EFFECTS OF DIETS, ANTIMICROBIALS AND MINERALS ON THE PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF FECAL BACTERIA IN FEEDLOT CATTLE

by

MEGAN E. JACOB

B. S., University of Wyoming, 2005

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2007

Approved by:

Co-Major Professor
T.G. Nagaraja

Approved by:

Co-Major Professor
Sanjeev Narayanan

Abstract

Antimicrobials are included in finishing cattle diets for growth promotion, feed efficiency, and protection against liver abscesses. The inclusion of in-feed antimicrobials at or below therapeutic concentrations may provide a selective pressure for antimicrobial resistant microorganisms. Additionally, heavy metals such as copper and zinc may be included in cattle diets because of growth-promoting effects. Heavy metal resistance genes are on transferable plasmids that also contain antimicrobial resistance genes. The objectives of this research were to 1) determine the prevalence of food-borne pathogens, *Salmonella* and *E. coli* O157, in cattle fed diets with or without monensin and tylosin and 0 or 25% wet corn distiller's grains (WDGS), 2) determine the prevalence of food-borne pathogens in cattle fed elevated concentrations of copper and zinc 3) evaluate the effect of antimicrobials on antimicrobial susceptibility of food-borne pathogens and commensal fecal bacteria, and 4) determine a possible association between in-feed antimicrobials and the concentration of antimicrobial resistance genes in the feces of cattle. Inclusion of 25% WDGS was associated with a higher prevalence of *E. coli* O157 on one of two sample collection days; however, there was no association between the use of monensin and tylosin, or copper and zinc on the prevalence of food-borne pathogens. Including monensin and tylosin in cattle diets was associated with an increased resistance of enterococci to macrolides, but was not related to concentration of the common macrolide resistance gene, *erm*B. In cattle fed diets with copper and/or zinc, no differences were observed in antimicrobial susceptibility or the concentration of antimicrobial resistance genes. In conclusion, results indicate that including growth-promoting antimicrobials in cattle diets at below therapeutic concentrations only limitedly impacted antimicrobial susceptibility and concentration of fecal antimicrobial resistance genes; however, this research encompassed only a select number of microorganisms. The positive association between WDGS and *E. coli* O157 prevalence in cattle has important implications for food safety, and warrants further investigation.

Table of Contents

List of Figures

List of Tables

Acknowledgements

My gratitude is extended to everyone who worked extremely hard to expose me to new concepts and techniques. These projects are the accumulation of efforts by many people. I would like to thank Dr. T. G. Nagaraja, Dr. Sanjeev Narayanan, Dr. Dave Renter, and Dr. Jim Drouillard for their guidance, advice, and patience. Neil Wallace, Xiaorong Shi, Heath Ritter, Trent Fox, and David George have also provided valuable contributions to these projects. This work would not be possible without the personnel at the K-State Beef Cattle Research Center and the College of Veterinary Medicine Pre-harvest Food Safety Laboratory. Finally, I would like to thank my family for continued support of me and my desire to learn.

CHAPTER 1 - Literature Review

Introduction

Antimicrobials are natural or synthetic compounds that inhibit or kill the growth of microorganisms by targeting cellular processes (Walsh, 2003; Giguère, 2006; Guardabassi and Courvalin, 2006). The Clinical and Laboratory Standards Institute (CLSI) and others have defined different applications of antimicrobial use in food animals that include therapy, prophylaxis, and growth promotion (USDA APHIS/VS/CEAH/CEI, 1999; CLSI, 2002). Therapeutic antimicrobial application involves administration of a compound to an animal(s) with apparent clinical disease. Prophylaxis is defined as antimicrobial administration to animals considered at risk for disease, without the identification of an etiologic agent. Finally, antimicrobials for growth promotion are given over time to improve physiologic performance and usually are administered as feed additives. Importantly, growth promoting antimicrobials are administered to food animals at smaller doses than therapeutic antimicrobials (USDA APHIS/VS/CEAH/CEI, 1999; Barton, 2000) to achieve the desired effects. Although growth promoting antimicrobials frequently are administered in-feed, therapeutic and prophylactic antimicrobials may be administered through the feed or through other routes such as injection (Schwarz and Chaslus-Dancla, 2001; McEwen and Fedorka-Cray, 2002). The focus of this review is to evaluate the effect of non-therapeutic, in-feed antimicrobial use on the susceptibility of fecal commensal and food-borne bacterial species from feedlot cattle.

Feedlot cattle are given antimicrobials in feed to prevent disease, increase feed efficiency and rate of weight gain, and/or to protect against liver abscesses (Nagaraja and Chengappa, 1998; NRC, 1999; McEwen and Fedorka-Cray, 2002; 2007 Feed Additive Compendium, 2006). Many

different antimicrobials are labeled for feed additive use in feedlot cattle (Table 1.1; 2007 Feed Additive Compendium, 2006). Chlortetracycline administered to feeder calves considered "high risk" improved average daily gain and feed conversion compared to control calves (Gallo and Berg, 1995). Rogers et al. (1995) reported that adding virginiamycin to the diets of cattle fed high-grain finishing rations improved animal growth and performance with minimal impact on feed intake. Another feed additive, the ionophore laidlomycin propionate, also was shown to increase gain and feed conversion compared to cattle not fed the ionophore (Spires et al., 1990). Russell and Houlihan (2003) stated that ionophores can improve feed efficiency by up to ten percent.

The mechanism for increasing cattle growth efficiency with antimicrobial use is believed to be multifaceted; some antimicrobials may alter normal microbial populations or metabolic processes in the gastrointestinal system, others may suppress disease, while additional mechanisms may still be unknown (USDA APHIS/VS/CEAH/CEI, 1999; Gustafson and Bowen, 1997; Barton, 2000; Phillips et al., 2004). The performance-enhancing benefits of these antimicrobials are believed to be associated with changes in microorganisms confined to the gastrointestinal tract, demonstrated by the absence of growth promotion in germ-free animals (Shryock and Page, 2006). Furthermore, in addition to traditional antimicrobials, cattle may also be administered anabolic compounds or mineral supplements to improve feed efficiency and gain (NRC, 1999; 2007 Feed Additive Compendium, 2006; Sapkota et al., 2007).

When fed to feedlot cattle as a means of growth promotion, antimicrobials are usually administered at dosage levels below those recommended for therapeutic purposes (Barton, 2000; Russell and Houlihan, 2003). Several antimicrobials have medicated feed additive claims for improved growth rate and feed efficiency as well as for prevention of bacterial diseases (2007

Feed Additive Compendium, 2006). It is generally accepted that any use of antimicrobial compounds applies selective pressure for resistant organisms (Teuber et al., 1999; American Academy of Microbiolgy, 2002). There currently is debate within the scientific community regarding the use of low-level, growth promoting antimicrobials, the subsequent development of antimicrobial resistant bacterial populations, and the impact of these resistant populations on animal and human health (Barton, 2000; American Academy of Microbiolgy, 2002). Multiple studies have identified associations between in-feed, growth promotant or prophylactic treatment and antimicrobial susceptibility of fecal bacteria from food animals. *Enterococcus* isolates from broilers fed treatments with or without virginiamycin had decreased susceptibility towards a related human antimicrobial, quinupristin/dalfopristin (McDermott et al., 2005). Additionally, enterococci isolates recovered from pigs during an on-farm epidemiologic study reported more erythromycin resistance from farms feeding tylosin for growth promotion than from farms not feeding tylosin (Jackson et al., 2004). Aarestrup et al. (2001) have shown that removing growth promotants (i.e., avilamycin and tylosin) from animal feed in Denmark decreased the incidence of antimicrobial resistance towards related compounds in *Enterococcus* isolates from broilers and pigs. Interestingly, the prevalence of resistance from the previous study never reached zero; some isolates seemed to maintain resistance even after a span of five years (Aarestrup et al., 2001). Dairy bull calves were shown to have an increased number of fecal *Escherichia coli* isolates resistant to two or more antimicrobials when given prophylactic, in-milk neomycin sulfate and tetracycline HCl, compared to control calves (Berge et al., 2006). Another study reported that ionophore supplementation did not impact the antimicrobial susceptibility of fecal coliforms from beef calves assigned to treatments with and without lasalocid, with the exception of an ampicillin association from one of two treatment years (Edrington et al., 2006). This study

suggests that the selective pressure for antimicrobial resistance and the ability to impact human health is probably somewhat compound specific. Ionophores, bambermycins, and quinoxalines all have a unique modes of action and no human antibiotic analogue (Boerlin and White, 2006); the risk of detrimental effects to humans from ionophore resistance in food animals is likely low (Russell and Houlihan, 2003). Measuring the ability of any of these organisms to infect humans and have detrimental effects on human health has not been as widely reported and is likely difficult to accomplish.

The potential repercussions from growth promoting antimicrobial use is not a recent concern; several expert committees, most notably the Swann Committee in 1969, have raised concerns regarding growth promotant use, antimicrobial resistance, and human health (Gustafson and Bowen, 1997). In 2001, the American Academy of Microbiology held a colloquium attended by representatives of academia, industry, and government research. Participants agreed that the use of any antimicrobial creates a potential for antimicrobial resistance. The use of growth-promoting antimicrobials alone is not responsible for the reservoir of resistance (American Academy of Microbiology, 2002). Additionally, it was their opinion that it is not important to identify where antimicrobial resistance initiated, but more so, how it is maintained and amplified (American Academy of Microbiology, 2002).

Definition of Antimicrobial Resistance

An interesting aspect in the study of antimicrobial resistance is that the term "resistance" is difficult to define. According to Guardabassi and Courvalin in *Antimicrobial Resistance in Bacteria of Animal Origin* (2006), there are different definitions of resistance depending on microbiologic, clinical, biochemical, and genetic criteria. Prescott (2000) describes multiple interpretations of resistance that include a specific strain's relation to the total population or in

relation to the mean tissue concentration of an antimicrobial when administered at a normal dose and route. Generally, laboratory *in vitro* tests are used to assess antimicrobial susceptibility (or resistance) under controlled conditions. The susceptibility of a bacterial strain towards a particular antimicrobial can be measured by microbroth dilution minimum inhibitory concentration (MIC) or agar disk diffusion method (Watts and Lindeman, 2006). The MIC method determines the lowest concentration of an antimicrobial that will completely inhibit the growth of an isolate. Standardized breakpoints allow for labeling strains as resistant, susceptible, or intermediate (Guardabassi and Courvalin, 2006). An advantage to this procedure is that the strain can be compared to others and result in a distribution of MIC values for a bacterial species (Watts and Lindeman, 2006).

Molecular methods are also available to detect antimicrobial resistance. There are several reported advantages to detecting antimicrobial resistance genes with molecular techniques: results are often obtained quickly, molecular detection allows for determining a risk of resistance when a strain has MIC values near the breakpoint, and the distribution and diversity of different resistance mechanisms can be better assessed (Aarts et al., 2006). Molecular detection methods include PCR, real-time PCR, and microarray analyses (Aarts et al., 2006). A limitation to molecular detection is that a resistance gene may be present, but may not actually be induced to provide resistance. Another potential downfall to defining resistance through laboratory procedures (MIC determination or molecular methods) is the lack of validity regarding *in vivo* complexities. Location, dosage, route of administration and other complexities can impact *in vivo* conditions and the ability of the organism to resist an antimicrobial compound (Guardabassi and Courvalin, 2006).

Mechanisms for Bacterial Antimicrobial Resistance

There are two broad categories of bacterial antimicrobial resistance, intrinsic and acquired. Intrinsic resistance reflects a bacterial genus or species lacking an appropriate target or the permeability needed for inhibition by an antimicrobial, whereas acquired resistance is observed once a particular strain has undergone chromosomal mutations or acquired genes encoding resistance (Schwarz and Chaslus-Dancla, 2001; Schwarz et al., 2006). More specifically, there are at least three general mechanisms by which bacteria resist antimicrobial activity: reduced compound accumulation, enzymatic inactivation, and modification of the target (Schwarz and Chaslus-Dancla, 2001; Walsh, 2003; Poole, 2005; Schwarz et al., 2006; Depardieu et al., 2007).

Reduced antimicrobial accumulation is mediated by decreased intake or increased export of the compound (Schwarz and Chaslus-Dancla, 2001). Bacterial efflux mechanisms can be encoded by either chromosomal or plasmid genes, and often belong to one of five classes of efflux pumps (Poole, 2005; Depardieu, et al., 2007). In addition, efflux mechanisms can be non-specific, allowing for the export of multiple antimicrobial compounds, or they may be compound and/or class specific (Walsh, 2003; Poole, 2005; Depardieu, et al., 2007). Plasmids, which are transferable between bacteria, often contain genes for specific efflux-mediated antimicrobial resistance, while multi-antimicrobial exporters normally are contained within the host genome. The effects of chromosomal efflux pumps typically occur after increased expression of the pumps (Walsh, 2003; Depardieu, et al., 2007). Several *in vitro* studies with common food-borne pathogens have shown that multi-drug efflux pumps also can provide resistance to common biocides and traditional antimicrobials (Poole, 2005).

Enzymatic inactivation, like efflux mechanisms, can be coded for by genes in the host chromosome or in plasmids, gene cassettes, or transposons (Walsh, 2003; Schwarz et al., 2006).

There are several broad mechanisms for enzymatic inactivation, including degradation and chemical modification of the antimicrobial (Schwarz, et al, 2006). Antimicrobial inactivating enzymes have been found in both Gram positive and Gram negative bacterial species, including *Staphylococcus aureus* and *Escherichia coli* (Walsh, 2003; Schwarz et al., 2006). Perhaps the best reported and well known inactivating enzymes are the β-lactamases. These enzymes, of which several hundred have been discovered and classified into four classes, cleave the β-lactam ring of multiple antimicrobials (Walsh, 2003; Schwarz et al., 2006).

The final mechanism for antimicrobial resistance is target modification, which can occur chemically or through mutation or protection of the target site (Schwarz et al., 2006). An example of a target site modification is seen in macrolide, lincosamide, and streptogramin B co-resistance encoded for by *erm* genes from multiple bacterial genera (Schwarz et al., 2006). The *erm* genes encode rRNA methylases (mono- or di-), specific for a single adenine residue (position 2,058) conserved within 23S rRNA (Leclercq and Courvalin, 1991; Weisblum, 1995; Schwarz et al., 2006). In contrast to target modification, tetracycline resistance frequently is a result of ribosomal protection. Tetracycline protection involves proteins with homology to elongation factors, which are produced and interact with the ribosome, preventing tetracycline binding (Schwarz, et al., 2006). Both the *erm* genes and tetracycline protection proteins are encoded on genes that are present on transferable elements (Leclercq and Courvalin, 1991; Schwarz et al., 2006).

Transfer of Antimicrobial Resistance

Mobile genetic elements can be passed through bacteria by vertical or horizontal transmission (Schwarz and Chaslus-Dancla, 2001). Mobile resistance determinants likely originated in antibiotic-producing organisms before therapeutic or non-therapeutic use of

antimicrobials (Boerlin and White, 2006). Populations of antimicrobial resistant bacteria are expanded by the acquisition of these resistance genes through horizontal transmission (Teuber et al., 1999). One specific type of mobile element, resistance-encoding plasmids, can be passed between bacterial types as seen by the presence of Gram-positive resistance determinants expressed in Gram-negative organisms (Courvalin, 1994). Doucet-Populaire and others (1992) have shown that plasmid DNA containing known kanamycin resistance could be transferred from *Enterococcus faecalis* to *Escherichia coli* isolates in the gastrointestinal tract of germ-free mice. Plasmids containing the *erm*B and *tet*M genes have the ability to transfer between *Lactobacillus plantarum* and *Enterococcus faecalis* within the gastrointestinal tracts of germ-free rats (Jacobsen et al., 2007). Movement of plasmids or other mobile elements is not limited to commensal bacterial species. Resistance genes located on mobile elements also can disseminate between commensal and pathogenic organisms (Boerlin and White, 2006). A precise determination of the rate at which horizontal transfer occurs within the complex gastrointestinal system of cattle has not been examined.

Food-safety Implications

According to National Cattlemen's Beef Association, the United States produced more than 26 million pounds of beef in 2006 (http://www.beefusa.org/uDocs/cattlenumbersandmeatproduction.pdf). Furthermore, a 2005 publication reported that 88% of U. S. households consumed beef at least once in two weeks (http://www.beefusa.org/udocs/beefbytescomplete03-28-05.pdf). The beef industry is important to the American economy and consumer assurance of a safe food supply is vital to the beef industry. An aspect of food safety is the emergence in and dissemination of antimicrobial resistance from food-borne bacteria. As previously discussed, antimicrobial resistance is not

only a problem of treatment failure, but also resistance genes that disseminate rapidly between bacterial strains. The interaction of human activity (consumption of food, contact with animals, etc.) and zoonotic bacteria creates a potential mixing pot for the spread of antimicrobial resistance.

The following sections outline specific antimicrobial resistance information on food-borne pathogenic and commensal bacteria of feedlot cattle as they relate to antibiotics included in cattle feed. These bacteria may serve as indicators for the prevalence of resistance in animals administered non-therapeutic antibiotics, and have important ramifications for food safety. Resistance to tetracycline and a select number of additional antimicrobials frequently is reported without information regarding therapeutic or non-therapeutic antimicrobial administration. For purposes of this review, unless a publication specifically references therapeutic use, antimicrobial susceptibility for compounds used both therapeutically and as growth promotants will be reported. In studies not specifically designed to assess growth-promotant use on antimicrobial resistance, susceptibly results will be reported only for compounds available for in-feed use, as correlations between other antimicrobials would be speculative. Additionally, the susceptibility of many Gram negative pathogens toward in-feed antimicrobials with Gram positive spectrum (i.e. macrolides, streptogramins) are not reported. Most commonly, antimicrobial susceptibility results are reported using *in vitro* laboratory methods for MIC determination and isolates are labeled susceptible, intermediate, or resistant according to CLSI criteria, which may or may not be designed for such isolates.

Antimicrobial Susceptibility of *E. coli* O157

Escherichia coli O157 is a significant food-borne pathogen for humans. Ruminants, primarily cattle, serve as an important reservoir for the organism (Gyles, 2007). The

contamination of beef carcasses is correlated to the fecal and hide prevalence of *E. coli* O157 (Elder et al., 2000); therefore, the impact of in-feed antimicrobials on resistance in *E. coli* O157 is a food safety concern. Sheep experimentally inoculated with *E. coli* O157 were given diets containing monensin, laidlomycin propionate or bambermycin, and concentration and antimicrobial susceptibility of *E. coli* O157 were examined (Edrington et al., 2003). Susceptibility was unaffected by ionophore use and only minimally impacted by bambermycin. Unfortunately, similar studies have not been reported in feedlot cattle.

Meng and others (1998) determined the susceptibility profiles of *E. coli* O157 isolates obtained from cattle, ground beef, milk and humans to sixteen different antimicrobials. They reported that cattle isolates were more frequently resistant to antimicrobials compared to food product or human isolates, and 38.1% of cattle isolates were at least co-resistant to tetracycline and the most common resistance profile included streptomycin, sulfisoxazole, and tetracycline resistance. Schroeder et al. (2002) received 133 *E. coli* O157 isolates from cattle and observed that 20% were resistant to tetracycline, the antimicrobial for which the expression of resistance was most prevalent. A 2004 Saskatchewan study examined 131 *E. coli* O157 feedlot cattle isolates and found 12% were resistant to tetracycline and 8% of isolates were multi-drug resistant, including resistance to tetracycline (Vidovic and Korber, 2006). The authors state that while most feedlots from which isolates were collected fed monensin, they do not believe the ionophore contributed to susceptibility profiles. Galland et al. (2001) reported antimicrobial susceptibility results from *E. coli* O157 isolates from large-scale feedlots in southwest Kansas; although no feedlots reported the use of antibiotics for growth promotion or prophylaxis, 8% of PCR confirmed isolates were resistant to tetracycline. In addition, all *E. coli* O157 isolates from this study were classified as intermediate to tylosin and resistant to erythromycin. Finally,

although *E. coli* O157 is known to carry a plasmid, Lim et al. (2007) conducted a study with a human clinical plasmid deletion mutant and found identical susceptibility profiles between wild-type and mutant isolates.

Antimicrobial Susceptibility of *Salmonella enterica*

There are more than 2,500 *Salmonella* serotypes, and the antimicrobial susceptibility profile of cattle *Salmonella* isolates is serotype dependent (Dargatz et al., 2003). One study collected *Salmonella* isolates from pen floor samples in 73 feedlots from 12 states and found the most frequent antimicrobial to which isolates were resistant was tetracycline (35.9%; Dargatz et al., 2003). This study did not report the use of in-feed antimicrobials at any feedlot from which isolates were obtained. *Salmonella Newport* isolates obtained from the study carried more resistance than any other serotype. Similarly, a 2002 publication reported that 23.2% of *Salmonella* isolates from cattle feedlots were resistant to tetracycline and 5.7% were resistant to sulfamethoxazole, while less than 5% of the isolates were resistant to any other antimicrobial evaluated (Dargatz et al., 2002). This study collected 50 fecal samples from 100 feedlots as part of the National Animal Health Monitoring System's Cattle on Feed Evaluation (NAHMS-COFE). The most common multi-drug resistant isolates (1.9% of isolates) in this study had reduced susceptibility towards ampicillin, neomycin, sulfamethoxazole, tetracycline, and ticarcillin. Neither therapeutic nor growth-promoting antimicrobial use was reported. Fluckey et al. (2007) described a study with sixty feedlot steers that were fed diets containing monensin and tylosin. Cattle were sent to slaughter in groups of 20, and fecal, hide and carcass samples were collected before or at arrival. *Salmonella* isolates (n = 101) were resistant to at least one antimicrobial (97% of isolates) and the most common resistance was to sulfamethoxazole (96%) followed by streptomycin (17.6%). No control group was included in this study. Dealy and

Moeller (1977b) conducted an experimental challenge study with *Salmonella Typhimurium*. Twenty Holstein calves were assigned to two treatments, non-medicated or medicated with bambermycin, and five isolates from each calf were tested for susceptibility. Calves were obtained from an auction, and information on previous antibiotic treatments was not available. The *S. Typhimurium* prevalence was negatively associated with bambermycin inclusion and calves fed bambermycin had a larger number of isolates susceptible to streptomycin, oxytetracycline, and ampicillin compared to the non-medicated calves. Treatment did not impact resistance to any other antimicrobial analyzed

Antimicrobial Susceptibility of *Campylobacter* species

Multiple studies have analyzed the antimicrobial susceptibility of *Campylobacter* isolates from beef and diary cattle; most studies examined isolates from cattle in their natural environment and determined MIC for antimicrobials commonly used in the therapeutic treatment of *Campylobacter* gastroenteritis in humans. The species of *Campylobacter* influences the frequency of antimicrobial resistance; *C. coli* are more frequently resistant to antimicrobials than *C. jejuni* (Bae et al., 2005; Englen et al., 2005; Inglis et al., 2006). In addition, tetracycline appears to be an antimicrobial to which cattle *Campylobacter* isolates commonly express phenotypic resistance (Sato et al., 2004; Englen et al., 2005; Inglis et al., 2005; 2006).

Inglis et al. (2005) assessed the effect of in-feed antimicrobials on the prevalence and antimicrobial susceptibilities of two cattle *Campylobacter* species, and found tetracycline resistance in 5.3 and 10.7%, and erythromycin resistance in 0.8 and 10.1%, of *C. jejuni* and *C. hyointestinalis* isolates, respectively. In addition, the study indicated that feeding chlortetracycline significantly increased the carriage rate of tetracycline resistant *C. jejuni* while also increasing the carriage rate of erythromycin resistant *C. hyointestinalis* (Inglis et al., 2005).

The therapeutic use of tetracycline, macrolides, or other antimicrobials was not reported in that study. Bae et al. (2005) isolated *Campylobacter* species from fifteen farms where 2.9% of *C. jejuni* were resistant to erythromycin and isolates obtained from feedlot cattle accounted for 62% of the erythromycin-resistant strains. A study comparing *Campylobacter* prevalence and antimicrobial susceptibility between organic and conventional dairy herds found no isolates resistant to erythromycin while approximately 45% of isolates were tetracycline-resistant; farm type was not associated with tetracycline resistance (Sato et al., 2004). Finally, Englen and others (2005) reported antimicrobial susceptibility results from *C. coli* and *C. jejuni* isolates collected from U. S. feedlot cattle for the National Animal Health Monitoring System study. Erythromycin, azithromycin, and tetracycline had resistance frequencies of 0.9, 0.9, and 51.6% from randomly selected *Campylobacter* isolates (Englen et al., 2005).

Antimicrobial Susceptibility of *Listeria* species

Callaway et al. (2006) observed a *Listeria* species prevalence of less than 4% in feedlot cattle. The antimicrobial susceptibility of these isolates was not determined, and other studies examining *Listeria* prevalence in feedlot cattle are rare. *Listeria* species are known to carry and transfer plasmids that contain resistance genes to antibiotics used as non-therapeutic feed additives (Roberts et al., 1996; Charpentier and Courvalin, 1999). Srinivasan et al. (2005), obtained thirty-eight *L. monocytogenes* isolates from dairy cattle and their environment and found resistance (determined by MIC) to tetracycline in 45% of isolates and the presence of *tet*(A) in 32% of isolates. In the same study, all isolates were susceptible to the macrolide antimicrobial, erythromycin. A study in bison (Li et al., 2007) revealed antimicrobial resistance to several non-therapeutic, in-feed antibiotics including bacitracin (88.3%), tetracycline (18.6%), tylosin (2.3%) and the related erythromycin (1.2%). Previous works from poultry and cheese

isolates reported a high prevalence of tetracycline resistance and a low prevalence of erythromycin resistance (Facinelli et al., 1991; Roberts et al., 1996). A more recent European study showed *Listeria* isolates from a variety of food products had no resistance to tetracycline or erythromycin (Aarestrup et al., 2007).

Antimicrobial Susceptibility of commensal *E. coli*

The effect of growth-promoting antimicrobials on the susceptibility of commensal bacteria in feedlot cattle has been more extensively studied than the relationship with food-borne pathogens. Edrington and colleagues (2006) fed diets with or without lasalocid to two groups of calves over two years and found little differences in antimicrobial susceptibility of putative fecal coliforms between the two groups of calves. In year one of the study, coliforms from calves fed lasalocid were resistant to more antimicrobials than calves in the control group, and more isolates were resistant to ampicillin in the lasalocid group. Inclusion of lasalocid did not have a significant effect in year two. Neomycin susceptibility also was assessed in this study, and only two isolates (one per year) were resistant, both from ionophore-fed calves. Additionally, resistance to oxytetracycline and chlortetracycline was the most common, yet was unaffected by treatment. A preliminary study involving oral administration of neomycin to feedlot cattle observed that the percent of neomycin resistant isolates increased more in calves given the treatment and was associated with an increase in MIC of all aminoglycosides (Chichester et al., 2006). The long-term effect of neomycin treatment (only fed for three days) or the length of time that isolates remained resistant was not examined in this study. A study previously described (Dealy and Moeller, 1977a) challenged Holstein calves with *S. typhimurium* and fed diets with or without bambermycin. During collection for *S. typhimurium, E. coli* isolates were also obtained and screened for antimicrobial susceptibility. The non-medicated (no

bambermycin treatment) had a higher percentage of isolates resistant to streptomycin and oxytetracycline compared to the treatment group. No susceptibility differences were reported for ampicillin, neomycin or sulfa-antibiotics. More *E. coli* isolates were resistant to bambermycin in the treatment group compared to the control group.

Fluckey and others (2007) conducted a study that included sixty feedlot steers fed diets containing monensin and tylosin. A total of 267 commensal *E. coli* isolates collected from feces, hides, and carcasses before shipping and at the abattoir were evaluated for antimicrobial susceptibility. Isolates were highly resistant to sulfamethoxazole (79%) and less resistant to tetracyclines (13%). A factor analysis study was designed to assess relationships between the MIC values of seventeen antimicrobials in commensal *E. coli* isolates from feedlot cattle (Wagner et al., 2003). Of 1,737 *E. coli* isolates obtained from 360 fecal samples, 66% were susceptible to all antimicrobials evaluated. The study concluded that MIC values were linked between antimicrobials within the same class, and between groupings observed in other studies. Finally, an interesting study was conducted in neonatal Holstein calves ($n = 27$) which were randomly allocated to three treatments: no dietary supplement, dietary supplement with oxytetracycline, and dietary supplement without oxtetracycline (Khachatryan et al., 2006). Fecal samples were collected once per week for three months and *E. coli* isolates were evaluated for susceptibility to six antimicrobials. The three treatments did not affect the levels of antimicrobial resistance to any one compound except chloramphenicol; the calves fed no dietary supplement had a higher level of chloramphenicol-resistant *E. coli* isolates. The multi-drug resistant phenotype streptomycin, sulfadiazine and tetracycline were more prevalent in *E. coli* isolates from calves fed either supplement, but were not dependent on oxytetracycline use.

Antimicrobial Susceptibility of *Enterococcus* species

The antimicrobial susceptibility of fecal *Enterococcus* species has been extensively studied in food-systems of poultry and swine (Aarestrup et al., 2001; Butaye et al., 2001; De Leener et al., 2004), but such work has not been commonly reported for feedlot cattle. In pigs, tylosin use has frequently been associated with macrolide resistance in *Enterococcus* isolates (Davies and Roberts, 1999; Aarestrup et al., 2001; Jackson et al., 2004). Butaye et al. (2001) sampled *Enterococcus faecium* and *E. faecalis* isolates from pet and farm animals with unknown previous antimicrobial usage, and described their susceptibilities to growth-promoting antimicrobials. Ten ruminant *E. faecium* and 25 ruminant *E. faecalis* isolates were obtained and none were resistant to monenesin, however, tylosin resistance was frequent. Ampicillin resistance was not commonly reported among ruminant samples; however, the percent of ruminant isolates susceptible to oxytetracyline was 20 and 29% for each *Enterococcus* species, respectively (Butaye et al., 2001). A descriptive study by Thal and others (1995) collected 34 cattle *Enterococcus* isolates and found none were resistant to the antimicrobials evaluated (ampicillin, gentamicin, streptomycin, and vancomycin); no information on the nature of these isolates was reported.

Molecular techniques have been used to catalogue the presence of common macrolide resistance genes (primarily the *erm* genes) in animals and humans (Jensen et al., 1999). One of these genes, *erm*X, and later *erm*B, were first identified in *Arcanobacterium pyogenes*, primarily isolated from cattle, and have been associated with decreased macrolide susceptibility (Jost et al, 2003; 2004). These genes also have been identified in enterococci from human and pig origin (De Leener et al., 2004), and it is likely they are present in cattle enterococci isolates as well.

Susceptibility to Metals

Heavy metals such as copper and zinc are added to cattle diets because of their antimicrobial and growth-promoting benefits (Hasman et al., 2006). More specifically, copper has been associated with finishing cattle performance at concentrations less than 20 mg Cu/kg DM, after which performance is reduced (Engle and Spears, 2000). The National Research Council recommends inclusion of copper (10 mg/kg), and zinc (30mg/kg), along with other microminerals, in the diets of finishing cattle (NRC, 1996). Resistance to heavy metals including arsenic and copper is widespread and the genes are often present on plasmids (Shryock and Page, 2006). Previous work in swine receiving copper in-feed revealed a copper resistance gene (*tcr*B) linked to antimicrobial resistance genes (*erm*B and others) on a transferable plasmid (Hasman and Aarestrup, 2002). This study acknowledged that although cattle are fed a lower concentration of copper than swine or broilers, 16% of *Enterococcus faecium* isolates from calves were resistant to copper. Antimicrobial susceptibility results from these isolates were not available. No other studies on the impact of in-feed heavy metals on antimicrobial and metal resistance in cattle are available.

Conclusions

The United States feedlot cattle industry frequently takes advantage of growth-promoting antimicrobials included in finishing cattle feed at concentrations below those used therapeutically. The effect of these antimicrobials on the susceptibility of both commensal and food-borne bacterial species is heavily debated and has potentially important ramifications for food safety. Most of the current literature reporting on antimicrobial susceptibility fails to include background information regarding both therapeutic and non-therapeutic in-feed antimicrobial use, and does not necessarily apply breakpoints meaningfully. Importantly, in

most studies from cattle feedlots, the majority of food-borne pathogens or commensal organisms are susceptible to the many of antimicrobials included within *in vitro* testing panels. The most common antimicrobials for which resistance is reported often includes antimicrobials used in-feed (i.e., tetracyclines and macrolides), however, because these antimicrobials also are used for therapeutic purposes, it is difficult to assess the impact of low-dose concentration alone. Again, it is generally accepted that any antimicrobial use will be a selective pressure for antimicrobial resistance. Because in-feed antimicrobials provide a continuous selective pressure, it is not unreasonable to believe they have an effect on the susceptibility of bacteria with which they associate; studies examining the effect of removing in-feed antimicrobials on bacterial susceptibility from feedlot cattle are not abundant.

Table 1.1 Approved antimicrobial feed additives and their indications for use in finishing cattle[a]

Antimicrobial[b]	Indications for use
Bacitracin Methylene Disalicylate	Reduction in number of liver condemnations due to abscesses
Bacitracin Zinc	Increased rate of weight gain and improved feed efficiency
Bambermycins	Increased rate of weight gain and improved feed efficiency
Chlortetracycline	Increased rate of weight gain and improved feed efficiency
	Reduction in number of liver condemnations due to abscesses
	Control of bacterial pneumonia associated with shipping fever caused by *Pasteurella* spp. susceptible to chlortetracycline
Laidlomycin	Increased rate of weight gain and improved feed efficiency
Lasalocid	Increased rate of weight gain and improved feed efficiency
Monensin	Improved feed efficiency
Neomycin	Treatment and control of colibacillosis (bacterial enteritis) caused by *Escherichia coli* susceptible to neomycin
Oxytetracycline	Increased rate of weight gain and improved feed efficiency
	Reduction of liver condemnation do to abscesses
Tylosin	Reduction in incidence of liver abscesses in beef cattle caused by *Fusobacterium necrophorum* and *Arcanobacterium pyogenes*
Virginiamycin	Improved rate of weight gain and improved feed efficiency
	Reduction of incidence of liver abscesses

[a] Adapted from the 2007 Feed Additive Compendium.

[b] Does not include feed additive combinations or anti-parasitic compounds.

References

Aarestrup, F. M., S. Knochel and H. Hasman. 2007. Antimicrobial susceptibility of Listeria monocytogenes from food products. Foodborne Pathog. Dis. 4(2):216-221.

Aarestrup, F. M., A. M. Seyfarth, H. D. Emborg, K. Pedersen, R. S. Hendriksen and F. Bager. 2001. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. Antimicrob. Agents Chemother. 45(7):2054-2059.

Aarts, H. J. M., B. Guerra, and B. Malorny. 2006. Molecular methods for detection of antibiotic resistance: p 37-48. *In* F. Aarestrup, (ed.), Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press, Washington D.C.

American Academy of Microbiology. 2002. The role of antibiotics in agriculture. American Academy of Microbiology, Washington, D.C.

Bae, W., K. N. Kaya, D. D. Hancock, D. R. Call, Y. H. Park and T. E. Besser. 2005. Prevalence and antimicrobial resistance of thermophilic Campylobacter spp. from cattle farms in Washington State. Appl. Environ. Microbiol. 71(1):169-174.

Barton, M. D. 2000. Antibiotic use in animal feed and its impact on human health. Nutrition Research Reviews. 13:279-299.

Berge, A. C., D. A. Moore and W. M. Sischo. 2006. Field trial evaluating the influence of prophylactic and therapeutic antimicrobial administration on antimicrobial resistance of fecal Escherichia coli in dairy calves. Appl. Environ. Microbiol. 72(6):3872-3878.

Boerlin, P. and D. G. White. 2006. Antimicrobial resistance and its epidemiology. *In* S. Giguère, (ed.), Antimicrobial therapy in veterinary medicine, 4th ed. Blackwell Publishing, Ames, IA

Butaye, P., L. A. Devriese and F. Haesebrouck. 2001. Differences in antibiotic resistance patterns of Enterococcus faecalis and Enterococcus faecium strains isolated from farm and pet animals. Antimicrob. Agents Chemother. 45(5):1374-1378.

Callaway, T. R., T. S. Edrington, A. D. Brabban, J. E. Keen, R. C. Anderson, M. L. Rossman, M. J. Engler, K. J. Genovese, B. L. Gwartney, J. O. Reagan, T. L. Poole, R. B. Harvey, E. M. Kutter and D. J. Nisbet. 2006. Fecal prevalence of Escherichia coli O157, Salmonella, Listeria, and Bacteriophage Infecting E. coli O157:H7 in feedlot cattle in the Southern Plains region of the United States. Foodborne Pathog. Dis. 3(3):234-244.

Charpentier, E. and P. Courvalin. 1999. Antibiotic resistance in Listeria spp. Antimicrob. Agents Chemother. 43(9):2103-2108.

Chichester, L. M., D. J. Kunze, G. H. Loneragan, T. M. Platt, L. L. Proffit, and M. M. Brashearsl. 2006. Oral administration of neomycin to feedlot cattle and susceptibility of *E. coli* and *Enterococcus* spp. Proc. 11th ISVEE. Available at: http://www.sciquest.org.nz.

Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards). 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard-second edition (NCCLS document M31-A2). Vol. 22 No. 6. National Committee for Clinical Laboratory Standards, Wayne PA.

Courvalin, P. 1994. Transfer of antibiotic resistance genes between gram-positive and gram-negative bacteria. Antimicrob. Agents Chemother. 38(7):1447-1451.

Dargatz, D. A., P. J. Fedorka-Cray, S. R. Ladely, K. E. Ferris, A. L. Green and M. L. Headrick. 2002. Antimicrobial susceptibility patterns of Salmonella isolates from cattle in feedlots. J. Am. Vet. Med. Assoc. 221(2):268-272.

Dargatz, D. A., P. J. Fedorka-Cray, S. R. Ladely, C. A. Kopral, K. E. Ferris and M. L. Headrick. 2003. Prevalence and antimicrobial susceptibility of Salmonella spp. isolates from US cattle in feedlots in 1999 and 2000. J. Appl. Microbiol. 95(4):753-761.

Davies, R. and T. A. Roberts. 1999. Antimicrobial susceptibility of enterococci recovered from commercial swine carcasses: effect of feed additives. Lett. Appl. Microbiol. 29(5):327-333.

De Leener, E., A. Martel, A. Decostere and F. Haesebrouck. 2004. Distribution of the erm (B) gene, tetracycline resistance genes, and Tn1545-like transposons in macrolide- and lincosamide-resistant enterococci from pigs and humans. Microb. Drug Resist. 10(4):341-345.

Dealy, J. and M. W. Moeller. 1977. Effect of bambermycins on Escherichia coli and antibiotic resistance in calves. J. Anim. Sci. 45(6):1239-1242.

Dealy, J. and M. W. Moeller. 1977. Influence of bambermycins on Salmonella infection and antibiotic resistance in calves. J. Anim. Sci. 44(5):734-738.

Depardieu, F., I. Podglajen, R. Leclercq, E. Collatz and P. Courvalin. 2007. Modes and

modulations of antibiotic resistance gene expression. Clin. Microbiol. Rev. 20(1):79-114.

Doucet-Populaire, F., P. Trieu-Cuot, A. Andremont and P. Courvalin. 1992. Conjugal transfer of plasmid DNA from Enterococcus faecalis to Escherichia coli in digestive tracts of gnotobiotic mice. Antimicrob. Agents Chemother. 36(2):502-504.

Edrington, T. S., T. R. Callaway, K. M. Bischoff, K. J. Genovese, R. O. Elder, R. C. Anderson and D. J. Nisbet. 2003. Effect of feeding the ionophores monensin and laidlomycin propionate and the antimicrobial bambermycin to sheep experimentally infected with E. coli O157:H7 and Salmonella typhimurium. J. Anim. Sci. 81(2):553-560.

Edrington, T. S., M. L. Looper, S. E. Duke, T. R. Callaway, K. J. Genovese, R. C. Anderson and D. J. Nisbet. 2006. Effect of ionophore supplementation on the incidence of Escherichia coli O157:H7 and Salmonella and antimicrobial susceptibility of fecal coliforms in Stocker cattle. Foodborne Pathog. Dis. 3(3):284-291.

Elder, R. O., J. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie and W. W. Laegreid. 2000. Correlation of enterohemorrhagic Escherichia coli O157 prevalence in feces, hides, and carcasses of beef cattle during processing. Proc. Natl. Acad. Sci. U S A. 97(7):2999-3003.

Engle, T. E. and J. W. Spears. 2000. Effects of dietary copper concentration and source on performance and copper status of growing and finishing steers. J. Anim. Sci. 78(9):2446-2451.

Englen, M. D., P. J. Fedorka-Cray, S. R. Ladely and D. A. Dargatz. 2005. Antimicrobial

resistance patterns of Campylobacter from feedlot cattle*. J. Appl. Microbiol. 99(2):285-291.

Facinelli, B., E. Giovanetti, P. E. Varaldo, P. Casolari and U. Fabio. 1991. Antibiotic resistance in foodborne Listeria. Lancet. 338(8777):1272.

Fluckey, W. M., W. G. Loneragan, R. Warner and M. M. Brashears. 2007. Antimicrobial drug resistance of Salmonella and Escherichia coli isolates from cattle feces, hides, and carcasses. J. Food. Prot. 70(3):551-556.

Galland, J. C., D. R. Hyatt, S. S. Crupper and D. W. Acheson. 2001. Prevalence, antibiotic susceptibility, and diversity of Escherichia coli O157:H7 isolates from a longitudinal study of beef cattle feedlots. Appl. Environ. Microbiol. 67(4):1619-1627.

Gallo, G. F. and J. L. Berg. 1995. Efficacy of a feed-additive antibacterial combination for improving feedlot cattle performance and health. Can. Vet. J. 36(4):223-229.

Giguère, S. 2006. Antimicrobial drug action and interaction: an introduction. *In* S. Giguère, (ed.), Antimicrobial therapy in veterinary medicine, 4th ed. Blackwell Publishing, Ames, IA

Guardabassi, L. and P. Courvalin. 2006. Modes of antimicrobial action and mechanisms of bacterial resistance: p 1-18. *In* F. Aarestrup, (ed.), Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press, Washington D.C.

Gustafson, R. H. and R. E. Bowen. 1997. Antibiotic use in animal agriculture. J. Appl. Microbiol. 83(5):531-541.

Gyles, C. L. 2007. Shiga toxin-producing Escherichia coli: an overview. J. Anim. Sci. 85(13 Suppl):E45-62.

Hasman, H. and F. M. Aarestrup. 2002. tcrB, a gene conferring transferable copper resistance in Enterococcus faecium: occurrence, transferability, and linkage to macrolide and glycopeptide resistance. Antimicrob. Agents Chemother. 46(5):1410-1416.

Hasman, H., S. Franke, and C. Rensing. 2006. Resistance to metals used in agriculture production: p 99-114. *In* F. Aarestrup, (ed.), Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press, Washington D.C.

Inglis, G. D., T. A. McAllister, H. W. Busz, L. J. Yanke, D. W. Morck, M. E. Olson and R. R. Read. 2005. Effects of subtherapeutic administration of antimicrobial agents to beef cattle on the prevalence of antimicrobial resistance in Campylobacter jejuni and Campylobacter hyointestinalis. Appl. Environ. Microbiol. 71(7):3872-3881.

Inglis, G. D., D. W. Morck, T. A. McAllister, T. Entz, M. E. Olson, L. J. Yanke and R. R. Read. 2006. Temporal prevalence of antimicrobial resistance in Campylobacter spp. from beef cattle in Alberta feedlots. Appl. Environ. Microbiol. 72(6):4088-4095.

Jackson, C. R., P. J. Fedorka-Cray, J. B. Barrett and S. R. Ladely. 2004. Effects of tylosin use on erythromycin resistance in enterococci isolated from swine. Appl. Environ. Microbiol. 70(7):4205-4210.

Jacobsen, L., A. Wilcks, K. Hammer, G. Huys, D. Gevers and S. R. Andersen. 2007. Horizontal transfer of tet(M) and erm(B) resistance plasmids from food strains of Lactobacillus plantarum to Enterococcus faecalis JH2-2 in the gastrointestinal tract of gnotobiotic rats. FEMS Microbiol. Ecol. 59(1):158-166.

Jensen, L. B., N. Frimodt-Moller and F. M. Aarestrup. 1999. Presence of erm gene classes in

gram-positive bacteria of animal and human origin in Denmark. FEMS Microbiol. Lett. 170(1):151-158.

Jost, B. H., A. C. Field, H. T. Trinh, J. G. Songer and S. J. Billington. 2003. Tylosin resistance in Arcanobacterium pyogenes is encoded by an erm X determinant. Antimicrob. Agents Chemother. 47(11):3519-3524.

Jost, B. H., H. T. Trinh, J. G. Songer and S. J. Billington. 2004. A second tylosin resistance determinant, Erm B, in Arcanobacterium pyogenes. Antimicrob. Agents Chemother. 48(3):721-727.

Khachatryan, A. R., T. E. Besser, D. D. Hancock and D. R. Call. 2006. Use of a nonmedicated dietary supplement correlates with increased prevalence of streptomycin-sulfa-tetracycline-resistant Escherichia coli on a dairy farm. Appl. Environ. Microbiol. 72(7):4583-4588.

Leclercq, R. and P. Courvalin. 1991. Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. Antimicrob. Agents Chemother. 35(7):1267-1272.

Li, Q., J. S. Sherwood and C. M. Logue. 2007. Antimicrobial resistance of Listeria spp. recovered from processed bison. Lett. Appl. Microbiol. 44(1):86-91.

Lim, J. Y., H. Sheng, K. S. Seo, Y. H. Park and C. J. Hovde. 2007. Characterization of an Escherichia coli O157:H7 plasmid O157 deletion mutant and its survival and persistence in cattle. Appl. Environ. Microbiol. 73(7):2037-2047.

McDermott, P. F., P. Cullen, S. K. Hubert, S. D. McDermott, M. Bartholomew, S. Simjee and D. D. Wagner. 2005. Changes in antimicrobial susceptibility of native Enterococcus faecium in chickens fed virginiamycin. Appl. Environ. Microbiol. 71(9):4986-4991.

McEwen, S. A. and P. J. Fedorka-Cray. 2002. Antimicrobial use and resistance in animals. Clin. Infect. Dis. 34 Suppl 3:S93-S106.

Meng, J., S. Zhao, M. P. Doyle and S. W. Joseph. 1998. Antibiotic resistance of Escherichia coli O157:H7 and O157:NM isolated from animals, food, and humans. J. Food Prot. 61(11):1511-1514.

Nagaraja, T. G., and M. M. Chengappa. 1998. Liver abscess in feedlot cattle: a review. J. Anim. Sci. 76:287-298.

National Cattlemen's Beef Association. 2005. Retrieved September 20, 2007, from http://www.beefusa.org/udocs/beefbytescomplete03-28-05.pdf.

National Cattlemen's Beef Association. 2007. Retrieved September 20, 2007, from http://www.beefusa.org/uDocs/cattlenumbersandmeatproduction.pdf.

National Research Council (U.S.). Board on Agriculture. Panel on Animal Health Food Safety and Public Health. Committee on Drug Use in Food Animals. 1999. The use of drugs in food animals: benefits and risks. National Academy Press, Washington, D.C.

National Research Council (U.S.). Subcommittee on Beef Cattle Nutrition. 1996. Nutrient requirements of beef cattle. National Academy Press, Washington, D.C..

Phillips, I., M. Casewell, T. Cox, B. De Groot, C. Friis, R. Jones, C. Nightingale, R. Preston and J. Waddell. 2004. Does the use of antibiotics in food animals pose a risk to human

health? A critical review of published data. J. Antimicrob. Chemother. 53(1):28-52.

Poole, K. 2005. Efflux-mediated antimicrobial resistance. J. Antimicrob. Chemother. 56(1):20-51.

Prescott, J. F. 2000. Antimicrobial drug resistance and its epidemiology: page 28. *In* J. F Prescott, J. D. Baggot, and R. D. Walker, (ed.), Antimicrobial therapy in veterinary medicine. Iowa State Press, Ames, IA.

Roberts, M. C., B. Facinelli, E. Giovanetti and P. E. Varaldo. 1996. Transferable erythromycin resistance in Listeria spp. isolated from food. Appl. Environ. Microbiol. 62(1):269-270.

Rogers, J. A., M. E. Branine, C. R. Miller, M. I. Wray, S. J. Bartle, R. L. Preston, D. R. Gill, R. H. Pritchard, R. P. Stilborn and D. T. Bechtol. 1995. Effects of dietary virginiamycin on performance and liver abscess incidence in feedlot cattle. J. Anim. Sci. 73(1):9-20.

Russell, J. B. and A. J. Houlihan. 2003. Ionophore resistance of ruminal bacteria and its potential impact on human health. FEMS Microbiol. Rev. 27(1):65-74.

Sapkota, A. R., L. Y. Lefferts, S. McKenzie and P. Walker. 2007. What do we feed to food-production animals? A review of animal feed ingredients and their potential impacts on human health. Environ. Health Perspect. 115(5):663-670.

Sato, K., P. C. Bartlett, J. B. Kaneene and F. P. Downes. 2004. Comparison of prevalence and antimicrobial susceptibilities of Campylobacter spp. isolates from organic and conventional dairy herds in Wisconsin. Appl. Environ. Microbiol. 70(3):1442-1447.

Schroeder, C. M., C. Zhao, C. DebRoy, J. Torcolini, S. Zhao, D. G. White, D. D. Wagner, P. F.

McDermott, R. D. Walker and J. Meng. 2002. Antimicrobial resistance of Escherichia coli O157 isolated from humans, cattle, swine, and food. Appl. Environ. Microbiol. 68(2):576-581.

Schwarz, S. and E. Chaslus-Dancla. 2001. Use of antimicrobials in veterinary medicine and mechanisms of resistance. Vet. Res. 32(3-4):201-225.

Schwarz, S., A. Cloeckaert, and M. C. Roberts. 2006. Mechanisms and spread of bacterial resistance to antimicrobial agents: p 73-98. *In* F. Aarestrup, (ed.), Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press, Washington D.C.

Shryock, T. R. and S. W. Page. 2006. Growth promotion uses of antimicrobial agents. *In* S. Giguère, (ed.), Antimicrobial therapy in veterinary medicine, 4th ed. Blackwell Publishing, Ames, IA

Spires, H. R., A. Olmsted, L. L. Berger, J. P. Fontenot, D. R. Gill, J. G. Riley, M. I. Wray and R. A. Zinn. 1990. Efficacy of laidlomycin propionate for increasing rate and efficiency of gain by feedlot cattle. J. Anim. Sci. 68(10):3382-3391.

Srinivasan, V., H. M. Nam, L. T. Nguyen, B. Tamilselvam, S. E. Murinda and S. P. Oliver. 2005. Prevalence of antimicrobial resistance genes in Listeria monocytogenes isolated from dairy farms. Foodborne Pathog. Dis. 2(3):201-211.

Thal, L. A., J. W. Chow, R. Mahayni, H. Bonilla, M. B. Perri, S. A. Donabedian, J. Silverman, S. Taber and M. J. Zervos. 1995. Characterization of antimicrobial resistance in enterococci of animal origin. Antimicrob. Agents Chemother. 39(9):2112-2115.

Teuber, M., L. Meile, and F. Schwarz. 1999. Acquired antibiotic resistance in lactic acid

bacteria from food. Antonie van Leeuwenhoek. 76:115-137.

USDA APHIS/VS/CEAH/CEI. 1999. Antimicrobial resistance issues in animal agriculture. Ed. March 2007. Retrieved September 20, 2007 from www.aphis.usda.gov/vs/ceah/cei.

Vidovic, S. and D. R. Korber. 2006. Prevalence of Escherichia coli O157 in Saskatchewan cattle: characterization of isolates by using random amplified polymorphic DNA PCR, antibiotic resistance profiles, and pathogenicity determinants. Appl. Environ. Microbiol. 72(6):4347-4355.

Wagner, B. A., M. D. Salman, D. A. Dargatz, P. S. Morley, T. E. Wittum and T. J. Keefe. 2003. Factor analysis of minimum-inhibitory concentrations for Escherichia coli isolated from feedlot cattle to model relationships among antimicrobial-resistance outcomes. Prev. Vet. Med. 57(3):127-139.

Walsh, C. 2003. Antibiotics: actions, origins, resistance. Washington, D.C., ASM Press.

Watts, J. L. and C. J. Lindeman. 2006. Antimicrobial susceptibility testing of bacteria of veterinary origin: p 29-35. *In* F. Aarestrup, (ed.), Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press, Washington D.C.

Weisblum, B. 1995. Erythromycin resistance by ribosome modification. Antimicrob. Agents Chemother. 39(3):577-585.

2007 Feed Additive Compendium. 2006. T. Lundeen, (ed.), The Miller Publishing Co. Minnetonka, MN.

CHAPTER 2 - Effects of feeding wet corn distiller's grains with solubles with or without monensin and tylosin on the prevalence and antimicrobial susceptibilities of fecal food-borne pathogenic and commensal bacteria in feedlot cattle

INTRODUCTION

The use of feed antimicrobials in livestock is controversial because of the potential selection pressure for resistant organisms and the subsequent risk to human health (Gustafson and Bowen, 1997; Barton, 2000). Monensin and tylosin are widely used in cattle diets to improve feed efficiency and reduce liver abscesses (Stock et al., 1995; Nagaraja and Chengappa, 1998). Because of concerns with antimicrobial resistance, there is interest in producing food animals without the use of antimicrobial feed additives. Wet distiller's grains with solubles (WDGS) are a co-product of ethanol production from cereal grains and are comprised principally of the bran, protein, and germ fractions (Spiehs et al., 2002). The high fiber content in distiller's grain makes their primary use in ruminant diets (Ham et al., 1994; Lodge et al., 1997; Kleinschimt et al., 2006). During ethanol production, antimicrobials, such as penicillin and virginiamycin, are used to suppress bacterial contamination (Narendranath et al., 2000). It is generally believed that the distillation process destroys these antibiotics (Shurson, 2005). The high energy density in distiller's grains provides an opportunity to reduce dependency on feed antimicrobials while achieving similar growth promotion.

Food-borne pathogens, such as *Escherichia coli* O157 and *Salmonella*, are important to the beef industry. The effect of antimicrobial additives and other feed ingredients on the

prevalence of food-borne pathogens has implications for food safety. Our objectives were to determine the effects of feeding WDGS with or without monensin and tylosin on the prevalence and antimicrobial susceptibilities of *E. coli* O157 and *Salmonella* and commensal *E. coli* and *Enterococcus* spp., in feces of finishing cattle. We hypothesized that feeding distiller's grains and feed antibiotics to cattle will exert pressure for antimicrobial resistance in the gut bacteria and increase the concentration of antimicrobial resistance genes.

MATERIALS AND METHODS

Animals, Diets and Sampling Schedule

The study was conducted in the summer of 2005. Three hundred-seventy crossbred yearling heifers were allotted to 54 concrete-surfaced feedlot pens (5.2 m^2 per animal) with 6 to 7 animals per pen. Diets were fed for 150 d and consisted of 6% (DM basis) alfalfa hay and steam-flaked corn (SFC; Table 2.1). The study was a randomized complete block design with a 2×3 factorial treatment arrangement (9 pens per treatment). Wet corn distiller's grain with solubles was included at 0 or 25% (DM basis) and antimicrobial feed additives were the second factor, included as none, monensin (Rumensin®, 300 mg·animal^{-1}·d^{-1}) alone, or monensin and tylosin (300 mg·animal^{-1}·d^{-1} and Tylan®, 90 mg·animal^{-1}·d^{-1}, respectively). Pens were blocked in a series of six sequential pens and treatments were randomly allocated within each block. There were common fence lines between adjacent pens. Fecal samples were collected rectally from each animal on d 122 and d 136 of the finishing period, placed in sterile bags and transported to the laboratory immediately.

Bacteriological Procedures

Unless otherwise indicated, all culture media were Difco brand (BD, Sparks, MD). Each fecal sample was cultured for *E. coli* O157 (Greenquist et al., 2005). Briefly, 1 g of feces was

enriched at 37°C for 6 h in 9 mL Gram Negative (GN) broth with cefixime (0.5 mg/L), cefsoludin (10 mg/L), and vancomycin (8 mg/L). Immunomagnetic bead separation (IMS) was performed, followed by plating onto sorbitol-MacConkey agar with cefixime (0.5 mg/L) and potassium tellurite (2.5 mg/L; ctSMAC). Plates were incubated overnight at 37°C, and sorbitol negative colonies (up to 6) were picked and re-plated onto blood agar plates (BAP; Remel, Lenexa, KS). Following an overnight incubation at 37°C, colonies were tested for indole production and latex agglutination for the O157 antigen (Oxoid, Remel; Lenexa, KS). Species were confirmed using the API 20E kit (bioMériux, Hazelwood, MO) and PCR was used to determine the presence of two Shiga toxin genes, s*tx*1 and *stx*2 (Fagan et al., 1999). The multiplex PCR program to amplify targets was: initial denaturation at 95ºC for 3 min, 30 cycles of 95ºC for 20 sec, 58ºC for 40 sec, and 72ºC for 90 sec, and a final elongation at 72ºC for 5 min.

In addition, 5 g of fecal sample from each animal was pooled by pen. Pooled samples were then cultured for *E. coli* O157, *Salmonella*, and commensal *E. coli* by procedures adapted from Barkocy-Gallagher et al. (2002). Briefly, 10 g of feces were enriched in 90 mL of tryptic soy broth (TSB) for 2 h at 25°C, 6 h at 42°C, and overnight at 4°C, followed by anti-O157 IMS and identification as described above. For the isolation of generic *E. coli*, 50 µL of enriched TSB were plated onto MacConkey agar and incubated at 37°C for 24 h. Up to two morphologically distinct, lactose-fermenting colonies were picked and re-plated onto BAP. After overnight growth at 37°C, colonies were tested for indole production and citrate tubes were inoculated to examine citrate utilization. For the isolation of *Salmonella,* 10 mL of enriched TSB was inoculated to 90 mL of tetrathionate broth (TTB) and incubated 24 h at 37°C. One milliliter of TTB was subjected to anti-*Salmonella* IMS, followed by enrichment of 100 µL in 10

mL Rappaport-Vassiliadis (RV) broth. The RV broth was incubated at 42°C for 16-18 h and 50 µL was plated on both Hektoen enteric agar supplemented with novobiocin (15 mg/L) and brilliant green agar with sulfadiazine (Sigma-Aldrich). The plates were incubated overnight at 37°C; up to three isolates from each plate were picked and re-plated onto BAP for overnight growth and latex agglutination. Species of *Salmonella* were serogrouped and sent to NVSL laboratories (Ames, IA) for serotyping.

Enterococcus isolates were obtained by diluting 1 g of pooled fecal sample in 10 mL of PBS and plating 50 µL onto M-Enterococcus agar. After 24 h growth at 37°C, characteristic colonies (metallic pink and pin-point) were picked and plated on BAP. Following overnight growth at 37°C, isolates were inoculated in 100 µL Enterococcosel broth and incubated at 37°C for 4 h to test for esculin hydrolysis. A commercial kit (20Strep API; bioMériux, Hazelwood, MO) was used for genus confirmation. All bacterial isolates were stored at -80°C for further use.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility patterns of all isolates were determined by microbroth dilution using the Sensititre automated antimicrobial system (Trek Diagnostic Systems, Cleveland, OH). Minimal inhibitory concentrations (MIC; µg/mL) were determined for antimicrobials in the standard bovine clinical panel (BOPO-1F) for gram negative organisms. The antimicrobials and highest concentrations evaluated in the gram negative panel were: ceftiofur (8 µg/mL), erythromycin (4 µg/mL), chlortetracycline (8 µg/mL), florfenicol (8 µg/mL), penicillin (8 µg/mL), ampicillin (16 µg/mL), danofloxacin (1 µg/mL), sulphadimethoxine (256 µg/mL), neomycin (32 µg/mL), sulphachloropyridazine (256 µg/mL), tylosin tartrate (20 µg/mL), sulphathiazole (256 µg/mL), spectinomycin (64 µg/mL), tilmicosin (32 µg/mL), clindamycin (2 µg/mL), tiamulin (32 µg/mL), enrofloxacin (2 µg/mL),

trimethoprim/sulfamethoxazole (2/38 µg/mL), gentamicin (8 µg/mL), and oxytetracycline (8 µg/mL). *Enterococcus* spp. were tested for antimicrobial susceptibility with the gram positive National Antimicrobial Resistance Monitoring System (NARMS) panel (CMV1AGPF), which included: bacitracin (128 µg/mL), chloramphenicol (32 µg/mL), erythromycin (8 µg/mL), flavomycin (32 µg/mL), penicillin (16 µg/mL), daptomycin (16 µg/mL), quinupristin/dalfopristin (32 µg/mL), tetracycline (32 µg/mL), vancomycin (32 µg/mL), lincomycin (32 µg/mL), tylosin tartrate (32 µg/mL), ciprofloxacin (4 µg/mL), linezolid (8 µg/mL), nitrofurantoin (64 µg/mL), kanamycin (1,024 µg/mL), gentamicin (1,024 µg/mL), and streptomycin (2,048 µg/mL).

Quantification of Antimicrobial Resistance Genes

Real-time PCR was used to quantify specific antimicrobial resistance genes, *erm*B and *tet*M. To accurately determine the concentration of these genes, standard curves were first generated using known concentrations of plasmid vectors with the antimicrobial resistance gene inserted. The quantification of genes in the pen floor fecal samples was conducted using the standardized real-time PCR after DNA had been extracted with a commerical kit.

Bacterial Strains and Plasmids.

Enterococcus faecalis MMH594 (Dr. L. Zurek, Kansas State University) was used as a template to amplify an erythromycin resistant gene (*erm*BCT) and a 16S rDNA gene (EUB). These fragments were cloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA) and transformed into One Shot Top10 *E. coli* cells (Invitrogen). An *E. coli* isolate (Dr. L. Zurek, Kansas State University) containing pFD310 which carries tetracycline, erythromycin, and ampicillin resistant markers (Smith et al., 1992) was used to amplify the *tet*M gene.

Primer Design.

Primers were designed or modified using Integrated DNA Technology PrimerQuest software (Coralville, IA; Table 2.2). The degenerate primer used to amplify *erm*BCT (product size 404 bp; Jost et al., 2004) for cloning procedures was redesigned for real-time PCR analysis to more precisely target the *erm*B gene (product size 175 bp; this study).

PCR Running Conditions.

The running conditions for PCR reactions were as follows: initial denaturation of 3 min at 94°C, 36 cycles of 30 s at 94°C, 30 s of annealing (Table 2.2), and 30 s at 72°C, final elongation of 2 min at 72°C with *Taq* DNA polymerase (Promega, Madison, WI). Real-time PCR running conditions were identical for each gene with the addition of a 15 min, 95°C initial activation and an increase to 40 cycles.

Cloning Techniques.

Products from *erm*BCT, *tet*M and EUB PCR were purified with Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI) and cloned in pCRII-TOPO. Transformation was performed by heat shock into *E. coli* with plating onto Luria-Bertani agar with ampicillin (100 μg/mL) and 5-Bromo-4-Chloro-3-Indolyl-β-D-galactopyranoside (X-gal) (40 mg/mL). Plasmids were purified from the white colonies, using Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). The concentration of DNA was determined using a nanodrop spectrophotometer (Nanodrop ND 1000, Nanodrop Technologies, Wilmington, DE) and confirmed with *Ecor*I (Promega, Madison, WI) digestions and sequencing (Beckman-Coulter CEQ™8000 Genetic Analysis System).

Real-time PCR Standardization.

Purified plasmid samples were serially diluted ten-fold. Five microliters of plasmid sample were added to Absolute QPCR SYBR Green Mix (ABgene, Epsom, UK) for a 25 μL

reaction. Real-time PCR was performed according to the manufacturer's instructions. Reliable gene products were available between 1×10^8 molecules/μL and 1×10^3 molecules/μL for EUB, and between 1×10^9 molecules/μL and 1×10^3 molecules/μL for *erm*B. Dilutions of *tet*M were reliable between 7.35×10^8 molecules/μL and 7.35×10^2 molecules/μL.

Extraction of DNA from Fecal Samples.

Pooled fecal samples (180-220 mg) that were frozen at -80°C immediately after collection were thawed on ice and DNA was extracted using the QIAamp DNA stool mini kit according to manufacturer's directions (QIAgen, Valencia, CA).

Real-Time PCR on Fecal Samples.

Real-time PCR was performed on 108 fecal samples to target *erm*B, *tet*M and EUB genes. Melting curves were generated (0.5 increments) to test uniformity and singularity of products. Electrophoresis on 1.2% agarose gels, cloning in pCRII-TOPO, and sequencing were used to confirm the composition and correctness of randomly selected PCR end products. The specific log copy number of genes was computed using the standardization curves.

Statistical Analysis

The prevalence of *E. coli* O157, estimated as the proportion of positive samples per pen, was analyzed with a pen-level logit model to test for effect of diet (with or without distiller's grains), antimicrobial feed additive (no antimicrobial, monensin only, or monensin and tylosin), sampling day, and interactions using PROC GENMOD in SAS (v. 9.1; Agresti, 1996). The probability of detecting food-borne pathogens (*E. coli* O157 and *Salmonella* spp.) from pooled pen samples were analyzed using pen-level logistic regression models in PROC GENMOD with diet, antimicrobial, and day as effects.

The prevalence of resistance to antimicrobials in the susceptibility panels was evaluated for *Enterococcus* and generic *E. coli* isolates using pen-level logistic regression models as described above. Isolates reported as "intermediate" were considered susceptible for the analysis. Initially, univariate models were used to screen for the effect of day on resistance to each antimicrobial compound. Day was included in final multivariate regression models containing treatment effects if $P \leq 0.15$ (Dohoo et al., 2003). Type 3 likelihood ratio statistics were used to test for treatment effects ($P < 0.05$), and least-square means tests were used to separate probability estimates when significant effects were observed. The MIXED procedure was used to evaluate mean differences in the log copy number of *erm*B and *tet*M, with the copy number of EUB control target as a covariate in the model. Identical effects as above were examined.

RESULTS

Prevalence of E. coli O157 and Salmonella

The overall prevalence of *E. coli* O157 in fecal samples collected from individual animals was 9.1% (67 of 738). Prevalence on d 122 was 8.4% (31 of 371) and d 136 was 9.8% (36 of 367). The *stx*2 gene was present in 94.0% of isolates (63 of 67), while the *stx*1 gene was present in 22.4% of isolates (15 of 67). Comparison of treatment groups revealed a significant WDGS effect ($P = 0.02$), however, a significant WDGS × sampling day interaction was also observed ($P = 0.02$). The WDGS effect was significant ($P < 0.001$) on d 122, but not on d 136 ($P > 0.2$; Figure 2.1). There was no feed antimicrobial effect ($P > 0.8$) or feed antimicrobial × WDGS interaction ($P = 0.19$) on the fecal prevalence of *E. coli* O157. Prevalence of *E. coli* O157 in pooled fecal samples collected from pens was 19.4% (21 of 108) with 85.7% (18 of 21) containing the *stx*2 gene and 23.8% (5 of 21) containing the *stx*1 gene. There were no effects of

WDGS ($P > 0.4$), feed antimicrobial ($P > 0.4$), or day ($P > 0.4$) on prevalence *E. coli* O157 in pooled pen samples. *Salmonella* prevalence in pooled pen samples was 19.4% (21 of 108) with no significant treatment effects. Serotypes recovered were Montevideo (16 isolates) and Muenster (5 isolates).

Antimicrobial susceptibility

Antimicrobial susceptibility patterns of all bacterial isolates are shown in Table 2.3. All *Salmonella* isolates (n = 21) were susceptible to gentamicin, neomycin, tetracyclines and ampicillin while resistant to clindamycin and macrolides. *Escherichia coli* O157 isolates (n = 21) were susceptible to aminoglycosides with only one isolate resistant to neomycin. The most frequent resistance in *E. coli* O157 isolates was to tetracyclines and sulfonamides. Chlortetracycline resistance was displayed in 33.3% of isolates, while 47.6% of isolates were resistant to oxytetracycline. Sulfonamide resistance ranged from 38.1% for sulfachlorpyridazine and sulfadimethoxine to 42.9% for sulfathiazole.

Similar to *E. coli* O157 and *Salmonella* serotypes, all generic *E. coli* isolates were largely susceptible to aminoglycosides. A total of 188 generic *E. coli* isolates were tested and only one isolate exhibited resistance to gentamicin, while seven isolates were resistant to neomycin. Feed antimicrobial treatment affected the susceptibility of *E. coli.* Cattle fed monensin and tylosin had a lower proportion of isolates resistant to chlortetracycline ($P = 0.008$) and oxytetracycline ($P = 0.002$) than cattle fed no antimicrobials. Cattle fed monensin and tylosin also had a lower proportion ($P = 0.04$) of isolates resistant to oxytetracycline than cattle fed monensin only. The resistance patterns to sulfonamides in generic *E. coli* were impacted by the inclusion of 25% WDGS. Cattle without WDGS had a lower proportion of resistance in sulfachloropyridazine (*P*

= 0.02), sulfathiazole (P = 0.02) and sulfadimethoxine (P = 0.02) than cattle fed diets with WDGS.

Among *Enterococcus* isolates (n = 96), 57.3% were resistant to macrolides (erythromycin and tylosin). *Enterococcus* isolates from monensin-fed (P = 0.01) or monensin and tylosin-fed (P = 0.01) cattle were more resistant to macrolides compared to isolates from cattle fed no antimicrobials (Figure 2.2). In cattle fed monensin and tylosin, 75% of *Enterococcus* isolates displayed resistance to tylosin and erythromycin and 66% of *Enterococcus* isolates from cattle fed only monensin were resistant to these antimicrobials. Only 36.8% of *Enterococcus* isolates were resistant in cattle fed no antimicrobials. There was no WDGS effect on the resistance or susceptibility of fecal enterococci to macrolides (data not shown). However, the resistance to several antimicrobials appeared to be influenced by WDGS treatment. Fewer *Enterococcus* isolates showed resistance to flavomycin in animals fed WDGS than those fed SFC only (P = 0.01). However, with quinupristin/dalfopristin there was a tendency for a higher proportion of resistant *Enterococcus* in cattle fed WDGS (P = 0.08).

Quantification of fecal ermB and tetM genes

The *tetM* gene was detected in every fecal sample, while the *ermB* gene was detected in 94 of 108 fecal samples (87%). Including 0 or 25% of WDGS did not effect the concentration of either *erm*B or *tet*M genes in the feces. There was no antimicrobial effect on the concentration of either *erm*B or *tet*M in the feces.

DISCUSSION

Distiller's grains with solubles with highly digestible bran, high ruminal escape protein, and energy dense germ fractions of the corn are well suited as a ruminant feed (Lodge et al., 1997; Kleinschimt et al., 2006). In the production of distiller's grains, antimicrobials, such as

penicillin G, streptomycin, tetracycline, monensin, and virginiamycin, are used to suppress the bacterial growth (Day et al., 1954; Aquarone, 1960; Narendranath et al., 2000). It is generally believed that temperatures achieved during the distillation are sufficient to destroy these antimicrobials (Shurson, 2005). There are no data to suggest that the byproduct has antibiotic residue. However, even if there is no residue, there is a possibility of genetic elements for antibiotic resistance being present in the byproduct. There is evidence that stored wet distiller's grains have a dense population of lactobacilli (Pederson et al., 2004), which could be a source of antibiotic resistance genes.

In our study, both susceptibility and real-time PCR results implied that WDGS was not associated with antimicrobial resistance. The proportion of *Enterococcus* isolates resistant to quinupristin/dalfopristin, a streptogramin antimicrobial compound like virginiamycin, was larger in cattle fed WDGS; however, this was only a trend and was not significant. No evidence of increased penicillin resistance was observed in *Enterococcus* isolates, which may have occurred if residual antibiotics remained. In addition, quantification of two resistance elements, *erm*B and *tet*M, by PCR revealed no differences in concentration between WDGS fed cattle and cattle fed no WDGS.

Additional antimicrobial susceptibility data from our study showed that monensin and tylosin use were associated with increased macrolide resistance in *Enterococcus* species, which was consistent with other studies reporting tylosin use in pigs (Aarestrup et al., 2001; Jackson et al., 2004). The most common mode of resistance to macrolides in enterococci of animal origin is the *erm*B gene (Jensen et al., 1999) which causes a 23S rRNA methylation (Roberts et al., 1999), thereby rendering the ribosomes tolerant to erythromycin. Furthermore, a previous study has shown that *erm*B and *tet*M can co-exist in a single transposon (De Leener et al., 2004). Although

our results showed the presence of both *erm*B and *tet*M resistance genes in every pooled pen sample tested, the concentration of each gene varied in samples.

Antimicrobial feed additives did not have a significant effect on *erm*B genes in feces; however, the results did not correlate with the macrolide susceptibility in *Enterococcus* isolates. Although macrolide resistance in enterococci isolates was more prevalent, the concentrations of *erm*B genes from pooled fecal samples were unchanged compared to cattle fed no antimicrobials. Because other genes can provide resistance to macrolides (Schwarz et al., 2006), all enterococci isolated in this study were screened by PCR and confirmed to contain the *erm*B gene. These results suggest that there might be additional mechanisms affecting macrolide resistance in these isolates.

An interesting observation of this study was the association between feeding WDGS and fecal prevalence of *E. coli* O157. The prevalence of *E. coli* O157 in cattle fed WDGS was higher compared to those not fed WDGS when individual cattle were sampled; however, this was only statistically significant on one sample day. Pooled pen samples did not reveal any difference in the prevalence of *E. coli* O157 or *Salmonella* species. The difference between individual animal samples and pooled pen samples is not surprising because the sensitivity in detection methods decreases when *E. coli* O157 positive fecal samples are pooled with *E. coli* O157 negative samples (Sanderson et al., 2005). In addition, the enrichment and isolation procedures were slightly different between the two samples. The role of diet on fecal shedding of *E. coli* O157 in cattle has been well studied (Buchko et al., 2000; Callaway et al., 2003; Berg et al., 2004; Van Baale et al., 2004). Dewell et al. (2005) have reported that fecal samples from feedlot pens fed brewer's grains, a similar fermentative product to distiller's grains, were six times more likely to be positive for *E. coli* O157 than feedlot pens not fed brewer's grains. Our

study is the first report of an association between feeding distiller's grains and prevalence of *E. coli* O157 in cattle. The reason for this possible association is not known. We hypothesize that higher prevalence associated with distiller's grains or brewer's grains is possibly due to the different hindgut environment created by the diets. Research has shown that the primary colonization site of *E. coli* O157 is the hindgut of cattle (Grauke et al., 2002; Naylor et al., 2003; Van Baale et al., 2004). The feeding of a byproduct with less starch results in higher ruminal pH than corn diet without distiller's grain (Firkins et al., 1985; Lodge et al., 1997). Additionally, replacing corn with a highly digestible fiber source in distiller's grain may cause a shift in digestion from the rumen to the hindgut (Ham et al., 1994). The higher fat content in distiller's grain, in addition to providing more energy, could have an effect of ruminal fermentation and rumen microbial population. Montgomery et al. (2005) have shown that feeding corn germ to cattle reduced the incidence of liver abscess; one of the reasons may be that fatty acids in the germ have an antibacterial effect, thereby suppressing the growth of *Fusobacterium necrophorum,* the causative agent of liver abscesses. Increased supply of fiber and possibly protein and germ in cattle fed distiller's grain could have a significant impact on hindgut fermentation. Possibly, the altered hindgut environment is more conducive to *E. coli* O157 colonization.

Monensin and tylosin in cattle diets had no effect of *E. coli* O157 prevalence. The effect of monensin on the prevalence of *E. coli* O157 has been studied and the results are conflicting. A positive association between feeding ionophores and the prevalence of *E. coli* O157 was noted in dairy cattle (Herriot et al., 1998). This was later contradicted by two studies that showed no effect of short-term monensin feeding on fecal shedding of *E. coli* O157 or *Salmonella Typhimurium* in lambs (Edrington et al., 2003) and a decreased duration of *E. coli* O157

shedding in forage-fed steers supplemented monensin (Van Baale et al., 2004). Recently, McAllister et al. (2006) found no association between monensin, tylosin, or monensin and tylosin on the prevalence of *E. coli* O157 in fecal samples of orally challenged animals.

Several limitations to our study include the small number of sample collection days, low statistical power to detect differences, and lack of baseline prevalence and antimicrobial susceptibility estimates. Effects that may be attributable to study duration may not have been seen with only two collection days. Additionally, the low prevalence of *E. coli* O157 and *Salmonella* in pooled-pen samples reduced our ability to statistically analyze antimicrobial susceptibility in these isolates, although our resistance patterns to tetracyclines and sulfonamides were in agreement with previous studies (Meng et al., 1998; Galland et al., 2001; Schroeder et al., 2002; Fitzgerald et al., 2003).

In conclusion, additional work should be conducted to confirm the increased prevalence of *E. coli* O157 when animals are fed WDGS. Feeding antimicrobials to cattle was associated with a higher prevalence of resistance towards related antimicrobials in commensal enterococci when individual isolates were characterized. However, quantification of resistance genes in cattle feces, regardless of bacterial species, may provide a better means to assess the impact of production practices on the dynamics of antimicrobial resistant bacterial populations. Antimicrobial feed additives did not appear to increase the presence or concentration of either *erm*B or *tet*M elements in cattle.

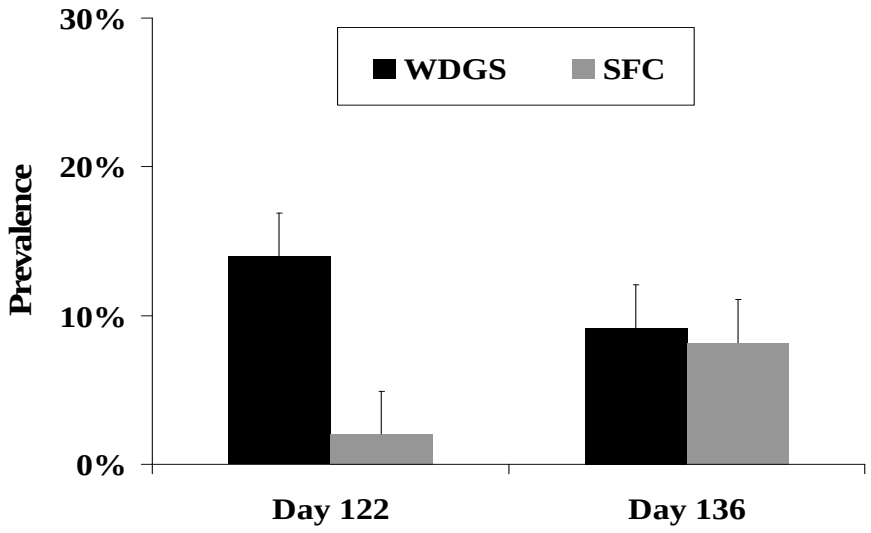

Figure 2.1 Prevalence and standard error of *Escherichia coli* O157 in fecal samples collected from individual animals on two collection days. The prevalence on d 122 was significantly greater ($P < 0.001$) in cattle fed steam-flaked corn (SFC) diet with wet distiller's grains with solubles (WDGS) compared to cattle fed SFC without WDGS. The difference was not significant on d 136 ($P > 0.2$).

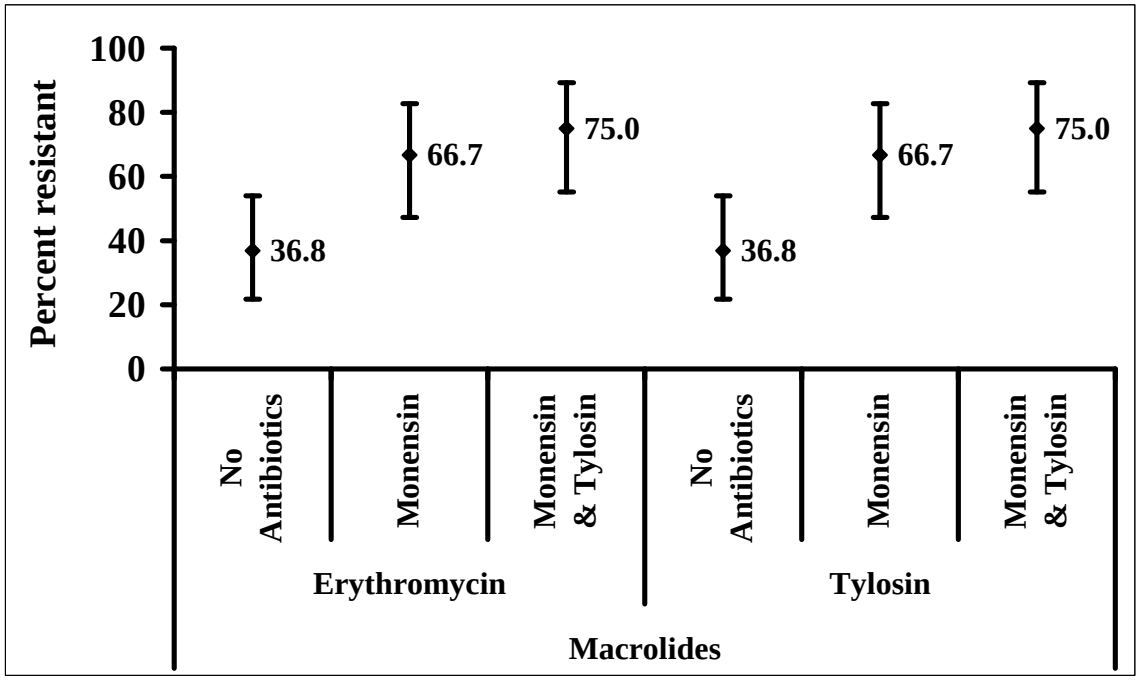

Figure 2.2 Percent (and 95% CI) of *Enterococcus* isolates resistant to erythromycin and tylosin. *Enterococcus* isolates from cattle fed monensin (*P* = 0.01) or monensin and tylosin (*P* = 0.01) were more resistant to macrolides compared to isolates from cattle fed no antimicrobials.

Table 2.1 Composition of diets

Ingredient	Steam-flaked corn diet	Steam-flaked corn diet with wet distiller's grains and solubles
	% of DM	
Steam flaked corn	83.8	64.8
Corn wet distiller's grains with solubles	-	25.0
Alfalfa hay	6.0	6.0
Supplement[1]	4.2	4.3
Corn steep liquor	5.0	-
Urea, 46% N	1.0	-

[1] Formulated with 0.7% Ca, 0.7% K, 0.3% NaCl, 0.3 mg/kg cobalt, 10 mg/kg copper, 0.5 mg/kg iodine, 60 mg/kg manganese, 0.25 mg/kg selenium, 60 mg/kg zinc, 0.055 mg/kg melengestrol acetate, 33 mg/kg monensin (if applicable), and 9.9 mg/kg tylosin (if applicable) in the final diet (DM basis).

Table 2.2 Primer Sequences

Primer	Sequence	Annealing temp.	Product size	Reference
*erm*BCT	F 5' GAAATTGGAACAGGTAAAGG 3'	43°C	404 bp	Modified from
	R 5' TTTACTTTTGGTTTAGGATG 3'			Jost et al., 2004
*erm*B	F 5' GAATCCTTCTTCAACAATCA 3'	45°C	175 bp	This study
	R 5' ACTGAACATTCGTGTCACTT 3'			
*tet*M	F 5' CTGTTGAACCGAGTAAACCT 3'	48°C	156 bp	This study
	R 5' GCACTAATCACTTCCATTTG 3'			
EUB	F 5' TGGAGCATGTGGTTTAATTCGA 3'	50°C	159 bp	Yang et al., 2002
	R 5' TGCGGGACTTAACCCAACA 3'			

Table 2.3 Antimicrobial resistance patterns of *Escherichia coli* O157, *Salmonella* spp., generic *E. coli*, and *Enterococcus* spp. isolates.

Class and antimicrobial compounds	Percent of isolates resistant			
	E. coli O157 (n=21)	*Salmonella* (n=21)	*E. coli* (n=188)	*Enterococcus* (n=96)
Aminocyclitols				
Spectinomycin	4.8	76.2	13.8	NA[1]
Aminoglycosides				
Gentamicin	0	0	0.5	0
Kanamycin	NA	NA	NA	12.5
Neomycin	4.8	0	3.7	NA
Streptomycin	NA	NA	NA	9.4
B-Lactams				
Ampicillin	38.1	0	4.8	NA
Penicillin	100	100	100	0
Cephalosporins				
Ceftiofur	NA[2]	NA[2]	NA[2]	NA
Glycopeptide				
Vancomycin	NA	NA	NA	0
Lincosamides				
Clindamycin	100	100	100	NA
Lincomycin	NA	NA	NA	96.9
Macrolides				
Erythromycin	100	100	100	57.3
Tilmicosin	100	100	100	NA
Tylosin	100	100	100	57.3
Phenicols				

Chloramphenicol	NA	NA	NA	0
Florfenicol	38.1	0	10.6	NA
Quinolones				
Ciprofloxacin	NA	NA	NA	1.0
Danofloxacin	0	0	0	NA
Enrofloxacin	0	0	0	NA
Sulfonamides				
Sulfachloropyridazine	38.1	0	9.6	NA
Sulfadimethoxine	38.1	23.8	10.1	NA
Sulfathiazole	42.9	7.1	9.6	NA
Trimethoprim/Sulfamethoxazole	0	0	0	NA
Tetracyclines				
Chlortetracycline	33.3	0	31.4	NA
Oxytetracycline	47.6	0	45.7	NA
Tetracycline	NA	NA	NA	80.2

[1] NA= Not applicable

[2] MIC values were available, however, Clinical and Laboratory Standards Institute interpretations (susceptible, intermediate, and resistant) were not reported

REFERENCES

Aarestrup, F. M., A. M. Seyfarth, H. D. Emborg, K. Pedersen, R. S. Hendriksen, and F. Bager. 2001. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. Antimicrob. Agents Chemother. 45:2054-2059.

Agresti, A. 1996. An Introduction to Categorical Data Analysis. Page 278 in SAS and SPSS for Categorical Data Analysis. V. Barnett, ed. John Wiley & Sons, Inc., New York.

Aquarone, E. 1960. Penicillin and tetracycline as contamination control agents in alcoholic fermentation of sugarcane molasses. Appl. Microbiol. 8:263-268.

Barkocy-Gallagher, G. A., E. D. Berry, M. Rivera-Betancourt, T. M. Arthur, X. Nou, and M. Koohmaraie. 2002. Development of methods for the recovery of *Escherichia coli* O157:H7 and *Salmonella* from beef carcass sponge samples and bovine fecal and hide samples. J. Food Prot. 65:1527-1534.

Barton, M. D. 2000. Antibiotic use in animal feed and its impact on human health. Nutr. Res. Rev. 13:279-299.

Berg, J., T. McAllister, S. Bach, R. Stilborn, D. Hancock, and J. LeJeune. 2004. *Escherichia coli* O157:H7 excretion by commercial feedlot cattle fed either barley- or corn-based finishing diets. J. Food Prot. 67:666-671.

Buchko, S. J., R. A. Holley, W. O. Olson, V. P. J. Gannon, and D. M. Veira. 2000. The effect of different grain diets on fecal shedding of *Escherichia coli* O157:H7 by steers. J. Food Prot. 63:1467-1474.

Callaway, T. R., R. O. Elder, J. E. Keen, R. C. Anderson, and D. J. Nisbet. 2003. Forage feeding to reduce preharvest *Escherichia coli* populations in cattle, a review. J. Dairy Sci. 86:852-860.

Day, W. H., W. C. Serjak, J. R. Stratton, and L. Stone. 1954. Antibiotics as contamination control agents in grain alcohol fermentations. J. Agric. Food Chem. 2:252-258.

De Leener, E., A. Martel, A. Decostere, and F. Haesebrouck. 2004. Distribution of the *erm*(B) gene, tetracycline resistance genes, and the Tn *1545*-like transposons in macrolide- and lincosamide-resistant enterococci from pigs and humans. Microbial Drug Resistance. 10:341-345.

Dewell, G. A., J. R. Ransom, R. D. Dewell, K. McCurdy, I. A. Gardner, A. E. Hill, J. N. Sofos, K. E. Belk, G. C. Smith, and M. D. Salman. 2005. Prevalence of and risk factors for *Escherichia coli* O157 in market-ready beef cattle from 12 U.S. Feedlots. Foodborne Pathog. Dis. 2:70-76.

Dohoo, I., W. Martin, and H. Stryhn. 2003. Veterinary Epidemiologic Research. Page 321 in Model-Building Strategies. S. McPike, ed. AVC Inc., Charlottetown, Canada

Edrington, T. S., T. R. Callaway, K. M. Bischoff, K. J. Genovese, R. O. Elder, R. C. Anderson, and D. J. Nisbet. 2003. Effect of feeding the ionophores monensin and laidlomycin propionate and the antimicrobial bambermycin to sheep experimentally infected with *E. coli* O157:H7 and *Salmonella typhimurium*. J. Anim. Sci. 81:553-560.

Fagan, P. K., M. A. Hornitzky, K. A. Bettelheim, and S. P. Djordjevic. 1999. Detection of Shiga-like toxin (stx1 and stx2), intimin (eaeA), and enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC hlyA) genes in animal feces by multiplex PCR. Appl. Environ. Microbiol. 65:868-872.

Firkins, J. L., L. L. Berger, and G. C. Fahey. 1985. Evaluation of wet and dry distiller's grains and wet and dry gluten feeds for ruminants. J. Anim. Sci. 60:847-?

Fitzgerald, A. C., T. S. Edrington, M. L. Looper, T. R. Callaway, K. J. Genovese, K. M. Bischoff, J. L. McReynolds, J.D. Thomas, R. C. Anderson, and D. J. Nisbet. 2003. Antimicrobial susceptibility and factors affecting the shedding of *E. coli* O157:H7 and *Salmonella* in dairy cattle. Lett. Appl. Microbiol. 37:392-398.

Galland, J. C., D. R. Hyatt, S. S. Crupper, and D. W. Acheson. 2001. Prevalence, antibiotic susceptibility, and diversity of *Escherichia coli* O157:H7 isolates from a longitudinal study of beef cattle feedlots. Appl. Environ. Microbiol. 67:1619-1627.

Greenquist, M. A., J. S. Drouillard, J. M. Sargeant, B. E. Depenbusch, X. Shi, K. F. Lechtenberg, and T. G. Nagaraja. 2005. Comparison of rectoanal mucosal swab cultures and fecal cultures from determining prevalence of *Escherichia coli* O157:H7 in feedlot cattle. Appl. Environ. Microbiol. 71:6431-6433.

Grauke, L. J., I. T. Kudva, J. W. Yoon, C. W. Hunt, C. J. Williams, and C. J. Hovde. 2002. Gastrointestinal tract location of *Escherichia coli* O157:H7 in ruminants. Appl. Environ. Microbiol. 68:2269-2277.

Gustafson, R. H. and R. E. Bowen. 1997. Antibiotic use in animal agriculture. J. Appl. Microbiol. 83:531-541.

Ham, G. A., R. A. Stock, T. J. Klopfenstein, E. M. Larson, D. H. Shain, and R. P. Huffman. 1994. Wet corn distiller's byproducts compared with dried corn distiller's grains with solubles as a source of protein and energy for ruminants. J. Anim. Sci. 72:3246-3257.

Herriott, D. E., D. D. Hancock, E. D. Ebel, L. V. Carpenter, D. H. Rice, and T. E. Besser. 1998.

Association of herd management factors with colonization of dairy cattle by Shiga toxin-positive *Escherichia coli* O157. J. Food Prot. 61:802-807.

Jackson, C. R., P. J. Fedorka-Cray, J. B. Barrett, and S. R. Ladely. 2004. Effects of tylosin use on erythromycin resistance in enterococci isolated from swine. Appl. Environ. Microbiol. 70:4205-4210.

Jensen, L. B., N. Frimodt-Møller, and F. M. Aarestrup. 1999. Presence of *erm* gene classes in gram-positive bacteria of animal and human origin in Denmark. FEMS Microbiol. Lett. 170:151-158.

Jost, B. H, H. T. Trinh, J. G. Songer, and S. J. Billington. 2004. A second tylosin resistance determinant, *erm*B, in *Arcanobacterium pyogenes*. Antimicrob. Agents Chemother. 48:721-727.

Kleinschmit, D. H., D. J. Schingoethe, K. F. Kalscheur, and A. R. Hippen. 2006. Evaluation of various sources of corn dried distillers grains plus solubles for lactating dairy cattle. J. Dairy Sci. 89:4784-4794.

Lodge, S. L., R. A. Shain, T. J. Klopfenstein, D. H. Shain, and D. W. Herold. 1997. Evaluation of corn and sorghum distiller's byproducts. J. Anim. Sci. 75:37-43.

McAllister, T. A., S. J. Bach, K. Stanford, and T. R. Callaway. 2006. Shedding of *Escherichia coli* O157:H7 by cattle fed diets containing monensin or tylosin. J. Food Prot. 69:2075-2083.

Meng, J., S. Zhao, M. P. Doyle, and S. W. Joseph. 1998. Antibiotic resistance of *Escherichia coli* O157:H7 and O157:NM isolated from animals, food, and humans. J Food Prot. 61:1511-1514.

Montgomery, S. P., J. S. Drouillard, J. J. Sindt, M. A. Greequist, B. E. Depenbusch, E. J. Good, E. R. Loe, M. J. Sulpizio, T. J. Kessen, and R. T. Ethington. 2005. Effects of dried full-fat corn germ and vitamin E on growth performance and carcass characteristics of finishing cattle. J. Anim. Sci. 83:2440-2447.

Nagaraja, T. G., and M. M. Chengappa. 1998. Liver abscess in feedlot cattle: a review. J. Anim. Sci. 76:287-298.

Narendranath, N. V., K. C. Thomas and W. M. Ingledew. 2000. Urea hydrogen peroxide reduces the numbers of Lactobacilli, nourishes yeast, and leaves no residues in the ethanol fermentation. Appl. Environ. Microbiol. 66:4187-4192.

Naylor, S. W., J. C. Low, T. E. Besser, A. Mahajan, G. J. Gunn, M. C. Pearce, I. J. McKendrick, D. G. E. Smith, and D. L. Gally. 2003. Lymphoid follicle-dense mucosa at the terminal

rectum is the principle site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host. Infect. Immun. 71:1505-1512.

Pederson, C., H. Jonsson, J. E. Lindberg, and S. Ross. 2004. Microbiological characterization of wet wheat distiller's grain, with focus on isolation of lactobacilli with potential as probiotics. Appl. Environ. Microbiol. 70:1522-1527.

Roberts, M. C., J. Sutcliffe, P. Courvalin, L. B. Jensen, J. Rood, and H. Seppala. 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob. Agents Chemother. 43:2823-2830.

Sanderson, M. W., J. M. Sargeant, and T. G. Nagaraja. 2005. Effect of pooling bovine fecal samples on the sensitivity of detection of *E. coli* O157:H7. Vet. Microbiol. 110:125-130.

Schroeder, C. M. C. Zhao, C. DebRoy, J. Torcolini, S. Zhao, D. G. White, D. D. Wagner, P. F. McDermott, R. D. Walker, and J. Meng. 2002. Antimicrobial resistance of *Escherichia coli* O157 isolated from humans, cattle, swine, and food. Appl. Environ. Microbiol. 68:576-581.

Schwarz, S., A. Cloeckaert, and M. C. Roberts. 2006. Mechanisms and spread of bacterial resistance to antimicrobial agents. Page 73 in Antimicrobial resistance in bacteria of animal origin. F. Aarestrup, ed. ASM Press, Washington D.C.

Shurson, J. 2005. Proposed recommended laboratory testing procedures for distiller's grains by-products. Available: http://www.ddgs.umn.edu/analytical_procedures/Final%20Recommended%20DDGS%20Testing%20Procedures%204-8-05.pdf. Accessed Jun. 22, 2005.

Smith, C. J., M. B. Rogers, and M. L. McKee. 1992. Heterologous gene expression in *Bacteroides fragilis*. Plasmid 27:141-154.

Spiehs, M. J., M. H. Whitney and G. C. Shurson. 2002. Nutrient database for distiller's dried grains with solubles produced from new ethanol plants in Minnesota and South Dakota. J. Anim. Sci. 80:2639-2645.

Stock, R. A., S. B. Laudert, W. W. Stroup, E. M. Larson, J. C. Parrott, and R. A. Britton. 1995. Effect of monensin and monensin and tylosin combination on feed intake variation of feedlot steers. J. Anim. Sci. 73: 39-44.

Van Baale, M. J., J. M. Sargeant, D. P. Gnad, B. M DeBey, K. F. Lechtenberg, and T. G. Nagaraja. 2004. Effect of forage or grain diets with or without monensin on ruminal persistence and fecal *Escherichia coli* O157:H7 in Cattle. Appl. Environ. Microbiol. 70:5336-5342.

Yang, S., S. Lin, G. D. Kelen, T. C. Quinn, J. D. Dick, C. A. Gaydos, and R. E. Rothman. 2002. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. J. Clin. Microbiol. 40:3449-3454.

CHAPTER 3 - Effects of Feeding Elevated Concentrations of Supplemental Copper and Zinc on Antimicrobial Susceptibilities of Fecal Bacteria in Feedlot Cattle

INTRODUCTION

In the United States, in-feed antimicrobials are included in cattle diets to aid in growth promotion, feed efficiency, and to decrease the incidence of liver abscesses (6, 17, 18). Compounds other than conventional antibiotics also are included in livestock diets to achieve growth promotion and feed efficiency. Such compounds include heavy metals like copper and zinc which are added to the diets at concentrations in excess of the nutritional requirements of the animals (12). The use of low level antimicrobials in livestock production is controversial because it may induce gut microorganisms to carry antimicrobial resistance genes (4, 8, 21). The emergence of bacterial populations resistant to copper and other metals also can occur (12, 22). A recent study by Hasman et al. (2006), described the association of an acquired transferable plasmid containing the copper resistance gene, *tcr*(B), in *Enterococcus faecium* isolates from pigs fed elevated levels of supplemental copper sulfate. The acquired *tcr*(B) gene was shown to provide resistance to copper at concentrations above those tolerated by normal cellular processes (11). Additionally, this plasmid carried genes for antimicrobial resistance (11, 13). Copper use in the environment has also been associated with bacterial antimicrobial resistance in soil (5).

Copper and zinc are currently recommended as nutritional supplements for cattle in the United States (20). Heavy metals fed at elevated concentrations may not only select for bacteria that are resistant to the respective heavy metals, but also to antimicrobials. The effects of such supplements on antimicrobial resistance in the U.S. cattle population have not been evaluated.

Antimicrobial resistance is a significant food safety risk, and factors contributing to antimicrobial susceptibility in the environment should be examined. The objectives of this study were to determine if feeding elevated concentrations of supplemental copper and zinc to feedlot cattle influenced the prevalence of food-borne bacterial pathogens and the susceptibility of fecal bacteria, food-borne and certain commensal, to antimicrobials and heavy metals.

MATERIALS AND METHODS

Study design and sampling

Twenty crossbred heifers (BW = 486 ± 79 kg) were randomly allocated to four treatments (five animals per treatment) and housed in individual concrete pens with a water fountain shared between adjacent pens. All animals within a treatment were penned next to each other with an empty pen between treatments to limit potential cross-contamination. Cattle were fed a standard steam-flaked corn-based high-grain diet (83%) with 6% alfalfa hay and without in-feed antimicrobials for the previous three months, except monensin, which was withheld for the previous two weeks. The four treatments of the study were: 1X National Research Council (NRC; 20) recommended copper (10 mg/kg diet) + 1X zinc (30 mg/kg diet), 10X copper (100 mg/kg diet) + 1X zinc (30 mg/kg diet), 1X copper (10 mg/kg diet) + 10X zinc (300 mg/kg diet), or 10X copper (100 mg/kg diet) + 10X zinc (300 mg/kg diet). Twenty grams of fresh fecal sample were collected by fecal grab from each animal on days 0, 14, and 32 of the feeding period. Cattle receiving the 10X copper + 1X zinc treatment were inadvertently fed diets containing only 3X copper (30 mg/kg diet) + 1X zinc (30 mg/kg diet) beginning on day 23 of the feeding period.

Bacteriological procedures

Each fecal sample was cultured to isolate *Escherichia coli, E. coli* O157, *Salmonella enterica*, and *Campylobacter*, *Listeria*, and *Enterococcus* species. Unless otherwise indicated, all media were Difco brand (Fisher Scientific, St. Louis, MO). Procedures modified from Barkocy-Gallagher et al. (2002) were used to isolate *E. coli, E. coli* O157, and *Salmonella* from fecal samples. Briefly, 10 g of feces were enriched in 90 ml of tryptic soy broth (TSB) with an incubation schedule of 2 h at 25°C, 6 h at 42°C, and overnight at 4°C. For the isolation of *E. coli*, 50 µl of enriched TSB were spread plated onto MacConkey agar and incubated at 37°C for 24 h. A lactose fermenting colony was picked and streaked onto a blood agar plate (BAP; Remel, Lenexa, KS), grown overnight at 37°C, and tested for indole production and citrate utilization. For the isolation of *E. coli* O157, anti-O157 immunomagnetic separation (IMS; Dynal, Inc., New Hyde Park, NY) was performed on 1 ml of enriched TSB, followed by plating onto sorbitol-MacConkey agar with cefixime (0.5 mg/L) and potassium tellurite (2.5 mg/L). Plates were incubated overnight at 37°C, and six sorbitol negative colonies were picked and re-plated onto BAP. Following an overnight incubation at 37°C, colonies were tested for indole production and latex agglutination for the O157 antigen (Oxoid, Remel; Lenexa, KS). Species were confirmed with the API 20E kit (bioMérieux, Hazelwood, MO) and multiplex PCR to detect *eae*, *stx1* and *stx2* genes (7). *Salmonella* isolation was performed by inoculating 10 ml of enriched TSB in 90 ml of tetrathionate broth (TTB), followed by incubation for 24 h at 37°C. Anti-*Salmonella* IMS (Dynal, Inc.) was performed with 1 ml of TTB, followed by enrichment of 100 µl in10 ml Rappaport-Vassiliadis (RV) broth. The RV broth was incubated at 42°C for 16-18 h and 50 µl was plated on Hektoen enteric (HE) agar supplemented with novobiocin (15 mg/L). The HE plates were incubated overnight at 37°C; up to three characteristic isolates

(colorless colonies with black centers) were picked and plated onto BAP for overnight growth and latex agglutination.

For the isolation of *Campylobacter* species, 1 g of fecal sample was diluted in 10 ml of 0.1% peptone water and swabbed onto *Campylobacter* blood-free selective medium (Oxoid). Plates were incubated for 48 h at 42°C utilizing a gas pack (Oxoid) to achieve microaerophilic conditions. Characteristic *Campylobacter* colonies (gray) were re-plated onto BAP and a gram stain and API-Campy kit (bioMérieux) were used to confirm the genus.

Listeria species were isolated by methods modified from Nightingale et al. (2004). One gram of fecal sample was added to 9 ml *Listeria* enrichment broth for 24 and 48 h at 37°C. From the enrichment broth at both time intervals, 100 µl were plated on Modified Oxford Medium. Colonies (black) were plated onto BAP and a *Listeria* API kit (bioMérieux) was used to confirm positive isolates.

Enterococcus species were isolated by diluting 1 g of fecal sample in 10 ml of phosphate buffered saline and plating 50 µl of the suspension onto M-Enterococcus agar. After 24 h growth at 37°C, up to two (pin-point, metallic pink) colonies that were morphologically different were picked and re-plated on BAP for overnight incubation at 37°C. For confirmation of genus, isolates were inoculated in 100 µl Enterococcosel broth and incubated at 37°C for 4 h to test for esculin hydrolysis, followed by analysis using the 20 Strep API kit (bioMérieux). If the two colonies picked from M-enterococcus agar had identical API biochemical profiles then only one isolate was used for further analyses. All bacterial isolates were frozen in protect beads (Cryo-Vac®; Key Scientific, Round Rock, TX) at -80°C for further use.

Antibiotic susceptibility determination

Microbroth dilution method was used to determine minimum inhibitory concentrations (MIC; μg/ml) of selected antibiotics for *E. coli* and *Enterococcus* species isolates. Reported susceptibility results were based on interpretations provided by the automated reader program (Sensititre®, Trek Diagnostic Systems, Cleveland, OH). *Escherichia coli* were tested for susceptibility to the BOPO-IF panel (Sensititre®). The antibiotics in this panel and the maximum concentration evaluated included: ceftiofur (8 μg/ml), erythromycin (4 μg/ml), chlortetracycline (8 μg/ml), florfenicol (8 μg/ml), penicillin (8 μg/ml), ampicillin (16 μg/ml), danofloxacin (1 μg/ml), sulphadimethoxine (256 μg/ml), neomycin (32 μg/ml), sulphachloropyridazine (256 μg/ml), tylosin tartrate (20 μg/ml), sulphathiazole (256 μg/ml), spectinomycin (64 μg/ml), tilmicosin (32 μg/ml), clindamycin (2 μg/ml), tiamulin (32 μg/ml), enrofloxacin (2 μg/ml), trimethoprim/sulfamethoxazole (2/38 μg/ml), gentamicin (8 μg/ml), and oxytetracycline (8 μg/ml). *Enterococcus* isolates were evaluated for susceptibility using the gram positive NARMS (National Antimicrobial Resistance Monitoring System) panel (CMV1AGPF; Sensititre®). The antibiotics in this panel and the maximum concentration evaluated included: bacitracin (128 μg/ml), chloramphenicol (32 μg/ml), erythromycin (8 μg/ml), flavomycin (32 μg/ml), penicillin (16 μg/ml), daptomycin (16 μg/ml), quinupristin/dalfopristin (32 μg/ml), tetracycline (32 μg/ml), vancomycin (32 μg/ml), lincomycin (32 μg/ml), tylosin tartrate (32 μg/ml), ciprofloxacin (4 μg/ml), linezolid (8 μg/ml), nitrofurantoin (64 μg/ml), kanamycin (1,024 μg/ml), gentamicin (1,024 μg/ml), and streptomycin (2,048 μg/ml).

Susceptibility determinations for copper and zinc

Minimum inhibitory concentrations of copper or zinc were determined with copper sulfate or zinc sulfate for *E. coli* and *Enterococcus* isolates using the agar dilution method (11).

Briefly, brain heart infusion plates containing 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 mM of copper sulfate ($CuSO_4 \cdot 5H_20$, pH = 7) or zinc sulfate ($ZnSO_4 \cdot 7H_20$, pH = 7) were prepared and inoculated with 20 µl of bacterial growth adjusted to standard concentration (McFarland = 0.5). Plates were incubated for 24 h at 37°C and growth of each isolate was recorded. The MIC value was defined as the lowest concentration of copper sulfate or zinc sulfate inhibiting visual growth.

Quantification of erm(B) and tet(M) genes in the feces

Bacterial strains and plasmids

Two genes, *erm*(BCT) and 16s rDNA (EUB), were amplified from a strain of *E. faecalis* MMH594 (Dr. L. Zurek, Kansas State University). Fragments recovered from PCR reactions were cloned into a commercial vector (pCRII-TOPO, Invitrogen, Carlsbad, CA) followed by transformation in One Shot Top10 *E. coli* cells (Invitrogen). The *tet*(M) gene was amplified from an *E. coli* isolate (Dr. L. Zurek) containing pFD310 carrying tetracycline, erythromycin, and ampicillin resistance (24).

Primers and PCR running conditions

Cloning and real-time PCR procedures utilized the same primers (Table 3.1), with one exception. Originally, primers for *erm*(BCT) (404 bp) were modified from Jost et al. (2004) for cloning procedures. To accurately determine the concentration of a gene using real-time PCR, a smaller PCR product (< 200 bp) was desired. A new primer was designed to target only *erm*(B) (175 bp; 14). The PCR running conditions for all four targets [*erm*(BCT), *erm*(B), *tet*(M) and EUB] were: 3 min of initial denaturation at 94°C; 36 cycles of 30 s at 94°C, 30 s at annealing temperatures (Table 3.1), 30 s at 72°C; and 2 min final extension at 72°C with *Taq* DNA polymerase (Promega, Madison, WI). Amplification protocols for real-time reactions were

identical for each target, but utilized the Absolute QPCR SYBR Green Mix (ABgene, Epsom, UK), which required the addition of a 15 min, 95°C initial activation.

Cloning techniques

Initial PCR products (*erm*(B), *tet*(M) and EUB) were purified with Wizard SV Gel and PCR Clean-up System (Promega) and cloned into the commercial vector pCRII-TOPO according to manufacturer's directions. Clones were selected by blue/white screening on Luria-Bertani agar, and the plasmids were purified using Wizard Plus SV Minipreps DNA Purification System (Promega). Nanodrop spectrophotometer (ND 1000, Nanodrop Technologies, Wilmington, DE) readings were used to determine DNA concentrations. The clones were further confirmed by digestion with *Eco*RI (Promega) and by sequencing (Beckman-Coulter CEQ™8000 Genetic Analysis System; Fullerton, CA).

Real-time PCR standardization

Serial ten-fold dilutions of purified plasmid were prepared. A 25 µl reaction utilizing Absolute QPCR SYBR Green Mix containing 5 µl of serially diluted plasmids were used to standardize each real-time PCR assay. Reliable products for EUB and *erm*(B) targets were detected between 1×10^8 and 1×10^3 molecules/µl, and 1×10^9 and 1×10^3 molecules/µl, respectively. The *tet*(M) target was reliably detected between 7.4×10^8 and 7.4×10^2 molecules/µl.

Extraction of DNA and real-time PCR of fecal samples

Fecal samples (180 to 220 mg) from days 0, 14, and 32 (previously frozen at -80°C) were thawed on ice and DNA was extracted using the QIAamp DNA stool mini kit according to manufacturer's directions (QIAgen, Valencia, CA). Antimicrobial resistance genes, *erm*(B) and *tet*(M), and conserved 16s rDNA (EUB) sequence were targets in real-time PCR reactions. A

melting curve was created at 0.5°C increments to determine if the right product was amplified during each run. In addition, PCR products from randomly selected samples were electrophoresed and sequenced to confirm the correctness of the targets amplified. The copy numbers of the targeted genes in the samples were calculated using the standardization curves.

Statistical analysis

The non-parametric, LIFETEST procedure in SAS (Version 9.1, SAS Institute, Cary, NC) was used to find differences in antibiotic, copper, and zinc MIC values with treatment and day effects for *E. coli* and *Enterococcus* isolates. Treatment × day interactions could not be evaluated with this test, which was chosen *a priori* to account for possible right-censored data (i.e. maximum concentration available in commercial microbroth dilution panels). The logrank test was used to determine significance. If treatment or day effects ($P < 0.10$) were observed, two way comparisons were used to evaluate specific mean MIC differences. Finally, the MIXED SAS procedure was used to evaluate differences in the mean proportion of real-time PCR products. The proportion was the log concentration of antimicrobial resistance genes [*erm*(B) or *tet*(M)] to the log concentration of 16s rDNA (EUB) control gene in each fecal sample. Copper or zinc treatment, sampling day and the treatment × day interaction were included as effects on the concentration of real-time PCR products.

RESULTS

This study examined the effects of feeding finishing cattle elevated concentrations of supplemental copper and zinc on food-borne pathogen prevalence and antimicrobial susceptibilities of food-borne and commensal bacteria. Possibly because of incorrect data entry of feed intake, cattle fed 10X NRC recommended copper with normal (1X) NRC recommended zinc were inadvertently fed a diet with a lower concentration of copper (3X NRC) beginning on

day 23 of the feeding period. The impact of altering this diet may include preventing some copper resistance events; however, copper was still included in the diet at elevated concentrations. In addition, two of the three sample collections (sampling days 0 and 14) had occurred prior to the inadvertent switch.

Prevalence of food-borne pathogens in the feces

The fecal prevalence of *E. coli* O157 was 5.0% (3 of 60). One *E. coli* O157 isolate was obtained from day 14 samples and two isolates were recovered from day 32 samples. Five *Salmonella* isolates were cultured, all from day 32 samples. No *Listeria* isolate was obtained from any fecal samples during the study. Additionally, only two *Campylobacter* isolates were cultured, both from samples collected on day 14 of the study. Because of the low prevalence and poor distribution, *E. coli* O157, *Salmonella, Listeria, and Campylobacter* were not included in further analyses. The numbers of *E. coli* and *Enterococcus* isolates obtained from fecal samples collected from all treatment groups were 60 and 69, respectively.

Antibiotic susceptibility

Escherichia coli isolates were resistant to clindamycin, erythromycin, penicillin, tiamulin, tilmicosin, and tylosin. Copper or zinc supplementation did not impact MIC differences for *E. coli* isolates, but, there was an effect of sampling day. Tilmicosin MIC values increased between day 0 and day 14 ($P = 0.03$) and day 0 to day 32 ($P = 0.04$), but were not different between day 14 and day 32 ($P = 0.94$; Table 3.2). As previously mentioned, however, all isolates still were classified as resistant by Clinical and Laboratory Standards Institute (CLSI) interpretations. The CLSI classifications for *E. coli* isolates towards other antibiotics were mostly susceptible or intermediate.

Enterococcus isolates were classified as susceptible or intermediate to chloramphenicol, ciprofloxacin, gentamicin, linezolid, penicillin, streptomycin and vancomycin. There were no effects of copper or zinc supplementation on MIC values, but effects of sampling day were observed (Table 3.2). The mean chloramphenicol MIC for *Enterococcus* isolates was greater from samples collected on day 32 than on day 14 ($P = 0.03$). The differences in MIC between day 0 and day 32, as well as day 0 to day 14 were not different ($P > 0.1$). Penicillin MIC values also differed between sampling days. The mean MIC of penicillin was lower on day 0 compared to day 14 ($P = 0.05$) and day 32 ($P < 0.01$). The MIC differences between isolates from days 14 and 32 were not different ($P > 0.4$). These isolates were not classified as resistant to these antibiotics by CLSI interpretations.

Copper and zinc susceptibilities

The copper and zinc MIC of *E. coli* isolates were affected by treatment or sampling day. Treatment was associated with zinc MIC ($P < 0.01$). Specifically, cattle diets containing 10X copper + 1X zinc were associated with a lower *E. coli* zinc MIC than either 1X copper + 1X zinc ($P = 0.02$) or 10X copper + 10X zinc ($P = 0.02$) treatments. The mean MIC values of *E. coli* isolates from both 1X copper + 1X zinc and 10X copper + 10X zinc treatments were identical. In addition, sampling day had an effect on the copper MIC values of *E. coli* isolates (Fig. 3.1). The mean copper MIC was higher in isolates from day 32 than from days 0 ($P < 0.01$) or 14 ($P = 0.02$). No other effects were seen on copper or zinc MIC of *E. coli* isolates. Copper and/or zinc concentration did not impact the MIC of these metals for *Enterococcus* isolates. There was a sampling day effect on the zinc MIC in these isolates. The mean zinc MIC was higher in isolates from day 0 than on day 32 ($P = 0.01$) samples. The copper MIC of *Enterococcus* isolates were not affected by sampling day ($P > 0.5$).

Real-time PCR

A total of 31 of 60 fecal samples were positive for *erm*(B). The proportions of *erm*(B) resistant elements to 16s rDNA were not different between treatments or between sampling days ($P > 0.05$). Fifty-three of 60 fecal samples were positive for the *tet*(M) gene but had no treatment effect ($P > 0.05$). A sampling day-effect ($P < 0.05$) was observed for *tet*(M) (Fig. 3.2). The concentration of *tet*(M) gene was higher ($P < 0.01$) in feces collected on day 14 compared to day 0. Day 32 samples were not different in concentrations of *tet*(M) when compared to sampling days 0 or 14.

DISCUSSION

Copper and zinc are essential to cattle, which require the metals for normal cellular processes (20). These metals also are added to cattle diets at concentrations above those physiologically required by the animal because of growth promoting effects, similar to those of some conventional antimicrobials (12). The mechanism for increased efficiency of cattle growth with antimicrobial use is multifaceted; some antimicrobials alter normal microbial populations or metabolic processes, others suppress diseases, while some mechanisms are unknown (4, 8).

Bacteria also require copper, and the copper homeostatic mechanisms have been well studied in *Enterococcus hirae*, which utilize CPx-type ATPases (16). The *cop* operon, which includes a repressor, chaperone, and two CPx-type ATPases, is encoded by four chromosomal genes and provides *E. hirae* growth in up to 8 mM of copper (25). A previous study reported the presence of additional acquired copper resistance determinants in *Enterococcus* sp. from different food animals (1) and more specifically, from pigs fed copper sulfate (13). The *tcr*(B) gene, which renders *Enterococcus* isolates more resistant to copper (above 8 mM), is part of an operon with similar function to the *cop* operon of *E. hirae* (10). The acquisition of *tcr*(B) may

also coincide with antimicrobial resistance, specifically the *erm*(B) gene, genetically linked to the copper resistance determinant on a plasmid (11). Zinc is an important regulator of the *cop* operon in *E. hirae* (25); however, the potential role of zinc in acquired copper resistance is not known.

Previous works have focused on feeding copper as a selective pressure for microbial copper resistance and resistance to antibiotics, specifically in commensal organisms (1, 13). In addition to determining the role of elevated copper and zinc on resistance traits, our study also examined their effect on prevalence of several food-borne bacteria. The impact of supplemental copper and zinc on the prevalence of food-borne pathogens has not been reported. Cattle are a primary source of food-borne bacterial pathogens, including *E. coli* O157 (2, 9). Poor distribution and low prevalence or absence of *E. coli* O157, *Salmonella, Campylobacter,* or *Listeria* isolates did not allow for statistical analyses of the treatment effects on prevalence. The timing of this study may have affected the prevalence of some of the organisms, particularly *E. coli* O157, since the study was conducted in October and November. Previous work has shown that prevalence of *E. coli* O157 in cattle peaks during the summer months of May through September (23).

Feeding cattle elevated concentrations of copper, zinc, or both did not have an effect on the antibiotic MIC of fecal *E. coli* or *Enterococcus* isolates. Resistance to antibiotics, specifically macrolides, was expected in *Enterococcus* isolates fed higher levels of copper because resistance genes for both copper (*tcrB*) and macrolides (*ermB*) have previously been linked (11). The mean MIC of macrolide antibiotics remained unchanged in *Enterococcus* species. Although there were no associations between copper and zinc supplementations and antibiotic susceptibility, sampling day effects were seen for tilmicosin in *E. coli* isolates and for

chloramphenicol and penicillin in *Enterococcus* isolates. In all cases, the mean MIC value increased across at least one sampling time interval. This may suggest that some selective pressure was present for these antibiotics, however, because treatment was not significant it was likely not because of copper or zinc supplementation.

In addition to antibiotic susceptibility, the MIC for copper and zinc sulfate were used to assess resistance towards these metals. Interestingly, the mean copper MIC of *E. coli* isolates increased over the course of the study. There was no effect of copper or zinc treatment on *E. coli* susceptibility to copper but there was an effect on zinc MIC. The mean MIC did not change between cattle fed 1X copper + 1X zinc and 10X copper + 10X zinc. Consistent copper MIC values for *Enterococcus* isolates were obtained for all treatment groups and across collection days. The MIC values of *Enterococcus* isolates were reasonable to account for a normal copper homeostatic mechanism (i.e. $\leq$ 8 mM) and not additional resistance genes (25). Although *E. faecium* in cattle have been shown to contain less copper resistance than isolates from other livestock, resistance has still been reported as high as 16% (11). The MIC of zinc for *Enterococcus* isolates was significantly different between sampling days, with the MIC decreasing as the study progressed.

Two gene targets, *erm*(B) and *tet*(M), were chosen for real-time PCR on fecal samples because of their potential proximity to *tcr*(B) gene on a horizontally-transferable plasmid. No treatment effects were seen for either *erm*(B) or *tet*(M) gene, which was consistent with antibiotic susceptibility results. Sampling day had a significant effect on *tet*(M) concentration. Initially, *tet*(M) gene increased in concentration, which may indicate some selective pressure. The same effect was not evident in the tetracycline antibiotic susceptibilities of bacterial isolates. This may be a result of *tet*(M) gene being present in bacterial species other than those isolated in

our study. No significant difference seen in the concentration of these elements between treatments is further justification that elevated copper and/or elevated zinc concentrations in cattle diets have little effect on acquired copper or antimicrobial resistance.

A previous study has reported that 175 ppm copper sulfate in the feed, equal to 2.8 mM Cu, would be enough to induce copper resistance in pigs (10). The author acknowledged that factors including pH, formation of copper complexes, and feed breakdown in the intestine may influence *in situ* copper concentration. The maximum concentration of copper suggested to avoid toxic effects in beef cattle is 100 mg/kg diet (20). Given the lower concentration of copper fed to cattle in this study compared to the concentration of copper fed to pigs in previous studies, and the more complex gastrointestinal environment in cattle compared to pigs, it is possible that the levels of copper may not have been sufficient to induce acquired copper resistance in gut bacteria. Finding no difference in the mean copper MIC between treatments in this study, along with finding little evidence of additional acquired antibiotic resistance, seems to support this hypothesis, although low statistical power may have decreased the ability to detect differences. In summary, feeding elevated copper and zinc concentrations to feedlot cattle did not appear to influence the prevalence of several food-borne pathogens, although the timing of the study may have impacted our ability to find prevalence differences. Furthermore, copper or zinc treatment did not significantly change the antibiotic susceptibility profiles of bacteria, change the susceptibility of fecal bacteria to copper (although *E. coli* zinc MIC was changed), or increase the concentration of a macrolide resistance gene previously linked to copper resistance.

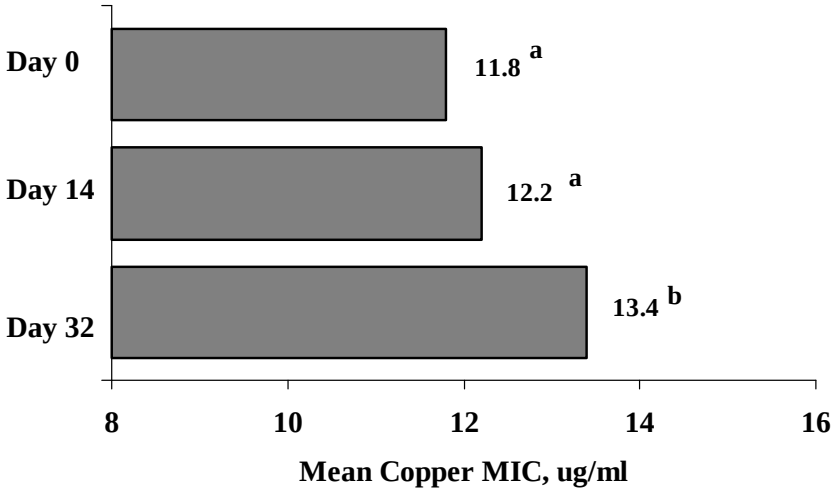

Figure 3.1 Copper minimum inhibitory concentration (MIC) of *Escherichia coli* isolates (n = 60) collected on different sampling days from cattle fed elevated concentrations of supplemental copper and zinc. Means with different superscripts indicate statistical significance ($P < 0.05$).

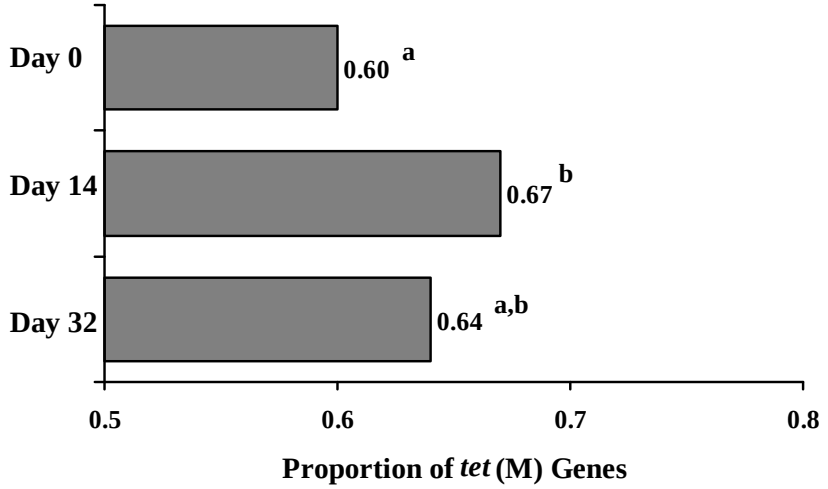

Figure 3.2 Mean proportion of real-time PCR amplified *tet*(M) to 16s rDNA (EUB) genes in feces collected on different sampling days in cattle fed elevated concentrations of supplemental copper and zinc. Means with different superscripts indicate statistical significance ($P < 0.05$).

Table 3.1 Primer Sequences

Primer name	Primer sequence	Annealing temperature	Product size	Reference
erm(B)	F 5' GAATCCTTCTTCAACAATCA 3'	45°C	175 bp	14
	R 5' ACTGAACATTCGTGTCACTT 3'			
tet(M)	F 5' CTGTTGAACCGAGTAAACCT 3'	48°C	156 bp	14
	R 5' GCACTAATCACTTCCATTTG 3'			
EUB	F 5' TGGAGCATGTGGTTTAATTCGA 3'	50°C	159 bp	26
	R 5' TGCGGGACTTAACCCAACA 3'			
erm(BCT)	F 5' GAAATTGGAACAGGTAAAGG 3'	43°C	404 bp	15
	R 5' TTTACTTTTGGTTTAGGATG 3'			

Table 3.2 The mean MIC of several antibiotics for fecal *Enterococcus* and *Escherichia coli* isolates from cattle fed different levels of supplemental copper and zinc

Bacterial species	Antibiotic	Mean MIC (μg/ml)		
		Day 0	Day 14	Day 32
Enterococcus (n = 69)	Chloramphenicol	5.4[a,b]	5.2[a]	6.7[b]
	Penicillin	0.5[a]	1.0[b]	1.1[b]
E. coli (n = 60)	Tilmicosin[c]	32[a]	32[b]	32[b]

[a, b] Row means within antibiotic with different superscripts indicate statistical significance ($P < 0.05$).

[c] Statistics were computed adjusting for right-censored data. Means do not reflect censored isolates.

REFERENCES

1. **Aarestrup, F. M., and H. Hasman.** 2004. Susceptibility of different bacterial species isolated from food animals to copper sulfate, zinc chloride and antimicrobial substances used for disinfection. Vet. Microbiol. **100**:83-89.

2. **Bach, S. J., T. A. McAllister, D. M. Veira, V. P. J. Gannon, and R. A. Holley.** 2002. Transmission and control of *Escherichia coli* O157:H7–a review. Can. J. Anim. Sci. **82**:475-490.

3. **Barkocy-Gallagher, G. A., E. D. Berry, M. Rivera-Betancourt, T. M. Arthur, X. Nou, and M. Koohmaraie.** 2002. Development of methods for the recovery of *Escherichia coli* O157:H7 and *Salmonella* from beef carcass sponge samples and bovine fecal and hide samples. J. Food Prot. **65**:1527-1534.

4. **Barton, M. D.** 2000. Antibiotic use in animal feed and its impact on human health. Nutr. Res. Rev. **13**:279-299.

5. **Berg, J., A. Tom-Petersen, and O. Nybroe.** 2005. Copper amendment of agricultural soil selects for bacterial antibiotic resistance in the field. Lett. Appl. Microbiol. **40**:146-151.

6. **Butaye, P., L. A. Devriese, and F. Haesebrouck.** 2003. Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on Gram-positive bacteria. Clin. Microbiol. Rev. **16**:175-188.

7. **Fagan, P. K., M. A. Hornitzky, K. A. Bettelheim, and S. P. Djordjevic**. 1999. Detection of Shiga-like toxin (stx1 and stx2), intimin (eaeA), and enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC hlyA) genes in animal feces by multiplex PCR. Appl. Environ. Microbiol. **65**:868-872.

8. **Gustafson, R. H., and R. E. Bowen.** 1997. Antibiotic use in animal agriculture. J. Appl. Microbiol. **83**:531-541.

9. **Gyles, C. L. 2007.** Shiga toxin-producing *Escherichia coli*: An overview. J. Anim. Sci. **85**(E. suppl.):E45-E62.

10. **Hasman, H.** 2005. The *tcr*B gene is part of the *tcr*YAZB operon conferring copper resistance in *Enterococcus faecium* and *Enterococcus faecalis*. Microbiol.?. **151**:3019-3025.

11. **Hasman, H., and F. M. Aarestrup.** 2002. *tcr*B, a gene conferring transferable copper resistance in *Enterococcus faecium*: occurrence, transferability, and linkage to macrolide and glycopeptide resistance. Antimicrob. Agents Chemother. **46**:1410-1416

12. **Hasman, H., S. Franke, and C. Rensing.** 2006. Resistance to metals used in agriculture production: p 99-114. *In* F. Aarestrup, (ed.), Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press, Washington D.C.

13. **Hasman, H., I. Kempf, B. Chidaine, R. Cariolet, A. K. Ersboll, H. Houe, H. C. B. Hansen, and F. M. Aarestrup.** 2006. Copper resistance in *Enterococcus faecium*, mediated by the *tcr*B gene, is selected by supplementation of pig feed with copper sulfate. Appl. Environ. Microbiol. **72**:5784-5789.

14. **Jacob, M. E., J. T. Fox, S. K. Narayanan, J. S. Drouillard, and T. G. Nagaraja.** 2006. Effects of feeding wet corn distiller's grains with solubles and monensin and tylosin on the prevalence and antimicrobial susceptibility of *E. coli* O157 in feedlot cattle. J. Anim. Sci. **84**(2):81.

15. **Jost, B. H., H. T. Trinh, J. G. Songer, and S. J. Billington.** 2004. A second tylosin resistance determinant, *Erm*B, in *Arcanobacterium pyogenes*. Antimicrob. Agents Chemother. **48**:721-727.

16. **Lu, Z. H., and M. Solioz**. 2002. Bacterial copper transport. Adv. Protein Chem. **60**:93-121.

17. **McEwen, S. A., and P. J. Fedorka-Cray.** 2002. Antimicrobial use and resistance in animals. Clin. Infect. Dis. **34** (Suppl 3):S93-106.

18. **Nagaraja, T. G., and M. M. Chengappa.** 1998. Liver abscess in feedlot cattle: a review. J. Anim. Sci. **76**:287-298.

19. **Nightingale, K. K., Y. H. Schukken, C. R. Nightingale, E. D. Fortes, A. J. Ho, Z. Her, Y. T. Grohn, P. L. McDonough, and M. Wiedmann.** 2004. Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. Appl. Environ. Microbiol. **70**:4458-4467.

20. **NRC.** 1996. Pages 62-64 and 68-69 in Nutrient requirements of beef cattle. 7th rev. ed. Natl. Academy Press, Washington D. C

21. **Phillips, I., M. Casewell, T. Cox, B. De Groot, C. Friis, R. Jones, C. Nightingale, R. Preston, and J. Waddell.** 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J. Antimicrob. Chemother. **53**:28-52.

22. **Sapkota, A. R., L. B. Price, E. K. Silbergeld, and K. J. Schwab.** 2006. Arsenic resistance in *Campylobacter* spp. isolated from retail poultry products. Appl. Environ. Microbiol. **72**:3069-3071.

23. **Smith, D. R., R. A. Moxley, S. L. Clowser, J. D. Folmer, S. Hinkley, G. E. Erickson, and T. J. Klopfenstein.** 2005. Use of rope devices to describe and explain the feedlot ecology of *Escherichia coli* O157:H7 by time and place. Foodborne Pathog. Dis. **2**:50-60.

24. **Smith, C. J., M. B. Rogers, and M. L. McKee.** 1992. Heterologous gene expression in *Bacteroides fragilis*. Plasmid **27**:141-154.

25. **Solioz, M., and J. V. Stoyanov.** 2003. Copper homeostasis in *Enterococcus hirae*. FEMS Microbiol. Rev. **27:**183-195.

26. **Yang, S., S. Lin, G. D. Kelen, T. C. Quinn, J. D. Dick, C. A. Gaydos, and R. E. Rothman.** 2002. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. J. Clin. Microbiol. **40**:3449-3454.