reducing milk replacer offered by 50%, feeding only at 0600 h. When starter grain consumption reached 1.81 kg daily, calves were weaned (59 ± 2 d of age). Post-weaning, calves were housed in the calf hutches for seven days then were housed in small groups in a 3-sided barn with access to a dry lot. Weaned calves were group-fed first cutting alfalfa hay and the starter grain described above ad lib.

Data Collection

Following the 0600 h feeding, body temperatures, fecal scores (1-4), and respiratory scores (1-6; (Larson et al., 1977) were recorded daily. Calves were weighed at birth and at weaning to calculate average daily gain (ADG).

Feces and Urine Collection

Beginning at 42 d of age feces and urine samples were collected from heifer calves at 0600 h, 1400 h, 2000 h, and 2400 h for 7 d. Heifer calves were stimulated to urinate, and the sample was collected once a steady stream of urine began. Fecal samples were collected from the gravel surface if a fresh sample was available at collection time. If no fresh sample was available, calves were rectally stimulated to produce a fresh sample.

Beginning at 39 d of age bull calves were housed in metabolism crates (1.2 m x 0.6 m) to facilitate total collection. The first 3 d were for adaptation to the stall and 7 d of total collection followed. Trays were placed under the metabolism crates to collect excreted feces and urine. Ice packs were used in the urine collection trays to cool urine to minimize N loss. Urine was collected four times daily, weighed, and subsampled. Feces were collected daily, weighed, and subsampled.

After the total collection period, samples were collected from calves (now group-housed) weekly. Calves were restrained with rope halters and feces (from bulls and heifers, via rectal palpation) and urine (from heifers only, via stimulation as before) were collected.

Removed Calves

A total of 12 calves were removed from the study (3, 7, and 2 were on control, subtherapeutic, and therapeutic treatments, respectively), because of health problems. Health problems that were observed in calves in the current study were largely due to protozoan and viral microorganisms, which are not susceptible to antibiotics. Table 1 describes the number, gender, breed, and reason each of each animal removed.

Table 1. Calves removed from the experiment

Number of Animals	**Breed**	**Gender**	**Reason for removal**
3	Holstein	Bull	Died; severe scours and dehydration
1	Holstein	Bull	Born with bowed tendons; treated with casts; treatment was not complete in time for total collection in metabolism crate
1	Crossbred	Heifer	Respiratory issues; treated with antibiotics
2	Crossbred	Bull	Respiratory issues; treated with antibiotics
1	Crossbred	Bull	High fever, navel ill; treated with antibiotics
1	Holstein	Heifer	Bacterial overgrowth in the GI tract, bloat; treated with

			antibiotics
2	Holstein	Heifer	Severe scours which required antibiotic treatment
1	Holstein	Heifer	Severe scours after weaning; treated with antibiotics

Sample Analysis

Feces samples were frozen immediately at -20^{o} C after collection. Samples collected from heifer calves were thawed, pooled by date collected on an equal wet weight basis. (Pooling was not necessary for feces samples from bull calves because one sample was collected of each day's total excretion.)

DNA Extraction

DNA was extracted from 500 mg of wet feces per calf from week 6, 7 and 12 using the FastDNA® Spin Kit for soil and the FastPrep® instrument (MP Biomedicals, Santa Ana, CA). Samples were extracted in batches of 4 to 12 samples. Feces samples were allowed to thaw for 30 m to 1 h at 20°C, depending on sample size and texture, and added to a lysing matrix E tube. Next, 978 µl of sodium phosphate buffer and 122 µl MT buffer were added to the sample in the lysing matrix E tube. Samples were homogenized for 40 s at a speed setting of 6.0. After homogenization, samples were centrifuged at 14,000 x *g* for 15 m to pellet debris. The supernatant was transferred to a clean 2.0 ml microcentrifuge tube, and 250 µl of PPS (protein precipitation solution) were added to the supernatant. Contents of the tube were mixed, then tubes were centrifuged at 14,000 x *g* for 5 m, and the supernatant was transferred to a clean 2.0

ml microcentrifuge tube. The binding matrix suspension was resuspended by gently shaking the bottle and 1.0 ml was added to 750 µl of supernatant in each tube. Tubes were vortexed for 2 min to promote binding of DNA and then stood undisturbed for 3 min for settling of the silica matrix. When the silica matrix settled, 500 µl of supernatant was removed and discarded and the binding matrix was then resuspended in the supernatant remaining in the tube. 600 µl of the mixture was transferred to a SPIN™ filter (MP Biomedicals, Santa Ana, CA). The tubes containing the filters were centrifuged at 14,000 x *g* for 10 m to force the liquid through the filter. The catch tubes were emptied and 600 µl of the original mixture was added to the filter again. The process was repeated until all of the liquid passed through the filter (usually 2 to 3 cycles). Then, 500 µl of prepared SEWS-M (a salt/ ethanol wash solution) were added to the filter. The pellet on the filter was resuspended using the force of the SEWS-M from the pipette tip. The filter tubes were centrifuged at 14,000 x *g* for 5 m. The catch tubes were emptied, and the tubes were centrifuged at 14,000 x *g* again for 5 m without adding any liquid to dry the matrix of wash solution. The catch tube was replaced with a new, clean catch tube. Filters were dried for 5 m at room temperature. The pellet above the filter was resuspended in 100 µl of DES (DNase/ Pyrogen-Free Water). Tubes were centrifuged at 14,000 x *g* for 5 m to bring the eluted DNA into the clean catch tube. The spin filter was discarded. DNA was allocated in 20 µl aliquots. The aliquots were placed in 0.5 ml cryovials, which were stored in a -80˚C freezer until use for qPCR.

qPCR Analysis

The DNA extracts were analyzed for ten genes: *erm*B, *erm*F, *int*I, *sul*I, *sul*II, *tet*C, *tet*G, *tet*O, *tet*W, and *tet*X. The *tet* genes were chosen to represent all main categories of resistance (efflux pumps, ribosomal protection, and degradation). The macrolide and sulfonamide genes were chosen to examine a variety of genes that the calves were not exposed to. Finally, *int*I was chosen to examine the presence of mobile genetic elements, and also because this gene is sometimes associated with *sul*I.

All supplies were sterilized using UV light prior to beginning the analysis. A 9 µl master mix containing 2.8 µl of sterile water, 0.6 µl of forward primer for the gene of interest, 0.6 µl of reverse primer for the gene of interest, and 5 µl of SsoFast EvaGreen mix for each well was prepared in a 2 mL centrifuge tube. Next, 9 µl of the mix was pipetted into each well of the qPCR plate. After the plate was prepared, 1 µl of diluted DNA extract was pipetted in triplicate into the qPCR plate. A dilution series qPCR was conducted for each gene and each sample matrix, through which it was determined that a 1:70 dilution provided the most consistent results across all samples. All qPCR were conducted in triplicate on a CFX-96 (Bio-Rad). Calibration curves were constructed from serial dilutions of positive controls over seven orders of magnitude. The qPCR plate was then sealed, and placed into the thermocycler. Positive controls were obtained from cloned PCR products of target genes verified by DNA sequencing. The

processes for the various genes are listed in Table 2. Gene copies for any samples with numbers of gene copies below the detection limit were recorded as 0 for the purposes of statistical analysis.

Table 2. PCR conditions for selected ARG analyses

*erm*B	95° C for 4 m, 94° C for 30 s, 63° C for 30 s. Decrement temperature by -1.0° C per cycle. 72° C for 30 s. Repeat 4 times. 94° C for 30 s. 58° C for 30 s, 58° C for 30 s, 72° C for 30 s. Plate read, and repeat 39 more times. Melt curve: 65° C to 95° C, with 0.5° C increment for 5 s, followed by plate read.
*erm*F	95° C for 4 m, 94° C for 30 s, 61° C for 30 s. Decrement temperature by -1.0° C per cycle. 72° C for 40 s. Repeat 4 more times. 94° C for 30 s, 58° C for 30 s, 72° C for 30 s. Plate read, and repeat 39 more times. Melt curve: 65° C to 95° C, with 0.5° C increment for 5 s, followed by plate read.
*int*I	98° C for 2 m, 98° C for 5 s, 66° C for 5 s. Plate read, and repeat 34 more times. Melt curve: 65° C to 95° C, with 0.5° C increment for 5 s, followed by plate read.
*sul*I	98° C for 2 m, 98° C for 5 s, 69.9° C for 5 s. Plate read and repeat 39 more times. Melt curve: 65° C to 95° C, with 0.5° C increment for 5 s, followed by plate read.
*sul*II	98° C for 2 m, 98° C for 5 s, 67.5° C for 5 s. Plate read, and repeat 39 more times. Melt curve: 65° C to 95° C, with 0.5° C increment for 5 s, followed by plate read. Melt curve: 65° C to 95° C, with 0.5° C increment for 5 s, followed by plate read.
*tet*C	98° C for 2 m, 98° C for 5 s, 70° C for 5 s. Plate read, and repeat 39 more times. Melt curve: 65° C to 95° C, with 0.5° C increment for 5 s, followed by plate read.
*tet*G	98° C for 2 m, 98° C for 5 s, 64.2° C for 5 s. Plate read, and repeat 39 more times. Melt

	curve: 65° C to 95° C, with 0.5° C increment for 5 s, followed by plate read.
*tet*O	98° C for 2 m, 98° C for 5 s, 50.3° C for 5 s. Plate read, and repeat 39 more times. Melt curve: 65° C to 95° C, with 0.5° C increment for 5 s, followed by plate read.
*tet*X	98° C for 2 m, 98° C for 5 s, 64.5° C for 5 s. Plate read, and repeat 39 more times. Melt curve: 65° C to 95° C, with 0.5° C increment for 5 s, followed by plate read.

Statistical Analysis

Fecal scores, respiratory scores, and body temperatures were analyzed using the Mixed procedure of SAS (9.2, 2008) with the model:

$$Y = \mu + D_i + T_j + B_k + G_l + BG_{kl} + TB_{jk} + TG_{jl} + e_{ijkl}$$

where:

μ = overall mean;

D = effect of day (i = 1 to 65)

T = effect of treatment (j = control, subtherapeutic, therapeutic);

B = effect of breed (k = crossbred, Holstein);

G = effect of gender (l = bull, heifer); and

e = error (interaction of day, treatment, breed, and gender).

Average daily gains were analyzed using the Mixed procedure of SAS (9.2, 2008) with the model:

$$Y = \mu + T_i + B_j + G_k + BG_{jk} + TB_{ij} + TG_{ik} + e_{ijk}$$

where:

μ = overall mean;

T = effect of treatment (i = control, subtherapeutic, therapeutic);

B = effect of breed (j = crossbred, Holstein);

G = effect of gender (k = bull, heifer); and

e = error (interaction of treatment, breed, and gender).

Because they were not normally distributed, ARG data were log transformed prior to statistical analysis to determine significance of main effects and interactions. Log-transformed values were used to calculate LSM. All gene data were analyzed using the Glimmix procedure of SAS (9.2, 2008) with the model:

$$Y = \mu + T_i + B_j + G_k + W_l + TB_{ij} + TG_{ik} + BG_{jk} + WT_{il} + WB_{jl} + WG_{kl} + e_{ijkl}$$

where:

μ = overall mean;

T = effect of treatment (i = control, subtherapeutic, therapeutic)

B = effect of breed (j = crossbred, Holstein);

G = effect of gender (k = bull, heifer);

W = effect of week (l = 6, 7, 12); and

e = error (interaction of treatment, breed, gender, and week).

After the log-transformed data set was used to determine significance, contrasts were performed to separate means for significant effects and interactions. All data are reported as LSM ± SE. Significance was determined at $P < 0.10$.

Results and Discussion

Growth and Health

There were no effects of treatment, breed or gender on ADG (1.30 ± 0.13), fecal scores (2.48 ± 0.17), respiratory scores (1.16 ± 0.04), or body temperature (102.2° ± 0.95; Table 3). The lack of effect of milk replacer medication on growth and health is in contrast to some observations (Quigley and Drew, 2000; Berge et al., 2006a; Stanton et al., 2010), probably because calves in the current study were fed a more nutrient-dense diet with higher intake than in most early research. Consequently, calves completing the study were relatively healthy and grew quickly. Langford et al. (2003) observed no effect of penicillin content of milk in calves fed ad libitum; they also attributed the lack of effect of antibiotic to the nutritional benefits to the calf of offering an unlimited supply of milk.

The lack of effect of treatment on fecal consistency is also contrary to published literature but, as is typical of calves fed intensive feeding programs, fecal consistency was relatively soft in all calves (mean 2.48 on a 4 point scale). Heinrichs et al. (2003) found that inclusion of neomycin and oxytetracycline in milk replacer increased the probability of normal fecal scores.

Also, Quigley et al. (1997) found that incidence of scours and number of days scouring were decreased in calves fed neomycin and oxytetracycline in the milk replacer. Donovan et al. (2002) found no differences in calves fed antibiotics or a probiotic supplement.

Also relevant to the lack of effect of treatment on growth and scouring in the current study is that every calf enrolled experienced a scour episode at some point during the trial. Unusual heat stress likely contributed to this pattern of wide spread scouring.

The lack of effect of orally fed antibiotics on respiratory health observed in the current study is typical (Casewell et al., 2003; Berge et al., 2009; Heinrichs et al., 2009). Beneficial effects on respiratory health are more often observed with long acting antibiotics such as tulathromycin (Stanton et al., 2010), or with therapeutic administration of an antibiotic such as florfenicol at the onset of symptoms (Scott, 2010; Felkel et al., 2011). Also, good ventilation at the site of the present study resulted in low incidence of respiratory disease regardless of treatment.

There is little published research on the relationship between antibiotics and body temperature in livestock. (Montgomery et al., 2009) found that rectal temperature was higher in heifers treated for apparent bovine respiratory disease than in healthy calves, but in cases where calves are infected with a scour-causing protozoon such as cryptosporidium they may or may not present with an increased body temperature (Lorenz, 2009).

Antibiotic Resistance Genes

Two of the analyzed genes (*tet*C and *int*I) were not detected in fecal samples of any calves. Four genes coding for resistance to tetracyclines were detected in feces (*tet*G, *tet*O, *tet*W, *tet*X; Figure 1) as were two genes coding for resistance to sulfonamides (*sul*I, *sul*II) and two genes coding for resistance to erythromycin (*erm*B, *erm*F). All calves had at least one of the *tet* genes present in the feces by week 12, and multiple ARG related to tetracyclines were present in nearly all of the fecal samples analyzed. Resistance genes associated with antibiotics not fed to calves were less abundant. About one third of the calves did not have any *sul*I present in their feces at each individual sampling point.

Feeding milk replacer with subtherapeutic or therapeutic doses of neomycin and oxytetracycline had no effect on the abundance of the ARG *tet*G, *tet*O, *tet*W, *tet*X, *sul*I, *sul*II, and *erm*B, but calves fed medicated milk replacers had reduced abundance of *erm*F as compared to control calves (Figure 3). This result was intriguing. Medicated milk replacer has been reported to decrease fecal shedding of *S. enterica* by dairy calves (Berge et al., 2006b). This could, in turn decrease the quantity of ARG being released in to the environment. Decreased fecal shedding of bacteria could explain the decreased numbers of gene copes of *erm*F with antibiotic feeding in the present study. Because the species of bacteria with erythromycin resistance being shed in the feces is unknown, it is probable that one of the two antibiotics fed in the milk replacer (neomycin

and oxytetracycline) were destroying the organism with resistance due to *erm*F. This would explain the control calves having greater gene abundance than calves fed the medicated milk replacers.

Abundance of the ARG analyzed was not different between genders or between breeds, but abundance of both *tet*G and *tet*W increased with time in all groups (Figure 1). For *tet*G, gene abundance increased between weeks 6 and 7, and stayed high through week 12. For *tet*W, the increase in abundance occurred during the period following weaning, and was detected at the d week 12 sampling point. Typically, antibiotic resistance in the gut bacteria increases in response to the dose of antibiotics to which the microbes are exposed (Langford et al., 2003). The increased abundance of both *tet*G and *tet*W with time was likely due to long term exposure of the gut microflora to these antibiotics.

Interaction of treatment with breed, gender, and week of treatment had no significant effects on gene abundance for the majority of the analyzed genes. The interactions of treatment and gender were significant for *tet*G (Figure 3) and *sul*I (Figure 4). Among control calves, heifers expressed *tet*G more than did bulls; that was reversed in the subtherapeutic group. There was no effect of gender on abundance of *sul*I among calves fed either of the medicated milk replacers, but among control calves, heifers had far more copies of *sul*I in their feces than did bulls.

The interaction of breed with gender was significant for *tet*G (Figure 5). Crossbred bulls had the higher abundance of *tet*G than crossbred heifers; there was no effect of gender among Holstein calves. The interaction of week and gender was significant for *sul*I. Heifers had higher abundance of *sul*I in day 42 and 49 than did bulls, but abundance of this gene was similarly low in bulls and heifers at 84 d (Figure 6). Biological explanations for these differences are not obvious. They may be attributed to small n among groups of calves.

Multiple drug resistance vs. abundance of multiple ARG

In the current study, 8 of the 10 target ARG were detected in the feces of the experimental calves. Fecal samples from all calves carried multiple types of ARG within the same sample. Also, all calves carried at least one gene coding for resistance to each the three classes of antibiotics of interest (tetracylines, sulfonamides, erythromycins). There is little published data related to the carriage of ARG by dairy calves, and little data examining the genetic basis for antibiotic resistance in the feces of young calves. Multiple antimicrobial resistance is not directly associated with therapeutic antibiotic use in calves (Berge et al., 2010) but is more likely to be encountered among calves than among adult cattle.

Class 1 integrons (*int*I), the mobile genetic elements, were not detected in any of the fecal samples in the current experiment. Class 1 integrons were detected in 10% of samples from scouring calves (Ahmed et al., 2009); calves on the current study were not diarrheic during

sample collections. That *int*I was not detectable but that multiple ARG were present in all calves is intriguing. It is plausible that the ARG identified were carried by different bacterial species in the feces sample, or the analyzed genes may be carried by the same bacterial species and located in proximity to each other on different genetic elements. Thus the abundance of multiple ARG in the same feces sample is not conclusive evidence of multiple drug resistance.

Conclusions

The lack of effect of antibiotic treatment on health measures is likely because calves on this experiment were closely monitored and fed a high plane of nutrition. In situations where management is not optimal, health differences may be more apparent.

Fecal abundance of genes related to tetracycline resistance increased over time regardless of treatment probably because tetracycline antibiotics were introduced into (or already present in) the environment, and even control calves were exposed to these antibiotics when they were handled. Genes related to antibiotics that were not fed were less abundant, but were still present in the feces. The timing of establishment of ARG in the intestine of newborn calves was not examined in this study but is of interest. These results suggest that techniques such as improved manure management and runoff prevention practices must be implemented to control the spread of ARG already present in the environment.

Table 3. Effects of treatment on various health and growth measures in calves fed medicated versus non-medicated milk replacers for 8 weeks

	Control[1]	Subtherapeutic[2]	Therapeutic[3]		*P* <		
				SE	Trt	Breed	Gender
n	12	7	9				
ADG, kg/d	1.4	1.3	1.3	0.13	0.46	0.23	0.46
Fecal score[4]	2.4	2.4	2.3	0.17	0.73	0.69	0.06
Respiratory score[5]	1.2	1.2	1.2	0.04	0.75	0.10	0.13
Body temperature,°	101.9	101.6	103.1	0.96	0.42	0.55	0.71

[1]Control milk replacer containing no antibiotics fed from d 2 to weaning

[2]Medicated milk replacer containing 10 mg/calf/d of tetracycline and neomycin fed from d 2 to weaning

[3]Medicated milk replacer containing 1000 mg/calf/d of tetracycline and neomycin fed from d 42 to weaning

[4]Feces scored from 1 (firm) to 4 (liquid; Larson et al., 1977).

[5]Respiration scored from 1 (normal) to 6 (cough and fever; Larson et al., 1977).

Table 4. Effect of milk replacer medication, breed, gender, week, and the interaction of milk replacer with these on abundance of selected antibiotic resistance genes in the feces of dairy calves

	Control[1]	Subtherapeutic[2]	Therapeutic[3]		*P* <				
	Gene copies/g wet feces (x 10^7)			SE (x 10^7)	Trt	Breed	Gender	Day	Interactions[4]
n	12	7	9						
*Tet*G	24.6	37.0	6.8	16.8	0.55	0.98	0.91	0.06	Trt*Gender (P<0.01); Breed*gender (P< 0.04)
*Tet*O	0.00012	1118	991	389	0.98	0.61	0.39	0.30	NS
*Tet*W	335	369	142	72.4	0.30	0.97	0.24	0.07	NS
*Tet*X	40.7	71.0	4.0	23.7	0.25	0.25	0.45	0.11	NS
Sul 1	1.7	0.0031	0.3	0.88	0.33	0.10	0.59	0.72	Trt*Gender (P<0.05); Gender*Week (P<0.06)
Sul 2	2.1	0.7	0.9	1.12	0.76	0.83	0.60	0.96	NS
*Erm*B	6.0	9.1	13.4	8.05	0.50	0.78	0.50	0.70	Trt*Breed (P<0.10)
*Erm*F	128.5	47.0	20.6	66.2	0.05	0.18	0.21	0.30	Trt*Breed (P<0.05)

[1]Control milk replacer containing no antibiotics fed from d 2 to weaning

[2]Medicated milk replacer containing 10 mg/calf/d of *tet*racycline and neomycin fed from d 2 to weaning

[3]Medicated milk replacer containing 1000 mg/calf/d of *tet*racycline and neomycin fed from d 42 to weaning

[4]Significant ($P < 0.10$) two and three way interactions of treatment, breed, gender and week

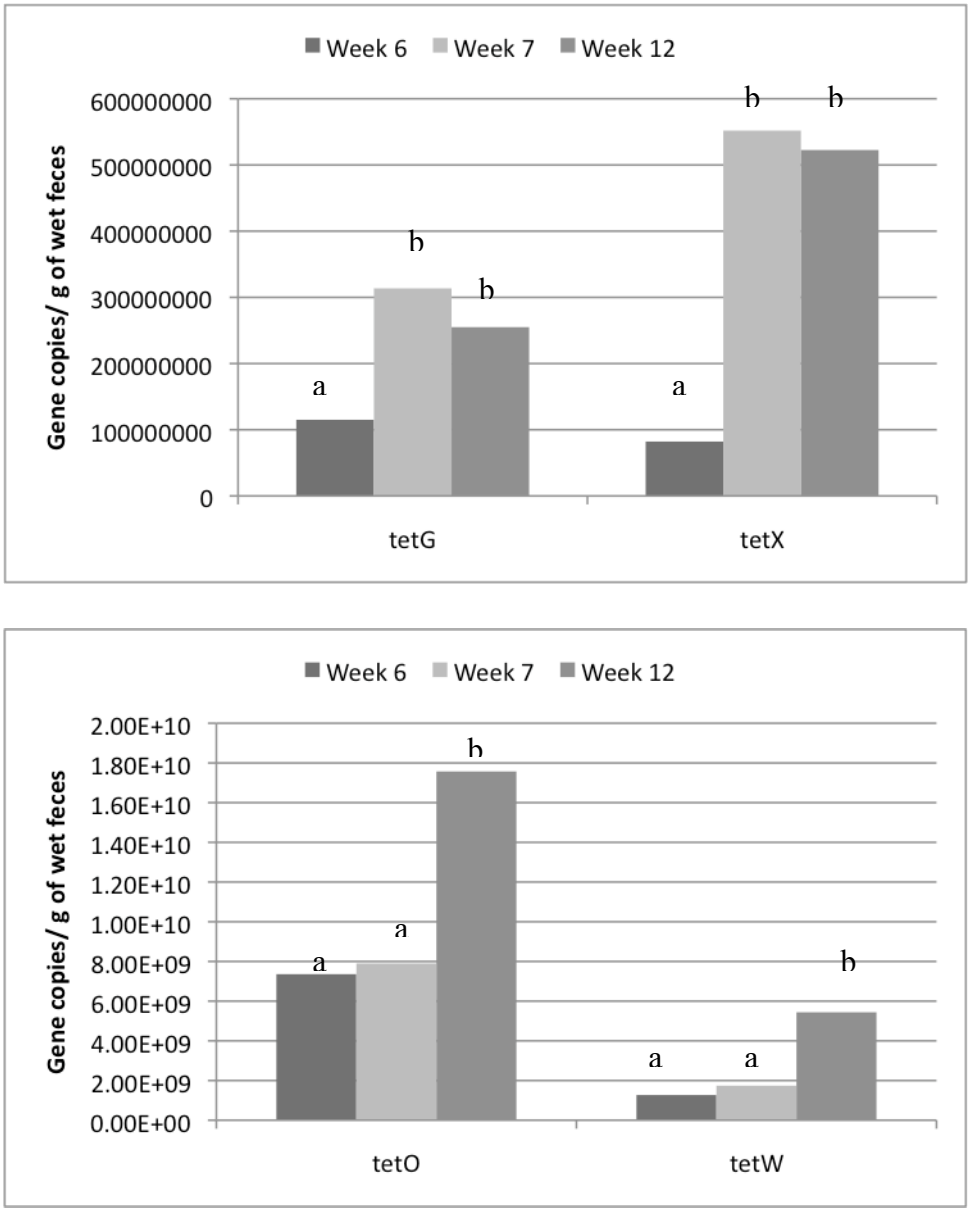

Figure 1. Effect of week on abundance of tetracycline ARG in feces of calves fed medicated or non-medicated milk replacers for 8 weeks.

Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

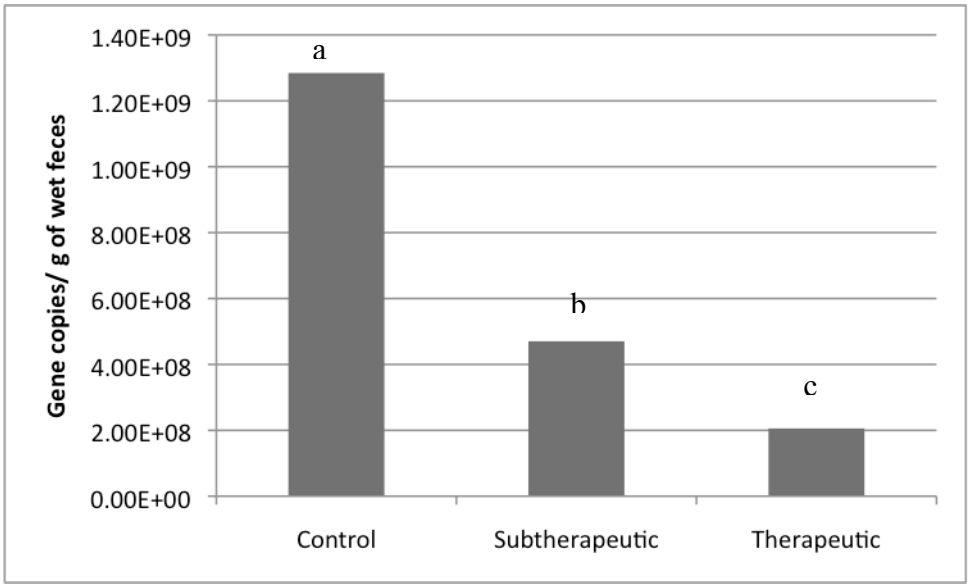

Figure 2. Effect of treatment on macrolide ARG *erm*F abundance in feces of calves fed medicated or non-medicated milk replacers for 8 weeks.

Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

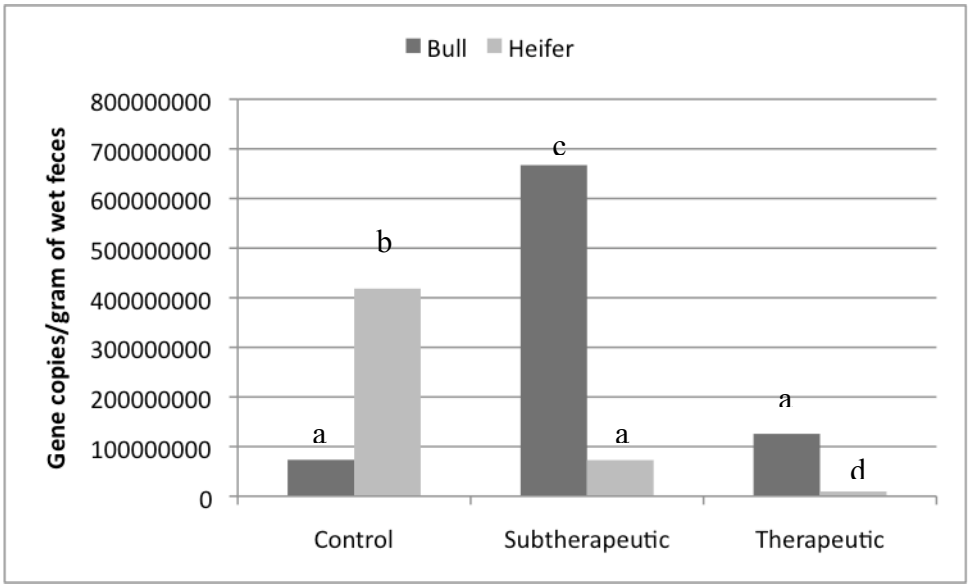

Figure 3. Effect of the gender by treatment interaction on abundance of tetracycline ARG *tet*G in feces of calves fed medicated non-medicated milk replacers for 8 weeks.

Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

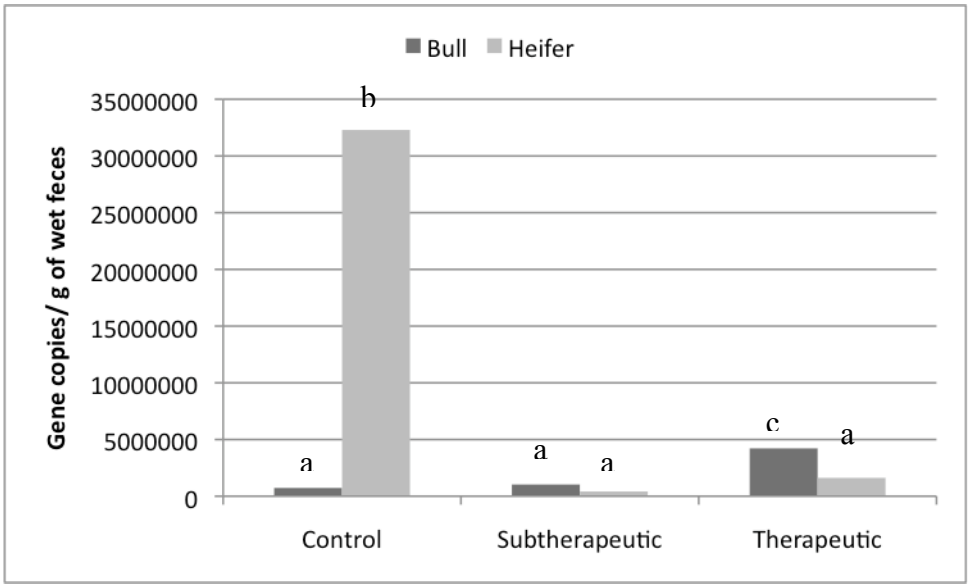

Figure 4. Abundance of sulfonamide ARG *sul*I in feces of calves fed medicated or non-medicated milk replacers for 8 weeks.

Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

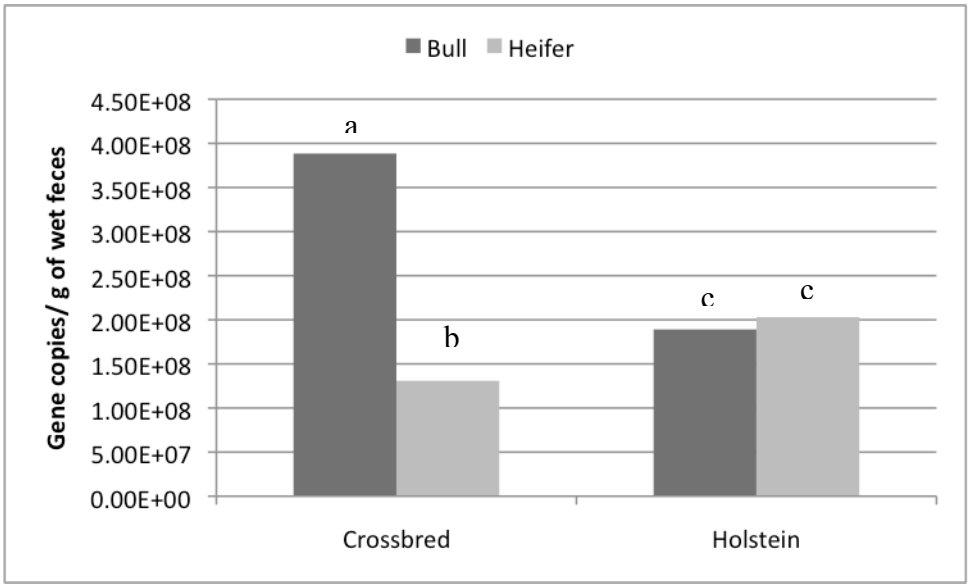

Figure 5. Effect of gender by breed interaction on abundance of *tet*G in feces of calves fed medicated or non-medicated milk replacers for 8 weeks.

Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

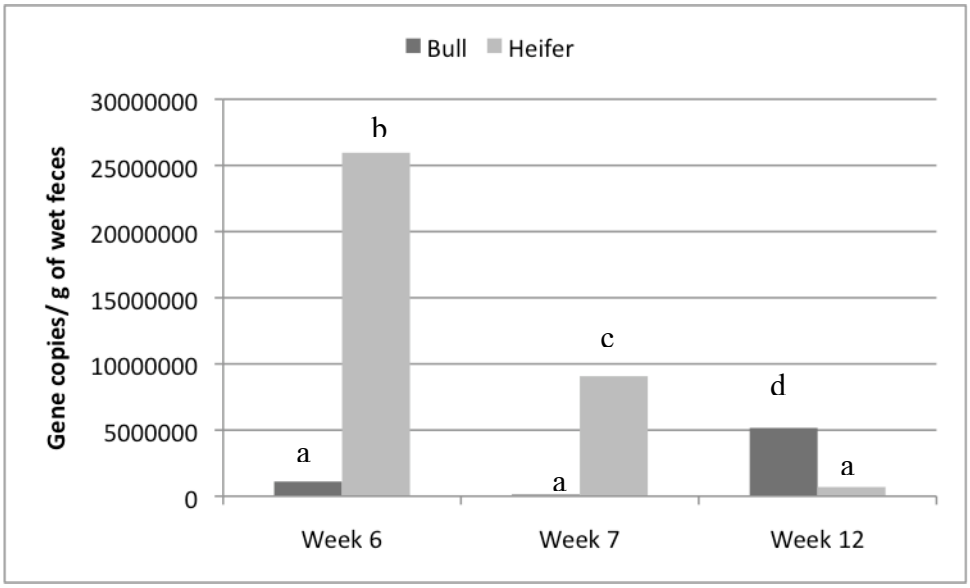

Figure 6. Effect of the gender by time interaction on abundance of sulfonamide ARG *sul*I in feces of calves fed medicated or non-medicated milk replacers for 8 weeks.

Letters above the bars indicate significantly different LSM groupings ($P < 0.10$).

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