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Investigation of the Co-occurrence of Zinc and Copper Resistance and Antimicrobial Resistance in Escherichia coli from Beef Cattle Production Systems

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Investigation of the Co-occurrence of Zinc and Copper Resistance and Antimicrobial Resistance in *Escherichia coli* from Beef Cattle Production Systems

by

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Abstract

The present set of studies was directed towards determining the relationship between zinc (Zn) and copper (Cu) resistance and antimicrobial resistance in *Escherichia coli* from beef cattle production systems. First, a spectrophotometric assay was developed to properly assess the resistance levels to both Zn and Cu in *Escherichia coli.* The method was standardized for *E. coli* and it displayed a good linear dynamic range (R^2>0.95), and precision (RSD< 35% in all but three Zn concentrations). The MIC for the *E. coli* reference strain (ATCC 25922) were 2.78 μmol/ml and 8.41 μmol/ml for Zn and Cu respectively. In the second study, we determined antimicrobial phenotypes and the Zn and Cu resistance levels for *E. coli* isolates from environmental samples obtained from W.A Ranches. The samples analyzed had a low prevalence of antimicrobial resistance as 31/39 isolates were susceptible to all the antimicrobials tested using the disc diffusion method. The most common resistance was ampicillin (4/39) and amoxicillin-clavulanic acid (4/39), with one isolate being resistant to doxycycline. All the isolates resistant to ampicillin were also resistant to amoxicillin-clavulanic acid. The isolates were then tested for Zn and Cu resistance using the assay developed. Seven of the eight isolates with resistant or intermediate antimicrobial resistance patterns showed higher optical density (OD) in the Zn and Cu resistance spectrophotometric assay when compared to the reference strain. Fisher's exact test was conducted to compare antimicrobial-resistant (all antimicrobials) and susceptible isolates in their resistance to Zn and Cu, with the results showing that the antimicrobial resistant isolates are also more likely to have a higher resistance to Zn (p value<0.001) and Cu (p value=0.013) as indicated by higher absorbance units (AU). A Principal Component Analysis showed the clustering of 6/8 antimicrobial-resistant isolates based on the Zn and Cu resistance meaning that resistance to these metals might be an indicator of antimicrobial resistance.

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Dedication

To my beloved parents Joaquina Servellón and Fernando Guardado Sr. for supporting me and letting my dreams come true.

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Symbol	Definition
AMR	Antimicrobial resistance
AMU	Antimicrobial usage
AU	Absorbance units
BRD	Bovine Respiratory Disease
CFU	Colony Forming Units
OD	Optical Density
Cu	Copper
mg	milligrams
MIAs	Medically Important Antimicrobials
MIC	Minimum Inhibitory Concentration
ml	milliliters
NaCl	Sodium Chloride
RSD	Relative Standard Deviation
Zn	Zinc
µmol	micromoles
µl	microliters
°C	Degree Centigrade

Chapter 1: Introduction

1.1 Beef cattle production systems in Alberta

Canada produces approximately 2% of the beef in the world, and 5% of the beef exports worldwide come from Canada (sixth place worldwide), generating around 2.3 billion a year for the Canadian economy(1). In Canada, the most prominent province involved in beef production is Alberta, which finishes 69% of the nation's beef cattle and accounts for more than 40% of the national herd(2). As of January 2020, approximately 2.5 million of the 4.3 million head of beef cattle in Alberta were in cow-calf operations(3). Out of 150 feedlots in Alberta surveyed by Canfax in 2018, 115 were below 10,000 head capacity, 23 between 10,000-20,000 and 12 above 20,000, the latter accounting for 35% of the total feedlot beef population within the province(4).

Cow-calf production systems are the backbone of the feedlot industry and represent more than 5 million head in Western Canada(3). The herd composition in Western Canada has been estimated as 86% commercial cross-bred and 14% purebred(5). Cow-calf herds are constituted of cow-calf pairs, heifers and bulls. The bulls do not roam on the same pastures as cows and are only introduced to the cow herd in early summer for breeding with a 1:25 bull-cow ratio for a span of 60-70 days(6). Approximately 270 to 300 days later calving season begins(6). The calving season in Western Canada starts in late March and spans for an average of 87 days in commercial herds(5). The calving season is usually timed just before or to coincide with high availability of grass to provide optimal nutrition for the cow that will then provide milk to the calf. Furthermore, the warmer weather during spring calving has been associated with lower calf mortality rates in comparison to winter calving in Alberta(7). Parturition is followed by a series calf processing procedures that include navel disinfection, ear tagging and in some cases early castration and dehorning(6). Vaccination during the suckling stage is also recommended as the low-stress levels

and time before weaning allows for an appropriate immune response(8), After six-to-eight months in the cow-calf operation, the calves are taken to auction markets and sold to feedlots when they, weigh between 600 and 900 pounds. The seasonal nature of the system means that the majority of the calves arrive during fall, with a smaller number arriving during the winter after backgrounding (pasture fed or grown in small feedlots with forage based diets until they reach one year)(9). A feedlot is an intensive feeding operation in which beef cattle or other livestock are housed to gain weight by consuming energy-rich diets before slaughter. Alberta Agriculture and Forestry defines a feedlot as "Any land enclosed by a fence or other means which is used or intended for use for the purpose of feeding cattle in confinement."(10). The primary purpose of this system is to boost the growth and weight gain by reducing the energy expenditure caused by foraging while increasing the intramuscular deposition of fat (marbling)(11).

Once in the feedlots, the calves' diet gradually changes from forage and some grains to almost entirely grain in the later stages. This diet produces tender and marbled beef while enhancing weight gain(12).The calves spend between 60-200 days in the feedlots, depending on their entry weight(13). The animals are usually ready for slaughter when they reach between 1100-1400 pounds depending on their frame(14).The vast majority of the calves purchased come from auction markets and are transported on livestock trucks to the feedlots(15). Studies have shown that stress suffered during transport affects the performance and health status of the calves(16). Additionally, calves are exposed to a group of management procedures referred to as processing during their first 24 hours in the feedlots. These procedures include ear tagging, vaccination against bovine respiratory disease (BRD) pathogens and clostridial diseases, deworming, mass medication of antimicrobials for disease prevention (metaphylaxis), and administration of abortion inductors to heifers(17, 18). Processing procedures have the objective of preventing disease and gradually

adapting the calves to the feedlot environment. However, the stress caused by them has a negative effect on calves which might be alleviated by using preconditioning as an alternative practice(19). Preconditioning is a term used to describe a set of practices at the cow-calf operation that prepare the calves while on pasture, before their transition to the feedlots(18). The practices include vaccination, deworming, castration, dehorning, soft weaning and eating from bunks, allowing time for the calves to adapt before being shipped. Studies have shown that preconditioned calves display less morbidity when compared to conventionally managed calves(20).

1.2 Causes of disease in cow-calf operations

Antimicrobials are used in cow-calf operations to treat bacterial diseases in cows, bulls and calves. The usage is directed to the affected individuals as the extensive nature of the operation reduces the spread of disease. Common causes for treatment in bulls include lameness, pneumonia, and infectious kerato-conjunctivitis(21). Causes of treatment in cows are similar to the ones in bulls with the addition of reproductive tract diseases and mastitis(21). Calves in cow-calf operations are treated for diarrhea, pneumonia and navel illness(22).The presence of newborn calves and the longer lifespan of both cows and bulls causes the presence of some diseases absent in feedlots. The diseases common on feedlots and also present in cow-calf operations will be addressed in Section 1.3.

1.2.1 Diarrhea in beef calves

Infectious diarrhea is one of the main challenges for the cow-calf industry with a prevalence of around 5% in western Canada(22). Calf diarrhea in beef calves can be caused by bacterial (*Escherichia coli),* viruses (rotavirus and coronavirus) and protozoal (*Cryptosporidium parvum*) with some cases being co-infected with more than one (23). A recent case-control study of calf

diarrhea across Midwest United States showed that diarrhea has the highest prevalence in calves between 0-4 weeks of age with most cases being clustered at less than 2 weeks of age (24). Regardless of the etiology, factors like dystocia, calf immunity, perinatal management and environmental contamination influence the likelihood of disease and its prognosis once established(25). Diarrhea can be caused by decreased absorption or increased secretion within the intestines(26). Bacterial pathogens like enterotoxigenic *E. coli* cause an increase in secretions by altering the membrane pumps within cells(27). Conversely, diarrhea caused by viral and protozoal pathogens is caused by the reduced absorption of fluids within the intestines by the destruction of villous epithelial cells (28). In the case of diarrhea caused by rotavirus and coronavirus, secretion overload is further increased by the compensatory hyperplasia of secretory crypt cells(29).

The treatment of calf diarrhea should be targeted towards reestablishing the acid-base balance and electrolyte levels, providing nutritional support and treat bacterial infections(30). The usage of antimicrobials in calf diarrhea has been associated with increased levels of antimicrobial resistance in dairy cattle farms, therefore, only animals suspected to be affected by bacterial pathogens should be treated(31). Antimicrobial treatments should be directed towards the bacterial pathogen isolated from diseased calves and to manage severely ill calves with risk of bacteremia(32). The selection of antimicrobials should be based in susceptibility testing results, however, empirically the treatment should target *E. coli (33).* The options for treatment include tetracyclines, sulfonamides, penicillins, phenicols and cephalosporins (21). However, the usage of critically important antimicrobials as cephalosporins for the treatment of calf diarrhea is controversial based on their importance for human medicine and the potential dissemination of antimicrobial resistance (33-35)

1.2.2 Umbilical infections in calves

The pathogenesis of umbilical infection in calves starts by the penetration of bacteria by the umbilicus in particular in calves with failure of passive transfer or those exposed to poor hygienic conditions. The invading bacteria can then infect the urachus (most commonly), umbilical arteries and umbilical vein(36). Bacteria affecting those structures can cause septicemia and migrate through the bloodstream affecting joints, liver, kidneys and lungs(37). Clinical signs of infection include heat, swelling, discharge and concurrent signs of pneumonia, joint infection and diarrhea(38). Common bacteria isolated include *E. coli*, *Trueperella pyogenes*, *Enterococcus* spp. and *Proteus*(38). The treatment of umbilical infections should be based on antimicrobial treatment in early stages and surgical removal of the structures once systemic compromise is present(39). A study in Western Canada reported that 68% of the cow-calf ranches use antimicrobials for the treatment of umbilical infections, the third cause of treatment in calves behind diarrhea and pneumonia(21). The same study showed that florfenicol, oxytetracycline and penicillin are the most common antimicrobials used for the treatment of navel infections in beef calves(21). The prevention of navel infections is based on proper navel disinfection shortly after birth, with studies showing that Navel Guard (SCG-Solutions Inc., McDonough, GA) 2% chlorhexidine gluconate and 7% iodine tincture are similarly effective for that purpose(40).

1.2.3 Transition of calves from cow-calf operations to feedlots

Calves born in cow-calf operations are sold to feedlots either on auction markets or, less commonly, directly from the ranch. The transportation and transition of calves from different origins to a confined environment poses challenges for the feedlot industry as those stressors increase both morbidity and mortality(18). Studies have shown that the origin of the calves has an

effect on the prevalence of antimicrobial resistance in BRD pathogens during the early feeding period (41). Consequently, the antimicrobial resistance prevalence at the cow-calf ranch has an effect on the prevalence of antimicrobial resistance at arrival to feedlots. A recent review article suggests that the morbidity and mortality caused by BRD has not changed dramatically over the last 45 years despite the availability of more effective treatment (42). Therefore, the emergence of antimicrobial resistance in feedlot cattle is a reason for concern for the beef industry. The integration of the management practices in all phases of the beef production cycle is necessary to reduce the prevalence of antimicrobial resistance and prevent the increase in morbidity and mortality during the early feeding period.

1.2.4 Bacterial diseases affecting mature beef cattle in pasture

1.2.4.1 Infectious bovine keratoconjunctivitis

Infectious bovine keratoconjunctivitis (IBK) or 'pinkeye' is the most common ophthalmic disease affecting both beef and dairy cattle herds. The clinical signs include conjunctivitis, blepharospasm, photophobia, corneal ulceration and lacrimation(43). Full recovery is expected in mildly affected individuals, while corneal scarring and vision loss has been observed in more severe IBK infections(43). *Moraxella bovis* is widely regarded as causal agent of IBK in cattle, however, the presence of other pathogens like infectious bovine rhinotracheitis (IBR) virus and *Mycoplasma* spp. predisposes cattle to corneal injury caused by IBK(44, 45). Furthermore, solar irradiation, mechanical trauma and flies are considered as risk factors for IBK(46). The prevalence of IBK in beef cattle is estimated at 5%, with lightly pigmented breeds as the Hereford being more susceptible to infection (47, 48). Affected calves often show reduced weight gains, a recent study revealing an association between IBK and lower weights at weaning with the affected group of

calves weighing 8.2 kg less in average than healthy calves (49). A study conducted in the 1970's shows similar results with 17 kg difference between affected and unaffected male calves at weaning(50). In pasture-raised calves followed over a 4-year production period, the average weight reduction was 5 kg for calves with unilateral IBK and 15.75 kg for calves with bilateral IBK (51). The treatment is based on antimicrobial therapy with either oxytetracycline, florfenicol or ceftiofur, all proven effective as a parenteral treatment(52). Additionally, NSAIDs like flunixin meglumine can be added to the treatment to reduce inflammation and ocular pain in more severe cases(43). The prevention of IBK is based on promptly vaccination (6 weeks before the expected peak of cases) and the control of risk factors associated with the disease.

1.2.4.2 Metritis in beef cows

Studies in western Canada haven shown that reproductive tract diseases and mastitis are the main bacterial diseases affecting beef cows (21, 53). Nevertheless, beef cows have shown a greater ability to clear uterine inflammation in comparison to dairy cows which reduces the effect of metritis on reproductive success(54). Metritis is general term used to describe the post-partum infections affecting the endometrium and deeper layers. The infection is caused by the contamination of the uterus by bacteria during the early post-partum period (1-10 days). *Escherichia coli* and anaerobes like *Fusobacterium necrophorum*, *Bacteroides* spp. and *Trueperella pyogenes* are commonly found as causal agents(55). Clinical signs of metritis include fever, depression, dehydration, diarrhea and uterine discharge(56). The treatment of the disease is based on systemic antimicrobials such as oxytetracycline, penicillin, ampicillin, or ceftiofur, with the latter being the most common (57). Intrauterine antimicrobials have also shown positive results (58, 59).

1.2.4.3 Mastitis in beef cows

Mastitis is the term used for the inflammation in the mammary gland and multifactorial disease affecting both dairy and beef cows. A number of different bacteria can cause mastitis including *Staphylococcus aureus*, *Streptococcus agalactiae*, *E. coli*, *Klebsiella pneumoniae,* and *Corynebacterium bovis* to name a few(60). Factors like environmental contamination, early lactation, poor udder conformation and deficient immune response predisposes cows to the disease (61). The profitability of cow-calf production system is affected by the presence of mastitis in beef cows which has been associated with decreased body weight gain in calves (62, 63). Beef cows are treated only for clinical mastitis as subclinical cases are usually not detected(64). Treatment of mastitis should be based on antimicrobial susceptibility results because resistance is common(65). As environmental pathogens are more common in beef cows, treatment with beta-lactam antimicrobials is common. Third generation cephalosporins appear to be effective in parenteral treatment of mastitis caused by coliforms(66). However, authors suggest that the risk of development of resistance and the importance of this class for human medicine, outweigh the benefits of this class of antimicrobials(60).

1.3 Causes of disease in feedlot cattle

The transportation, subsequent change of environment, and the management procedures that calves undergo at arrival at the feedlot cause impairment of the immune response to pathogens (67). Opportunistic pathogens like the ones responsible for BRD (*Pasteurella multocida, Mannheimia haemolytica, Histophilus somni*) are commonly the first cause of morbidity and mortality on feedlots at arrival(68). As the production cycle advances, other diseases influenced by management practice, and environmental conditions become more common. A clear example

are liver abscesses occurring later in the feeding cycle associated with grain-heavy diets that predispose cattle to rumen acidosis(69). Other diseases, such as footrot and digital dermatitis, are influenced by environmental and management factors, causing lameness and morbidity in feedlot cattle(70). Common bacterial diseases in feedlot cattle are summarized in Table 1.1.

Table 1.1 Common causes of bacterial disease in feedlot cattle

Disease	Causal agent(s)	Treatment/Prevention
Bovine Respiratory Disease	*Mannheimia haemolytica* *Pasteurella multocida* *Histophilus somni* *Mycoplasma bovis* *Trueperella pyogenes*	Metaphylaxis In-feed antimicrobials Parenteral antimicrobials
Liver Abscesses	*Fusobacterium necrophorum*	In-feed antimicrobials
Infectious Lameness (footrot and digital dermatitis)	*Fusobacterium necrophorum* *Treponema* spp.	Parental antimicrobials In-feed antimicrobials

Information for the elaboration of this table was obtained from the Compendium of Veterinary Products- Canada Edition(71).

1.3.1 Bovine Respiratory Disease (BRD)

Bovine Respiratory Disease (BRD) is the most devastating and costly disease affecting beef cattle, increasing the cost of production by 7% ,with the cost increasing depending on the number of treatments needed(72, 73). The decrease in carcass quality in animals that have been affected also affects the cost of production, increasing the importance of the prevention of this disease(74). The prevalence of this respiratory disease complex has been estimated as high as 16.2% in feedlots of over 1000 head in the US, which can be extrapolated to roughly150 feedlots in Alberta based on herd sizes(75). However, there are inherent differences between the US and Canada feedlot systems limiting our ability to extrapolate the prevalence of BRD. The calves placed in stocker operations before getting to the feedlots, and are heavier and better prepared for the feedlot environment(76). In contrast, calves placed in Canadian feedlots are mostly auction

market derived and newly weaned which predisposes them to by causing stress through transport, change in feed, lack of familiarity with feedbunks and waterers etc… or exposure to calves from different origins(77). Researchers have found that more than 50% of the mortalities in feedlots are related to BRD(78). A series of factors including stress, immunological status , and the presence of viral diseases cause the manifestation of BRD and its multi-agent etiology make the prevention and treatment extremely difficult(79).

The term BRD is used to categorize any undifferentiated respiratory disease, and it is usually accompanied by fever, cough, and other respiratory signs(80). The pathogens that cause this disease can be divided into viral and bacterial. On the viral side, bovine viral diarrhea virus (BVDV), infectious bovine rhinotracheitis (IBR), bovine respiratory syncytial virus (BRSV), and bovine parainfluenza-3 have been identified in cases(80). The bacteria that may contribute to this disease complex include *Pasteurella multocida*, *Mannheimia haemolytica*, *Mycoplasma bovis* and *Histophilus somni*, all of which are normal inhabitants of the upper respiratory tract(81). The proliferation of bacteria from the upper respiratory tract in the lower airways is aided by the impairment of the immune system caused by both transport and viral infections. The presence of BVDV has been related to increased severity of bacterial bronchopneumonia; this has been associated with the immunosuppression effects caused by the virus(82).

1.3.2 Liver Abscesses in feedlot cattle

Liver abscesses are the most frequent cause of condemnation of the liver at slaughter(83). Liver abscesses can occur during any point of the feeding period, and have a high economic impact on the feedlot industry(69). According to the National Beef Quality Audit in 2011, liver condemnation percentage was 20.9%, with two-thirds of livers affected by liver abscesses(84).

The losses caused by liver discounts amassed to 61.2 million CAD in Canada during the 2016-2017 period(85). Despite those numbers, the biggest economic impact caused by liver abscesses is the reduced carcass quality and decreased average daily gains during the production period; these effects are only noticeable in cattle with severe abscesses(83).

The pathophysiology of the disease requires the proliferation of *Fusobacterium necrophorum,* either alone or in conjunction with other pyogenic bacteria, in the liver(86). Bacteria can migrate to the liver by various routes, including the portal vein, hepatic artery, or rumen perforation. The main risk factor is the grain-rich low forage diet on feedlots as this ferments rapidly in the rumen causing rumen acidosis, leading to rumenitis. *Fusobacterium necrophorum* from the rumen microflora can colonize, proliferate and enter the rumen wall(87, 88). Subsequently, the bacteria migrate via the portal vein to the liver and cause hepatic abscessation; this has been confirmed by genotyping of the bacteria from both organs(89).

1.3.3 Lameness in beef cattle

Lameness is the term use to define the manifestation of pain inducing alterations that result in changes in the normal locomotion of the animal(90). Lameness is a concern for the beef cattle industry from both an economic and a welfare standpoint. In a study that focused on estimating the prevalence of lameness on feedlots in Alberta, 32.3% of the all cattle suffered lameness (85). The results suggest that lameness is the second most common cause of treatment on feedlots behind BRD(91). The economic impact of lameness on the feedlot industry is well described, with costs being incurred through treatment and losses by discarding cattle before they reach ideal weight(92). Lameness affects the feeding behavior in cattle which causes losses by reducing average daily gains and increasing the mean days of feed (90, 93). Early detection and accurate

treatment measures for lameness were associated with positive net returns when compared to later treatments(92) The causes of lameness on beef cattle can be divided into infectious and non-infectious(94). The infectious disease conditions include footrot, digital dermatitis, and joint infections (95, 96). Bovine foot rot is a bacterial disease that affects the subcutaneous tissue and interdigital skin. The anaerobic bacterium *Fusobacterium necrophorum* is described as the main bacteria associated with the disease(97). Lesions can progress to affect bone and ligamentous structures leading to joint infections(98) The treatment of foot rot consists in antimicrobial therapy and NSAIDs to control the pain and inflammation. Common used antimicrobials for the treatment of footrot in beef cattle include oxytetracycline, procaine penicillin, ceftiofur and tilmicosin(96). A study conducted in feedlot cattle in Canada showed that oxytetracycline and ceftiofur are comparable in their effectiveness as a treatment options for footrot(99).

Digital dermatitis differs from foot rot both in the etiology and epidemiology. The first fundamental difference is that despite the isolation of *Treponema* spp. in 70% of the lesions, the inoculation of the agent is not sufficient to cause the disease experimentally(100). It has been hypothesized that the impairment of the innate immune response is a key factor for the development of lesions(101).The disease also has a contagious nature which poses challenges for both treatment and prevention(102).Several studies have suggested that environmental factors like wet pastures and floors predispose cattle to infectious causes of lameness(91, 103). The wetness in ground surfaces causes softness in the hoof and adjacent skin which then predisposes to lesions and penetration of environmental bacteria.

Trauma, musculoskeletal disorders, injury, and toe-tip necrosis syndrome are examples of non-infectious causes(104). The tip-toe necrosis syndrome seems to be caused by the constant wear of the solar horn leading to the formation of a space in the apical portion of the white line

allowing for bacterial proliferation(104).Hence, non-infectious causes of lameness can also lead to bacterial infections. Factors like inappropriate handling before and after arrival and cattle temperament have been associated with non-infectious causes of lameness as this makes them prone to injuries(105).

1.4 Antimicrobial usage in cow-calf operations

A recent survey compiling the antimicrobial usage patterns in cow-calf operations in western Canada showed that the most common antimicrobial classes used in treatments are tetracyclines, phenicols, macrolides, penicilins and sulfonamides in descending order(106). Most of the antimicrobials are administered parenterally with a few oral formulations used commonly for the treatment of diarrhea in calves and disease prevention(106).

1.5 Antimicrobial usage in feedlots

The usage of antimicrobials in feedlots is necessary to treat or prevent diseases for which vaccination is unavailable or ineffective(107). Consequently, four different kinds of usages of antimicrobials occur in feedlots(108). Aside from the treatment of clinical disease, antimicrobials are used for metaphylaxis and prophylaxis. Therapeutic usage of antimicrobials in beef cattle is directed towards the treatment of diseases such as BRD, histophilosis, footrot, liver abscesses, and infectious kerato-conjunctivitis(107). Currently, a variety of antimicrobials are available for the treatment of BRD including: oxytetracycline (tetracycline), tulathromycin, gamithromycin, tildipirosin and tilmicosin (macrolides), ceftiofur (β-lactam), enrofloxacin (fluoroquinolone) and florfenicol (phenicol)(109). The majority of those above are administered parenterally, with tilmicosin and oxytetracycline having oral formulations(71).

1.5.1 Metaphylaxis

Metaphylaxis is the parenteral mass medication of a group of susceptible or high-risk animals upon arrival to prevent the manifestation of disease(110). In feedlots, metaphylaxis is used to decrease the incidence of acute BRD and for reducing mortality in high-risk calves(111). The category of high-risk calves is used for animals in which it is likely that acute disease might happen before vaccination is able to raise immunity(15, 111, 112). Calves that are underweight, recently weaned, unused to eating from bunks, auction market derived and excessively commingled can be classified as high-risk(112, 113). Metaphylaxis has gained popularity as studies have shown that clinical signs of BRD (depression, nasal discharge, fever) are not reliable indicators of the disease(114-118). Other early detection methods (accelerometers, ultrasonography) have drawn mixed results (119). Consequently, we use methaphylaxis as a management protocol not only for disease prevention but to treat diseased animals that are not identified by clinical diagnoses(120).

Various studies have supported the use of metaphylaxis for prevention and control of BRD and histophilosis in feedlots, some going back to the 1980s(121-123). From the antimicrobial options available, long-acting oxytetracycline(tetracycline), florfenicol, tulathromycin, and tilmicosin (macrolides) are the most used in Canadian feedlots(124, 125). However, not all the antimicrobials used in metaphylaxis appear to have the same effect on reducing the prevalence of disease or retreatment rates(126, 127). A study compared the effect of tulathromycin, tilmicosin, and oxytetracycline used for metaphylaxis; the findings indicated that tulathromycin reduces both initial treatments for undifferentiated fever and overall mortality rates. Tulathromycin also showed to be most cost-effective alternative, as the net advantage was 3.79 CAD/animal those obtained by using tilmicosin and 16.96 CAD/animal compared to those obtained by using oxytetracycline (123). Similarly, Nickell and colleagues compared the morbidity and mortality in groups treated

with tulathromycin or tilmicosin at day one after arrival. The study showed that cattle in the tulathromycin group displayed lower BRD morbidity (32.8% to 68%) and lower mortality (3.6% to 13.5%). In addition, average daily gains were greater in the tulathromycin treatment group (128).

Using combinations of antimicrobials for metaphylaxis has also been explored. in one study, randomly allocating calves in three different groups, they were treated metaphylactically with one of three different treatment protocols(129).The first protocol included a dose of ceftiofur and a subsequent dose tulathromycin 8 days later, the second group received ceftiofur only and the third group received ceftiofur with tilmicosin. Results indicated that calves treated with the combination of ceftiofur with tulathromycin showed significantly lower morbidity when compared to the other treatments. Nevertheless, the usage of ceftiofur in mass medication is highly controversial as cephalosporins continue to be extremely important for treatment in human patients(130). This matter prevents the widespread usage of ceftiofur in metaphylaxis protocols in feedlots.

Conversely, another study favored the use tildipirosin over tulathromycin in metaphylaxis based on the scoring of lung lesions and minimizing clinical disease after the inoculation of *Histophilus somni(131).* This seems to be related to the fact that tildipirosin has a longer half-life in the lungs than tulathromycin(71). There is a gap in the effect that metaphylaxis has on histophilosis given the fact that the TME and myocarditis presentations of the disease aren't accounted for in the studies(117). This may not be important as, evidence suggests that the clinical presentation of histophilosis in Canada is changing with TME becoming less prevalent(132).

Evidence suggests that metaphylaxis is an economically feasible way to reduce the losses caused by BRD and histophilosis(133). A recent meta-analysis compared 37 metaphylaxis trials

conducted either at arrival on feedlots or in stocker cattle(134). The results showed that cattle treated with tulathromycin have lower odds of mortality from day 1 to finish compared to those treated with tilmicosin (4 times greater) or oxytetracycline (5 times greater). The meta-analysis also suggested that there exists no significant difference between metaphylactic administration of oxytetracycline and controls. The results from the meta-analysis further support the clear differences in effectiveness existing between drugs used for metaphylaxis for control of BRD.

The selection of resistant bacteria in cattle treated by mass medication methods has been observed in both feedlots and stocker beef cattle(135). The exposure of bacteria to antimicrobials will naturally contribute towards the selection of resistant strains(136). However, the selection pressure appears to vary between different antimicrobial classes and stages during the feeding period. The clearest example is the transient increase of tetracycline resistance in cattle administered tetracyclines during the early stages of the feeding period (137-139). As the feeding period wanes the tetracycline resistance levels between control and treatments appear to be comparable(139). Development of resistance to macrolides, the most frequently used class in metaphylaxis, has also been explored. Several studies have shown that the administration of tulathromycin has a minimal effect on the selection of resistant bacteria early during the feeding period(140, 141). Based on the effectiveness of the drug class and the evidence of low resistance selection capacity, macrolides appear to be a safer choice for metaphylaxis in feedlot cattle. Though it is important to keep in mind that bacterial communities vary greatly between management systems and geographical areas, meaning that susceptibility testing is always the preferred mean to choose a therapeutic regime.

Table 1.2 Injectable antimicrobials labelled for metaphylaxis

Antimicrobial	Commercial name	Dosage (mg/kg)	Route
Florfenicol	Florkem® Nuflor®	40	SC
Gamithromycin	Zactran®	6	SC
Tildipirosin	Zuprevo®	4	SC
Tilmicosin	Micotil™ TilcoMed®	10	SC
Tulathromycin	Draxxin®	2.5	SC

1.5.2 Antimicrobial added in-feed for prophylaxis

The usage of medically important antimicrobials in feed is a widespread practice in the Canadian beef cattle industry as they have been used to treat or prevent disease. The use of non-medically important antimicrobials like ionophores (monensin, lasalocid) is still allowed either to manage coccidiosis (invasion of the intestinal tract by protozoa) or to enhance feed conversion efficiency (142, 143).

Most feedlots in Alberta add the antibiotic tylosin to feed throughout the feeding period to prevent liver abscesses caused by *Fusobacterium necrophorum* and *Trueperella pyogenes(125).* Tylosin is used to decrease losses caused by abscesses at slaughter and improve feed conversion efficiency (69, 84, 144). In the past chlortetracycline has been an alternative for the prevention of liver abscesses; however, it has now proven to be less effective than tylosin(145). A dosage of 60-90 mg/head of tylosin on feed is used in feedlots(87).Despite the constant usage of in-feed treatment, there is currently no evidence of an increase of antimicrobial resistance in *F. necrophorum.*(146).

Other indications for antimicrobials in feed or water in Canada are for foot rot, BRD, and histophilosis prevention and treatment(71). There is evidence of the negative effect caused by lameness on average daily gains and losses caused by death or railing of cattle(92, 147, 148).

Consequently, chlortetracycline may be added to feed for the prevention of foot rot in Canadian feedlots at a dosage of 70 mg/ head/day (149). In Alberta, chlortetracycline is pulsed in-feed for two separate five-day periods at a dosage of 6 grams/head/day with a day in between to prevent and reduce mobility caused by Histophilosis during the early feeding period (150).

Although there is evidence suggesting a reduction in morbidity, there is an increasing concern over the use of antimicrobials in feed as it may select for antimicrobial-resistant bacteria(151, 152). A study evaluated the effect of chlortetracycline in feed on the abundance of 10 antimicrobial resistance genes in feces; six of ten genes were significantly higher in the treatment group(153). In contrast, other studies have found non-significant differences between conventional antimicrobial usage and antimicrobial-free pens (154, 155). It is imperative to limit the usage of antimicrobials to treatment and prevention of disease as resistance seems to be on the rise in bacterial pathogens in feedlots(107, 156, 157).

1.6 Antimicrobial resistance

Antimicrobial resistance is an emerging threat to both human and animal health. The evolutionary changes in bacteria over time are one of the reasons for the reduction of the efficacy of antibiotics(158). Bacteria have outstanding genomic plasticity which facilitates the adaptation to overcome environmental threats including antimicrobials(159). As a result, the bacterial exposure to antimicrobials contribute towards the acquisition of resistance determinants from exogenous sources (horizontal gene transfer) or caused by mutations within the bacterial genome. The inappropriate use of antimicrobials in human medicine has accelerated this process to the point in which routine surgical procedures and organ transplants have become a considerable risk (160). The economic burden caused by this threat has been estimated at over 30 billion USD in the

United States alone (161). In Canada, 26% of all the infections treated in 2018 at human hospitals were reported to be resistant to antimicrobials with the cost of treatment totalling approximately 1.4 billion CAD to the healthcare system, with the frequency of resistance expected to reach 40% by 2050 and associated treatment costs increasing to 7.6 billion CAD(162). Studies have shown a link between antimicrobial usage in food animals and antimicrobial-resistant infections in humans(163, 164). Cases of haemolytic uremia have been traced back to ground beef consumption in the US and Argentina(165, 166). Unfortunately, the burden caused by mild zoonotic diseases on humans is not well understood as surveillance does not usually account for follow-up of the patients(167). Furthermore, the development of new antimicrobials is a slow process affected by scientific hurdles and unclear financial feasibility(168). Measures need to be taken to maintain the effectiveness of the available antimicrobials for both human and veterinary medicine and prevent resistant zoonotic infections.

Judicious use and responsible stewardship is required to maintain the effectiveness of antimicrobials, especially in veterinary medicine as monitoring of usage has been an issue (169). Good Stewardship Practice (GSP) in veterinary medicine encompasses the multidisciplinary practices required to maintain the efficacy of antimicrobial drugs(170). The implementation of GSP is based on a dynamic approach in which practitioners, policymakers, diagnostic laboratories and pharmacologists collaborate to ensure the responsible usage of antimicrobials(171).Throughout the process different sectors cooperate to develop antimicrobial usage guidelines, implement antimicrobial usage monitoring and educate both veterinarians and farmers on the importance of stewardship(172). In the case of production animals, the emphasis has been placed on antimicrobial usage guidelines and susceptibility testing resulting in a reduction of antimicrobial resistance (173, 174). Recent regulatory changes in antimicrobial usage in Canada

include the restriction of medically important antimicrobials to those prescribed by veterinarians and the regulation of pharmaceutical imports. Those changes are expected to cause a more judicious usage of antimicrobials as approximately 95% of antimicrobials used in livestock were not prescribed by veterinarians in 2018 (175).

Resistance determinants are disseminated in the environment, increasing the risk for pathogenic bacteria from animals or humans to acquire them; this transfer of genes has been demonstrated by previous studies (176, 177). A multisystemic approach is required to tackle the issue, considering not only the animals and humans but the environment as a potential source of resistance genes and resistant bacteria.

1.6.1 Antimicrobial resistance mechanisms

Bacteria are complex microorganisms. They can respond to and adapt to stressors using a variety of different mechanisms. As a result, antimicrobial resistance exhibited by bacteria can be intrinsic, adaptative, or acquired(178). Intrinsic resistance can be defined as an antimicrobial resistance shared within a bacterial species, independent of horizontal gene transfer or previous exposure to the antimicrobial (179). Examples of intrinsic resistance include the lack of a cell wall in *Mycoplasma* species conferring resistance to beta-lactams and glycopeptide resistance in Gram-negative bacteria due to the impermeability of the outer membrane(180, 181). Acquired resistance is defined as the antimicrobial resistance exhibited by previously susceptible bacteria after mutations or the acquisition of genes(182). The acquisition of genes is mediated by horizontal gene transfer, which can occur by transformation, transduction, or conjugation(183).

Molecular mechanisms like transformation and transduction are important but limited in their spread as the first one is restricted to naturally transformable bacteria as the process is initiated by the host and the latter requires a bacteriophage to facilitate the transfer (184, 185). On the other

hand, conjugation has recently has recently being identified as the most important mechanism of antimicrobial resistance transfer between bacteria in agricultural production systems(186, 187). Conjugation involves the transfer of genetic material from one bacteria to another; the mechanism requires physical contact between bacteria and mediation by mobile genetic elements(188). Mobile genetic elements able to mediate conjugation include plasmids and integrative conjugative elements (ICEs) both of which have been found in bacteria from beef cattle origin(156, 189, 190)

Adaptative resistance mechanisms are the most concerning for both human health and veterinary medicine. The exposure of bacteria to antimicrobials can lead to ab alteration in protein expression or genes enabling resistance in a previously sensitive bacterium(191). This phenomenon can be demonstrated by exposing bacteria to gradually increasing concentrations of antimicrobials; after a couple of days, the bacteria are able to survive concentrations 20-fold higher than the initial exposure (192). However, this mechanism is unstable as bacteria start to become sensitive after a couple of generations without exposure to antimicrobials(193). Exposure to antimicrobials can also cause selection of resistant strains and mutation in bacteria with both potentially increasing antimicrobial resistance. The absence of exposure to antimicrobials also has an apparent effect on the expression of phenotypes *in vivo*. A study looked at the cephalosporin resistance phenotypes of *Salmonella enterica* serovar Heidelberg and *E. coli* after a voluntary withdrawal of ceftiofur in hatcheries(174). The annual report from the CIPARS in 2003 showed that cephalosporin resistance was more common in chicken meat than in humans, this triggered voluntary cease of *in-ovo* administration of ceftiofur in hatcheries between 2005-2006. Subsequently, ceftiofur was partially reintroduced to treat chick omphalitis between 2007-2008. The study compared the resistance to cephalosporins in the three periods of time, with the results showing an association between ceftiofur usage with higher levels of resistance to cephalosporins.

Conversely, another study found resistance genes in soil from backgrounding beef operation two years after closure (141). Concentrations of *Enterococcus* spp. and resistance gene copies from the soil in the feeding area were significantly higher than the ones on the grazing area, suggesting the accumulation of feces caused by confinement might have on persistence of AMR(194). Based on the current body of evidence, it is clear that acquired resistance to antimicrobials and its persistence is influenced by AMU but also by the inherent factors of each bacterium.

1.7 Antimicrobial resistance in beef cattle

Antimicrobial resistance in feedlots is a huge concern based on the hazards it poses to both animal and human health(107). Bacteria of beef cattle origin can be divided into zoonotic and bovine pathogens. Zoonotic bacteria are key for monitoring antimicrobial resistance in beef cattle production based on their relevance to human health and isolation from healthy animals or sub-products(195). Resistance in bovine pathogens has also been reported, particularly in bacteria that cause BRD(196).

1.7.1 Antimicrobial resistance in zoonotic bacteria from beef cattle origin

Enteric bacteria as *Campylobacter, Salmonella*, *E. coli*, and *Enterococcus* spp. are the major zoonotic foodborne bacteria from feedlot origin(107). Resistance determinants to various antimicrobials have been detected in samples from feedlots and ground beef. A recent study looked at the resistance determinants found in *E. coli* from both fecal samples from feedlots and human wastewater, with results demonstrating that they share the same pool of extended-spectrum cephalosporin-resistance genes in both sample types (142). However, the multilocus sequence typing conducted on both sets of genomes shows little similarity between groups of strains, which might indicate horizontal gene transfer or mutations in different loci within the different *E. coli*

strains(197). A similar study was conducted on *Campylobacter jejuni*, and *Campylobacter coli* isolated from feedlot cattle feces with 35.4% and 74.4% respectively having a resistance phenotype for fluoroquinolones(198). Based on multilocus sequence typing, the mechanism behind the dissemination of resistance seems to depend on clonal expansion for the *C. coli* with *C. jejuni* isolates being genetically independent which suggests horizontal gene transfer as the dissemination mechanism(198).

A cross-sectional study in the US looked at the prevalence and susceptibility of *Salmonella* spp. from feedlot origin. The results showed that the fecal prevalence of *Salmonella* spp. from preharvest cattle is highly variable between feedlots, with the susceptibility profiles being sensitive for fluoroquinolones on all feedlots despite the usage of antimicrobials(199). A study conducted by a group in the US looked at the resistance determinants in *E. coli*, *S. enterica*, and *Enterococcus* spp. in a cow-calf operation finding no differences between treated and untreated animals (145). The results suggest that other factors apart from usage have an effect on the dissemination of resistance determinants, though the results include just one cow-calf operation, which limits the scope of the study(200).

1.7.2 Antimicrobial resistance in bovine only pathogens

The term bovine only pathogens is used to define bacteria without zoonotic potential; the bacteria causing cattle diseases were discussed extensively in **Sections 1.2**. and **1.3.** Extensive research has been conducted on both the prevalence of and patterns of antimicrobial resistance in BRD pathogens(201). Most of the research has been focused on *Mannheimia haemolytica* based on the more virulent nature of the bacterium(202, 203).Nonetheless, studies suggest that outbreaks are primarily caused by commensal strains *M. haemolytica* and not by a single virulent strain(204,

205). A recent study estimated that 130/233 *M. haemolytica* isolated from morbid and dead cattle in Alberta were resistant to at least 4 classes of antimicrobials. (196).

Another cause for concern is the recent increase of multidrug resistance on BRD pathogens. A study showed that the treatment of pen-mates increases the likelihood of obtaining multidrug resistance isolates in untreated animals(125). Recent findings suggest that the spread of resistance in BRD causing bacteria may be related to horizontal gene transfer. A study looked at the prevalence of BRD pathogens and their respective antimicrobial resistance patterns in samples from Alberta, Texas, and Nebraska (135).The results showed that a group of 18 isolates coming from both Texas and Nebraska were carrying an integrative conjugative element that provided resistance for up to seven antimicrobials(190). Different integrative conjugative elements have also been identified in *Pasteurella multocida* and *Histophilus somni* which highlights their importance in the spread of AMR(206).

1.8 Supplementation of trace minerals in beef cattle operations

The term "essential element" is used to describe minerals that have a proven physiological role in the body. They can be classified as major or trace elements based on their concentrations in the animal and the quantities required in diet(207). Most of the essential elements required by ruminants are found on adequate concentrations in feed, though some of them should be supplemented to reach the dietary requirements (208). In the case of beef cattle nutrition, the goal is to provide enough minerals to exceed the maintenance level and fulfill the requirements needed for optimal growth and performance. Cattle ingesting mineral levels below the required minimum show deleterious effects on growth and immune responses before showing more apparent signs of deficiency(209). Despite this, economic losses in beef cattle nutrition are usually related to mismanaged or overpriced supplementation plans(210) A recent survey directed towards

determining the professional recommendations of nutritionists in the US found that values of microminerals supplemented were at least twice those recommended by The National Research Council(NRC) (208, 211). It is vital to maintain a balance between feeding supplements to meet the requirements without reaching toxicity, especially in the case of microminerals(212).

1.8.1 Zinc supplementation in beef cattle

Zinc (Zn) is a micromineral required for structuring and regulation of proteins and catalysis (213). In livestock, these functions are related to gene expression, appetite control, fat absorption, and antioxidant defense(214). Gene expression is interrelated to fetal growth and development as the reproductive cells differentiate and proliferate rapidly during gestation(215). Zinc deficiency has been related to abortions and retarded growth in neonates by the effects it causes on the metabolism of androgen hormones (216). Zn deficiency also causes increases in the levels of cholecystokinin, an appetite-regulating hormone(217). The supplementation of Zn has been associated to increased immune response in humans and animal models by Zn reducing the effects of oxidative stress on cells(218).

The Zn requirement set for beef cattle is 30 mg/kg of feed (208). The amount of zinc to be supplemented should be calculated, taking the basal zinc concentration into account, yet a survey shows that this is not happening with trace minerals(211). The vast majority of zinc supplementation to beef cattle in Canada is inorganic (sulfate, oxide), though it seems that there is a changing tendency in the US as the same survey showed that 45.4 % of the nutritionists preferred organic sources of trace minerals in finishing diets. Conversely, none of the respondents based their supplementation only on inorganic sources(211).

1.8.2 Copper supplementation in beef cattle

Copper (Cu) is a trace mineral vital for the activity of a variety of proteins, co-factors, and enzymes. Those enzymes play a role in iron transport, protection from antioxidants, and cellular respiration(214). One of those enzymes is cytochrome c-oxidase, an enzyme involved in the last phase of cellular respiration(219). Another enzyme called ceruloplasmin is essential for the mobilization and absorption of iron used for the synthesis of hemoglobin(207). Cu deficiency affects the immune system, by decreasing the amount of interleukin 2, which reduces T-cell proliferation and by reducing the availability of neutrophils(220).

The Cu requirement on feedlot cattle diets is 10 mg/kg(208). This requirement increases depending on the amount of molybdenum and sulfur in feed (221). Thiomolybdate is formed when sulfur from the diet is turned to sulphide in the rumen and reacts with molybdenum; this compound joins the available copper to form copper thiomolybdate which limits the absorption of the trace mineral(207, 222). The form of inorganic Cu supplemented in beef cattle diets are usually accompanied by sulfur (copper sulphate), forming thiomolybdate(223). Consequently, diets with high concentrations molybdenum require increased supplementation of Cu. Organic compounds have yielded higher absorption rates and are not affected by the presence of inhibitors in diet which reduces the environmental burden caused by supplementation(224).

1.8.3 Environmental residuals of trace minerals

Essential elements like Zn and Cu are vital for the regular function of an animal organism since they can form metallo-organic complexes to assemble proteins and enzymes, although this property might also cause the formation of toxic compounds(225). As a result, the organism regulates the absorption of these elements via transport proteins. This mechanism causes all the non-absorbed minerals to be excreted to the environment. Those factors determine that the

absorption percentage of Zn and Cu is inversely proportional to the supplementation amount. With the activity of the transporters increasing in the presence of deficiency(226).

The supplementation of trace minerals above required levels in beef cattle is a common practice in North America(211). Nevertheless, there is no research regarding the levels of Zn and Cu residuals on the environment. An experimental study in the US quantified the fecal excretion of both Zn and Cu in Angus steers, between two different inorganic supplementation schemes, with values for the excreted portion Zn being between 80-85% and 90-95% for Cu(227). A study by Zhou and colleagues looked at the levels of Zn and Cu both in soil and feces in dairy cattle farms in China, with the lowest values being inside the requirement range and the highest above five-fold the requirement (228). The relative abundance of resistance genes including tetracycline, aminoglycosides and beta-lactams in that study showed a correlation with the quantity of Zn and Cu in the environment (228). Another study found similar correlations between feces and soil samples from poultry, swine, and beef cattle origin(229).Nevertheless, the difference between production systems makes the extrapolation of the results complicated manner. It is imperative to obtain estimates of the levels of Zn and Cu excretions in beef cattle production systems to further assess the potential for co-selection of antimicrobial resistant bacteria.

1.9 Antimicrobial properties and mechanisms of zinc and copper

Metals fulfill cellular functions that organic compounds are not able to satisfy(230). As a result, metals are necessary for the biochemistry of living organisms(231). Around 25 percent of the proteins require metals atoms for their functionality and structure; those elements include iron, manganese, magnesium, zinc, and copper(232). Conversely, essential metals can also cause toxicity if present in surplus amount(233). In the case of non-essential elements like mercury and

silver, toxicity is reached at lower concentrations(234, 235). The bactericidal activity caused by metals has triggered the recent popularity of their use as antimicrobials, either on surfaces, coatings, or by direct application of the compounds(236).

Metals have different chemical properties that contribute towards bacterial toxicity; the most common are reduction potential, donor atom selectivity, and speciation(231). Donor atom selectivity is based on the premise that the atomic structure in metals determines their donor ligand affinity. In the case of transition metal ions the order that determines the preference for donor ligand is called the Irving-Williams series (Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II)(237). The donor ligand affinity provides proteins with selection parameters to ensure that the right metal is incorporated for protein function or folding(238). Another concept that determines the reactivity of metals is the Hard and Soft Acid Base (HSAB) principle. This principle classifies acids and bases either as soft, hard, or borderline. Based on the classification, the principle predicts that soft acids prefer soft bases, borderline acids prefer borderline bases, and hard acids prefer hard bases(239). Since Cu(II) and Zn(II) are both borderline acids, their bacterial toxicity will be less than soft acids (silver) based on their ability to form bonds with thiol groups (R-SH) found in bacterial proteins(240, 241).

Copper(II) is one of the most stable transition metals meaning that if other transition metals are present in similar amounts, proteins will most likely form a bond with Cu(242). Consequently, the levels of intracellular Cu need to be regulated to prevent toxicity(243). Channels such as OmpC porins are likely responsible for the passage of Cu(II) and Cu(I) through the outer membrane(244). The outer membrane protein ComC decreases permeability to Cu in *E. coli*, the expression of this protein is reduced in the presence of low copper availability to allow uptake(245).

Zinc is the second most crucial transition metal ion for living organisms behind iron based on its relative abundance(245). This metal plays a vital role in general cellular metabolism, bacterial expression, and as a cofactor for virulence factors. Bacteria incorporate Zn into 5-6% of all proteins(246). ZupT and ZnuACB are in charge of the uptake of zinc across the cytoplasmic membrane, with low and high affinity, respectively. In conditions of low Zn availability ZnuACB is in charge of the uptake; in the case of high zinc availability, the uptake is carried out by ZupT(247).

Several mechanisms have been cited as physiologically relevant causes of metal toxicity. The mechanism of toxicity depending on the metal in question(231). Transition metal ions like Cu have the potential to participate in redox reactions and trigger the synthesis of reactive oxygen species (ROS) (248). Copper (II) can be reduced to copper (I) in the presence of reducing agents like superoxide, once reduced copper is able to catalyze the formation of hydroxyl radicals from hydrogen peroxide by the Haber-Weiss reaction(249). Metals also have the ability to catalyze site-specific damage on bacterial proteins with the damage caused contributing towards bacterial toxicity(250). Studies *in E.coli* suggest that one or at most a few amino acids in any given protein are susceptible to metal-catalyzed oxidation(251). Carbonyl groups are the result of metal-catalyzed oxidation of amino acid chains; therefore, the level of carbonyl groups is used as an indicator of oxidative protein damage(231). Evidence from previous studies indicates that the family of Fe-S dehydratases can be inactivated by metals(252). The access of metals to sulfur atoms causes binding to sulfur and exerts toxicity by replacing iron (200). The replacement of iron leads to the inactivation of the protein and the arrest of key metabolic pathways causing toxicity(253).In addition, free iron within the cell causes the production of ROS. (252). Studies have shown that both Zn and Cu can exert toxicity by this mechanism in *E. coli* (252, 253).

1.9.1 Bacterial resistance mechanisms to zinc and copper

Extensive research has been conducted on copper surfaces as a bactericidal in touch surfaces at hospitals and other medical environments(254-256). It has been hypothesized that the bactericidal properties of copper surfaces are likely caused by the release of copper cations(257). Nonetheless, bacterial cells are able to regulate the levels of copper that enter the cell. Those mechanisms include relative impermeability of both internal and external membranes, restricted access by extracellular sequestration, and active extrusion from the cell(258). Studies have shown the *Cue* (copper efflux) system is responsible for resistance to copper in *E. coli(244, 259).* The transport from the cytoplasm to the periplasm is caused by ATP hydrolysis, which is dependent on the ATPase Cop-A gene(260). The remaining part of the *cue* system is *CueO*, a multicopper oxidase enzyme that oxidizes Cu(I) to the less toxic Cu(II) (261). The oxidation reactions caused by *CueO* are oxygen-dependent, with them terminating in the absence of this component(262). In the absence of oxygen, the *cus* system is in charge of removing periplasmic copper (259). The *cus* system is integrated into four proteins: CusA, CusB , CusC and CusF (263). CusCBA acts as a powerful efflux system acting around the entire cell envelope(264). In contrast, the CusF is a chaperone in charge of the transport of periplasmic copper to provide full resistance(265). Experiments with *Escherichia coli* suggest that the cell's ability to regulate the levels of copper is enough to prevent killing but not inactivation(266). Other resistance mechanisms include multicopper-oxidase (MCO), which can oxidize Cu(I) into Cu(2); this reaction requires the presence of oxygen(267). The presence of this gene has been confirmed in *Histophilus somni* (156).

Zinc resistance in bacterial organisms seems to be related to the efflux of the metal aided by transporters(268). Various systems have been identified in bacteria and can be part of the

chromosome or encoded on plasmids. The three most common transporter systems are P_1Btype ATPase, CDF transporters, and CBA efflux systems. The most efficient transporters are those from the P_1Btype ATPase family(269). The ZntA protein from *E. coli* is the most well-described member and has the ability to transport not only Zn but other metals like Cd (Cadmium) and Pb (Lead)(270). Another member of this group (CzrC) has been found in methicillin-resistant *Staphylococcus aureus* from swine-origin(271). The family of cation diffusion facilitator (CDF) is also linked to bacterial zinc export. Studies have shown that the ZitB transporters increase *E. coli* resistance when compared to bacteria carrying only ZntA transporters(272).

1.9.2 Zinc and copper resistance in bacteria from food animal origin

Resistance to zinc and copper in bacteria from food animals has been shown in various production systems and has been related to supplementation of minerals on feed. Most of the metal resistant bacteria identified are from fecal origin. A study conducted in Denmark looked at the susceptibility of strains from cattle, swine, and poultry origin to copper, zinc, and disinfectants(273). In that study, *Enterococcus faecium* and *Enterococcus faecalis* showed a marked bimodal distribution when tested for copper susceptibility, which indicates the acquisition of resistance determinants. The samples were either susceptible at 8mM or susceptible at 16 mM almost no isolates susceptible at 12 mM. As early as 1993, copper resistance has been observed in *E .coli* from food animal origin(274). In that study, four out of thirty-three strains showed resistance to copper on concentrations higher than 18 mM, and the resistance phenotype was also transferable by conjugation. Sequencing methods have been used to further characterize the resistance determinants for copper in *E .coli* from food animal origin, with results indicating

resistance to other metals like mercury and tellurium (275). Studies have also shown copper resistance and resistance determinants in *Salmonella* spp. obtained from copper-fed pigs(276).

Administration of increased doses of zinc has been a common practice as an alternative for the prevention of diarrheal diseases on piglets. As a result, the prevalence of resistance determinants has increased in bacteria originating from swine (271, 277). Studies have shown zinc resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) from swine-origin, which is of great concern as swine are considered a reservoir for MRSA infection on humans(278, 279). The resistance gene *czrC* appears to be the determinant that confers zinc resistance in MRSA from both swine and human origin(280).

1.10.3 Metal resistance testing in bacteria from food animal origin

The excretion of metals in feces resulting from mineral supplementation in food animal production systems affects bacterial metal resistance and may have a role in the co-selection of antimicrobial resistance(281). As a result, the detection of metal resistance in bacteria from food animal origin has gained importance (282). The fundamental principle of metal resistance testing is similar to testing conducted for antimicrobial resistance, the exposure of bacteria to increasing concentrations, and the determination of growth inhibition(283). Methods used include agar dilution, disc diffusion, and microbroth dilution(282). Factors like the media selection and pH may affect the availability of metal ions and the outcome of the test(282). Frequently, LB broth has been used as the media for metal resistance testing in *E. coli(284, 285).* However, a study shows that this media reduces the duration of the exponential growth phase in *E. coli* which might affect the outcome of the test(286). Other particularities apply to metal resistance testing and may vary depending on the bacteria in question. This topic will be further addressed in **Chapter 2.**

1.10 Zinc and copper selection of antimicrobial-resistant bacteria

Bacteria, like any other living organism, adapt, and evolve to ensure survival. Therefore, the presence of stressors cause bacteria to maintain or develop acquired resistance to antimicrobials(287). Several factors are able to cause stress on bacteria, though compounds differ in having specific targets for their action(288). Essential minerals like copper and zinc are also capable of inducing stress when present in concentrations higher than the bacterial requirements(289). Subsequently, there exists a growing concern that metals can be responsible for selecting resistant pathogenic bacteria.

The process by which copper and zinc indirectly select antimicrobial-resistant bacteria is referred to as co-selection. The mechanism of co-selection depends on a link between metal and antimicrobial resistance, which can be physiological (cross-resistance) or genetic (co-resistance and co-regulation)(290). Cross-resistance happens when one determinant is responsible for resistance to different compounds; the most common example is the presence of efflux pumps responsible for extrusion of metals and antimicrobials(291). A study showed that antifouling paint from ships selects for heavy metal and tetracycline resistance in marine biofilms(292). Furthermore, the presence RND efflux system which has been associated with decreased metal and antimicrobial susceptibility was also observed in those marine biofilm communities(292). Conversely, co-resistance is a mechanism of co-selection in which a genetic linkage exists between metal resistance and antimicrobial resistance determinants within the same mobile genetic element(291). It has been shown that the presence of copper resistance determinant *tcrB* is linked to genes that encode resistance for glycopeptide and macrolide antibiotics in feedlots and swine production systems(293, 294). The last mechanism of co-selection is co-regulation, which is based

on the stress response to metal exposure resulting in the upregulation of both metal resistance and antimicrobial resistance(295).

The supplementation of copper and zinc above requirement levels has become a common practice on production animal systems both in North America and Europe(294, 296). Several studies have highlighted the effect that the increased supplementation has on antimicrobial phenotypes. A trial conducted on feedlot cattle showed that the supplementation of higher concentrations of copper increases the prevalence of antimicrobial resistance determinants in *Enterococcus* spp. two groups were exposed to 100 mg/kg and 10 mg/kg of copper sulphate respectively, with the former showing a 6.9% higher prevalence of macrolide resistance(297). Another study conducted on swine drew similar results with a dose of 125 mg/kg of copper, causing an increase in tetracycline and tylosin resistance in *Enterococcus* spp.(298). However, some studies have found inconclusive differences in antimicrobial resistance phenotypes between groups of feedlot cattle being fed a conventional dose and an increased dose of minerals. The first study classified the groups by doses of copper 10 mg/kg and 100 mg/kg with no significant differences in the antimicrobial resistance phenotypes of *E. coli* and *Enterococcus* spp. (299). A drawback of the study is the fact that the *E. coli* obtained from both control and experiment groups were resistant before the start of the trial. On that same note, *E. coli* isolated from feedlot cattle that were supplemented with zinc up to 90 mg/kg showed no effect on the susceptibility to tetracyclines and ceftriaxone (300). Based on the current body of evidence, it seems clear that associations between metals and antimicrobials are also dependent on the antimicrobial in question.

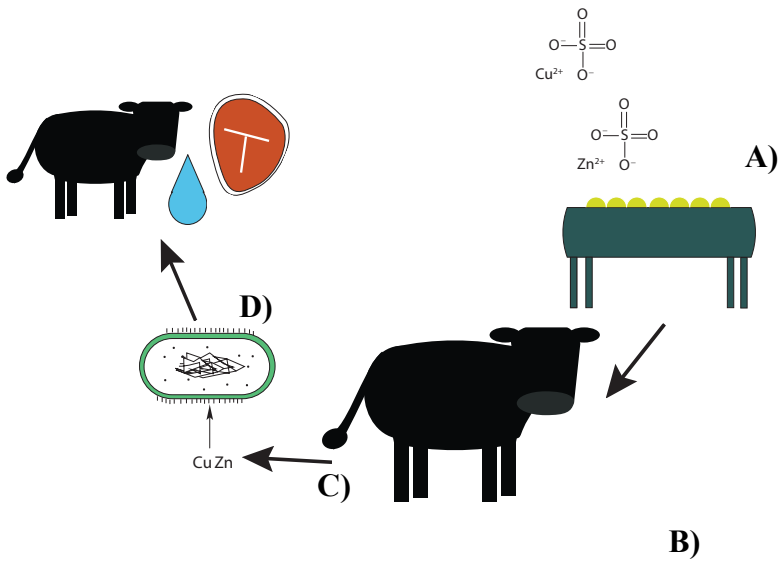

Figure 1.1 Copper/zinc supplementation and the pathways to co-selection in beef cattle production systems.

A) Zinc and copper are added to beef cattle feed. **B)** Beef cattle ingest feed with supplements absorbing between 5-15%. **C)** The non-absorbed portion of the supplements is then excreted to feces, exposing *E. coli* and selecting for antimicrobial resistant colonies. **D)** Resistant *E. coli* can then contaminate beef and water for human consumption or infect other animals.

1.11 Non-antimicrobial factors involved in the selection of antimicrobial-resistant bacteria

Modifiable non-antimicrobial factors are those outside of antimicrobial use that may affect the levels of antimicrobial resistance on bacterial populations. In the case of food animal production systems, those factors include but are not limited to hygiene, diet, health status, and stock density(301). Research in this topic has mainly focused on comparing groups with conventional antimicrobial usage and reduced antimicrobial usage while accounting for other managing factors(302). A scoping review recently addressed the body of literature available on the topic, concluding that more research is required to explore management practices that could reduce antimicrobial resistance as the magnitude and direction of the associations was not consistent(301).One of the studies addressed, compared the effect on *E. coli* resistance determinants, by exposing feedlot cattle to subtherapeutic doses of chlortetracycline plus

sulfamethazine and compare them with a control group(303). The results suggested that exposure to antimicrobials increased the levels of resistance; however, a grain-based diet also had an association with resistance when compared to a silage diet (248). It has been shown that colonic acidification has an effect on *E. coli* populations by increasing their acid resistance and increasing the production of membrane-bound transporters(304). Another study explored the difference in resistance determinants between feedlot cattle in conventional management and those raised without antimicrobial exposure(155). As expected, the exposure to antimicrobials increased the amount of resistance present (104). Nonetheless, other factors, like the resistance present in the microbiota before exposure, affected the resistance levels (104). A study compared the resistance determinants and changes in genetic composition of *E. coli* from conventional and organic dairy in a six month period (305). The results showed that *E. coli* populations in organic farms were more stable and carried less resistance determinants that conventional dairy farms (250). In general, it seems clear that more research needs to be conducted before considering the widespread implementations of practices that may reduce antimicrobial resistance(301).

1.12 Research questions and hypothesis

The present study aims to answer the following question:

How is resistance to Cu and Zn associated with antimicrobial resistance in *Escherichia coli* from beef cattle production systems?

We hypothesize:

Resistance to Cu and Zn will be associated with antimicrobial resistance phenotypes. The statistical association indicates the co-occurrence of both resistances within the same isolates

which might be caused by the presence of antimicrobial/metal co-selection mechanisms (co-resistance, cross-resistance or co-regulation) in the isolates.

1.13 Study objectives

The objectives of this study are:

1. Develop an assay to measure the resistance of *E. coli* to Cu and Zn.
2. Describe antimicrobial resistance and metal resistance (Cu and Zn) of *E. coli* in strains isolated from samples collected from a cow-calf ranch.
3. Evaluate the association between phenotypic resistance to antimicrobials and resistance to Cu and Zn in *E. coli*.

1.14 Significance of the study

This project will fill the gap in knowledge that exists in the field of antimicrobial resistance in beef cattle production and environmental stewardship. As the supplementation of minerals will continue in the years to come, it's imperative to understand the effects that mineral residues might have on the resistance phenotypes of bacteria in cattle production environments. Currently, no absorbance spectrophotometric microbroth dilution method has been used to evaluate Zn and Cu resistance in bacteria from beef cattle origin. *Escherichia coli* is the ideal bacteria for this purpose as it is exposed to residuals in feces and can also become a health concern for both animals and humans.

Chapter 2 will describe the development of the Zn and Cu resistance microdilution assay. Chapter 3 will describe the results from testing of Zn and Cu resistance in *Escherichia coli* isolated from fecal samples of cattle origin and the comparison between those results with the AMR phenotypes. Chapter 4 will discuss the topics in the thesis and compare them with existing literature.

Chapter 2: Spectrophotometric microtiter-plate assay for determination of zinc and copper resistance in *Escherichia coli*

2.1 Abstract

The development of microdilution method with a fine scale to test Zn and Cu resistance in *Escherichia coli* will provide a more efficient tool for quantification of resistance. Here, bacteria were inoculated into microtiter plates with defined concentrations of Zn and Cu, and the absorbance was measured before and after 24 hours of incubation. The method was standardized for *E. coli* as it displayed a good linear dynamic range (R^2>0.95), and precision (RSD< 35% in all but three Zn concentrations).

Keywords: Mineral supplementation, metal resistance, *Escherichia coli*, copper, zinc, antimicrobial resistance, antibiotic resistance

2.2 Introduction

Authors have previously tested metal resistance in bacteria from agricultural origin (282). The (almost) ubiquitous presence of *E. coli* in different environments, pathogenic potential and the exposure to minerals in feces makes it the most useful bacteria to test. The most frequent method used for testing metal resistance in bacteria is agar dilution (306-308). Alternative methods like disc diffusion and microbroth dilution have also been used (309, 310). Only a few studies have looked at the implementation of optical density as an indicator of bacterial growth in metal resistance assays (285, 310, 311).

The microdilution methods offer clear advantages over agar dilution including enhanced reproducibility, less space requirements, easy generation of reports and reduced cost of reagents (312). Factors like culture media used, inoculum density, and incubation time need to be standardized to assure the reproducibility of microdilution assays (313, 314). Metal resistance assays based upon optical density currently available lack standardization for *E. coli* which limits their applicability as a testing tool (285, 311). The development of a reliable quantitative metal resistance method will prove useful as very little is known about the extents to which mineral residuals affect fecal bacteria.

2.3 Materials and Methods

The standard reference strain of *Escherichia coli* (ATCC 25922) was used. *Escherichia coli* was incubated at 37°C in trypticase soy agar (Becton Dickinson, Sparks, MD, USA). The inoculum was prepared by adding 3-4 separate colonies from cultures with a period of growth of 22 hours to a 5 ml tube of sterile demineralized water and adjusting optical density equivalent to 0.5 on the McFarland turbidity scale. Using a 100 µL micro-pipettor, 50 µL of the inoculum

suspension was transferred to a 5 mL of Cation Adjusted Muller-Hinton Broth (CAMHB) (Becton Dickinson, Sparks, MD, USA) tube to give an inoculum of 1×10^5 colony forming units (CFU)/ml.

The stock solution of Zn was prepared by adding 200 mg of zinc sulfate heptahydrate ($ZnSO_4+7H_2O$) (Sigma Aldrich, St. Louis, MO, USA) to 10 ml of sterilized distilled water with a final pH of 7. The stock solution of Cu was prepared by adding 300 mg of copper sulfate pentahydrate ($CuSO_4+ 5H_2O$) (Sigma Aldrich, St. Louis, MO, USA) to 7.5 ml of sterilized water, 2.5 ml of 10% Tris Base (VWR, Solon, OH, USA) was added to reach a pH of 7.0. Both stock solutions were filter sterilized by passing through 0.22 μm filters. Then, 1 ml of the stock solutions were added to 9 ml of sterilized distilled water to make a working solution with a concentration of 12.01 μmol/ml for Cu and 6.95 μmol/ml for Zn.

Ninety-six well microplates (Thermo Scientific, MA, USA) with serial dilution concentrations of Cu and Zn were prepared. To obtain a more accurate estimate, concentrations of the solutions were prepared with the metal concentration decreasing by 120 μmol/mL for Cu and 70 μmol/mL for Zn between wells with a range of 0-6.95 μmol/mL of Zn and 0-12.1 μmol/ml of Cu. Subsequently, between 1000 and 0 μl of the solution was added to twelve different 1.5 mL microcentrifuge tubes for both Zn and Cu. Then, each tube was filled to 1000 μl with sterilized distilled water. Each tube was vortex mixed and 50 μl of the contents from each tube was transferred to an assigned well. 50 μl of the previously inoculated CAMHB were also added to each plate using the Sensititre auto-inoculation system (Trek Diagnostics Systems, Cleveland, OH, USA) so each well was inoculated with $1x10^5$ CFU/ml. The absorbance of each plate was recorded before incubation, to adjust for the turbidity of the metal solutions. The plates were then incubated at 37°C for 24 hours and the final absorbance of each well was read using a microtiter plate reader (Spectramax M2, Molecular Devices, USA) at 600 nm.

Both the initial and final absorbance of the plates were collected and graphed using RStudio version 1.1.463 (315). The mean absorbance units (AU) of each assay for five biological replicates, which were done in two technical replicates were graphed. To determine the MIC, we performed a one-way ANOVA to compare all the wells below 0.1 OD to the first concentration above 0.1 OD for both the Zn and Cu resistance assay. Once a difference was detected a post-hoc Tukey Test HSD was conducted to compare all the groups analyzed and determine the lowest concentration showing a difference, that concentration was labelled as the MIC of *E. coli* for both Zn and Cu.

A linear regression model was developed to assess the linearity of the method proposed with viable cells count of *E. coli* for wells for five concentrations of both Zn and Cu, modelled alongside the absorbance values. The counts were converted to CFU/mL (x) and modeled against the absorbance units (AU) (y) obtained on the assay. The goal was obtaining a determination coefficient (R^2) with a value greater than 0.95 as considered acceptable for microbiological methods (316). The viable cells count from wells 1, 4, 6, 8, and 12 were obtained by collection of 10 ul from each final well mixture and performing a serial 10:100 dilution in normal saline (0.9% NaCl). Ten ul of the diluted inoculum were then spread on trypticase soy agar with 5% defibrinated sheep blood and incubated in the same conditions as the microplates. All counts were done in quadruplicate with the means serving as the result for each well.

To assess the replicability of the results, the precision of the assay was estimated. This was accomplished by calculating the RSD (Relative Standard Deviation) for each well by dividing the standard deviation of the five biological replicates by the absolute value of the mean and multiplying by 100. Values between 15-35 % are considered acceptable for microbiological methods (316).

2.4 Results

The method showed a linear relationship between AU and CFU/ml (**Figure 2.1C and 2.1D**). The R^2 for both Zn and Cu are >0.95, which satisfies the requirements for microbiological methods(316). All but three RSD values in the Zn assay were inside the acceptable range (15-35%) (**Table 2.2, Table 2.3).** The Cu assay values were all inside the acceptable range(316). The AU values for both the Zn and Cu resistance assay were graphed (**Figure 2.1A and 2.1B**). The MIC breakpoints for both Zn and Cu were obtained. (**Table 2.1**). The results demonstrate this assay can classify isolates as susceptible or tolerant when compared to the reference.

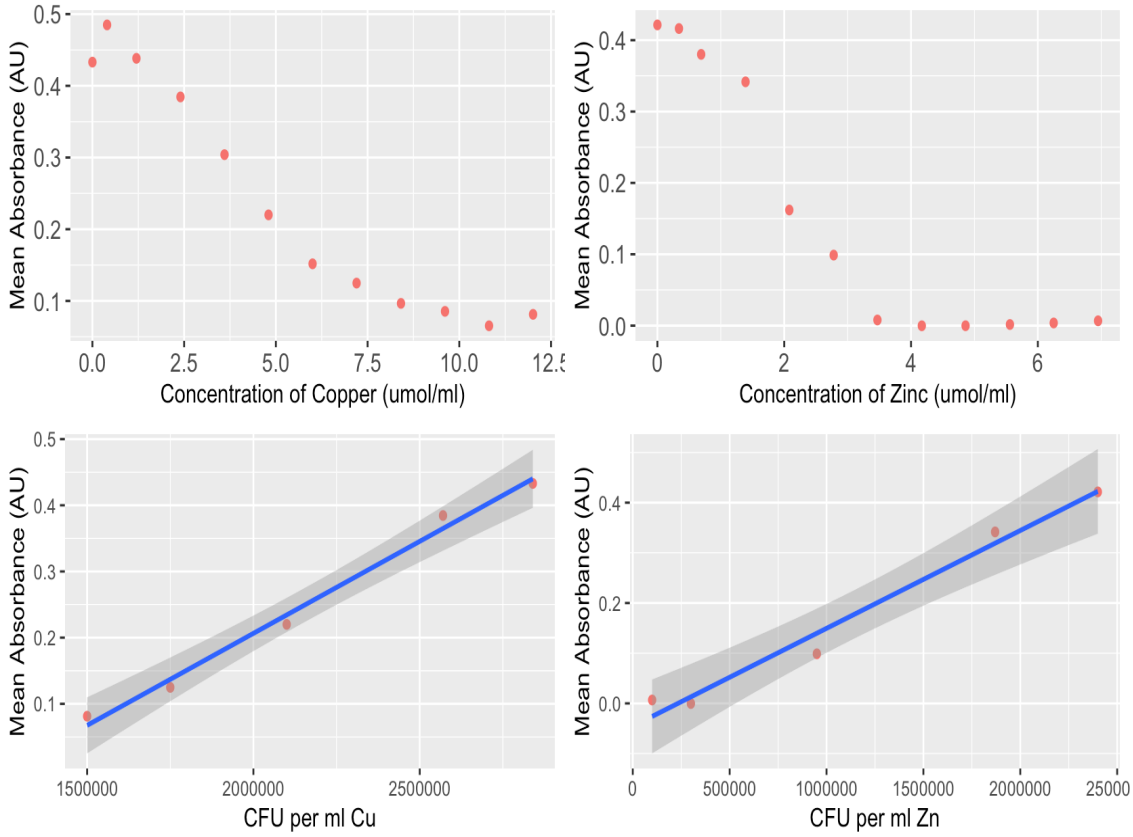

Figure 2.1A, Figure 2.1B. Metal resistance assay results for *E. coli.*

Shown are the metal resistance assay results for zinc and copper in *Escherichia coli*, with each point representing reading from one well of the plate. (A) Mean absorbance obtained (N=5) for each one of the concentrations of copper. (B) Mean absorbance obtained (N=5) for each one of the concentrations of zinc.

Figure 2.1C, Figure 2.1D. Correlation between absorbance values and colony forming units (CFU) in *E. coli.*

(A) Copper resistance assay (y=3.56e06x + 1.26e06) R^2=0.9866. (B) Zinc resistance assay (y=5.01e06x+2.53e-05) R^2=0. 9885. USP considers $R^2 > 0.95$ as acceptable.

2.5 Discussion

The metal resistance assay developed has particular relevance for food animal production systems as a great portion of supplemented Zn and Cu is excreted. A study showed that more than 80% of the supplemented Zn and more than 90% of the supplemented Cu are excreted by fecal route from Angus steers(227). Based on the supplementation recommended in North America (30 mg/kg for Zn and 10 mg/kg for Cu) it is plausible to infer that in this environment the *E. coli* exposure to Zn and Cu are well above the breakpoints established on the present study. Moreover, authors have shown that selection for Cu resistance can be triggered at concentrations up to 10 fold below the MIC *in vitro* which suggests the potential for selection of resistant *E.coli* within beef cattle production environments as concentrations in those are higher than the ones in that study (317). Further research needs to be pursued to confirm if this is the case in beef cattle operations, outlining the applicability and need for a fast and accurate high throughput assay method proposed.

The results obtained align with previous observations in which Zn sulfate causes a greater effect on *E.coli* growth when compared to Cu sulfate(284). However, a few viable bacteria were recovered from even the highest concentration of Zn and Cu used in this assay. We hypothesize that this is caused by subpopulations of persister cells that were dormant during the exposure and became metabolically active after culturing on media without the metal challenge (318, 319).

The turbidity and color of Zn and Cu does not allow for the use of colorimetric metabolic indicators of growth like 2,3,5-triphenyltetrazolium chloride (TTC)(320). As a result, the main

limitation of the method is the lack of specificity for live/viable cells. Optical density measurements before incubation are made to adjust for density and carry over of particulates from the residue sample being tested. Dead cells, cell shape, cell debris, as well as metabolic products (glycocalyx, outer membrane vesicles, or exopolymetric matrix) can all contribute to the optical density at 600 nm. However, the background absorbance appears to be negligible given the correlation observed here between OD and CFU. The convenience, low cost, and reliability of the facile method proposed here provides a great tool for environmental monitoring of the effect of Zn and Cu residuals on bacterial metal resistance.

Table 2.1 Minimum inhibitory concentrations for Zn and Cu for *E. coli*

Bacteria	Zn (μmol/ml)	Cu (μmol/ml)
E.coli (ATCC 25922)	2.78	8.41

Table 2.2 Relative Standard Deviation (RSD) for each one of the wells in the *E. coli* Zn resistance assay.

Concentration of Zn (μmol/ml)	RSD (%)
0	8.62
0.34	7.30
0.69	9.98
1.39	11.35
2.08	33.21
2.78	31.23
3.47	309.73
4.17	291.34
4.86	355.07
5.56	28.96
6.25	24.47
6.95	30.59

Table 2.3. Relative Standard deviation (RSD) for each one of the wells in the *E. coli* Cu resistance assay.

Concentration of Cu (μmol/ml)	RSD (%)
0	5.47
0.40	6.86
1.20	7.60
2.40	10.52
3.60	11.16
4.80	11.23
6.00	20.25
7.20	14.82
8.41	18.22
9.61	21.52
10.81	32.08
12.01	22.50

Chapter 3: WA Ranches Study

3.1 Abstract

Essential minerals are supplemented in beef cattle production with the absorption rate being poor and resulting in high excretion levels. As a consequence, bacteria from the gastrointestinal tract can be exposed to high concentrations of metals. This study focused on determining the association between zinc (Zn) and copper (Cu) resistance and antimicrobial resistance phenotypes in *Escherichia coli* isolated from environmental samples obtained from a cow-calf ranch. *E. coli* was isolated from water, soil and manure. The results showed a low prevalence of antimicrobial resistance as 31/39 isolates were susceptible to all the antimicrobials tested using the disc diffusion method. The most common resistance patterns were ampicillin (4/39) and amoxicillin-clavulanic acid (4/39), with one isolate being resistant to doxycycline. The Zn and Cu resistance profiles were obtained by using the spectrophotometric microdilution assay described in the previous chapter. Seven of the eight isolates with resistant or intermediate antimicrobial resistance patterns showed Zn and Cu resistance when compared to the reference strain. Fisher's exact test was conducted to compare antimicrobial-resistant and susceptible isolates in their resistance to Zn and Cu, with the results indicating that the antimicrobial resistant isolates are also more likely to be tolerant to Zn (p value<0.001) and Cu (p value=0.013). Our findings suggest that the antimicrobial resistant *E. coli* isolates are also co-resistant to Zn and Cu and this could be due to co-selection.

3.2 Introduction

The emergence of antimicrobial resistance is a multifactorial phenomenon posing a challenge to both human and veterinary medicine. The development of resistance in pathogens reduces the effectiveness of antimicrobials in disease treatment. As a result, antimicrobial stewardship practices have been implemented in human and veterinary medicine. Antimicrobial stewardship practices in veterinary medicine include regulation, development of clinical guidelines, and the surveillance of antimicrobial usage and resistance(172). In Canada, the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) reports annual antimicrobial sales and antimicrobial resistance patterns from the livestock industry(321). The surveillance of antimicrobial resistance focuses almost exclusively on fecal bacteria as they are often found in products for human consumption and are potential foodborne pathogens.

The persistence of antimicrobial resistance in livestock can be facilitated by several factors, including antimicrobial usage, horizontal gene transfer, and co-selection. The latter factors can disseminate resistance determinants already present in bacterial populations with or without the presence of antimicrobials(322). Antimicrobial usage is capable of generating mutations and maintaining them in bacterial populations by exerting selective pressure(323). Recent changes in Canadian legislation have restricted the usage of Medically Important Antimicrobials (MIAs) for human medicine to veterinary prescription only, with the primary objective of reducing the spread of resistance in animal production(143). Studies have shown the effectiveness of policy changes to reduce the prevalence of antimicrobial resistance in bacteria from livestock(173, 174). The effect of policies is less evident in environments with low antimicrobial usage, such as cow-calf operations, which also have a significantly lower prevalence of resistance than feedlots and dairy farms(324).

Supplementation of essential minerals is a widespread practice across beef cattle production systems. Ideally, cattle will get the amount of essential minerals required from their base diet(325). However, poor mineral content in soil is a frequent cause of mineral deficiencies(210). The need for supplementation combined with the poor absorbance rate of the compounds available results in the excretion of about 90% of copper (Cu) and 80% of zinc (Zn) of the amount supplemented (227). The excreted Zn and Cu are not affected by degradation, which causes metals to accumulate the environment over time. Studies suggest that exposure of bacteria to Cu and Zn can select for antimicrobial resistance (290, 306). Consequently, the potential for transmission of antimicrobial-resistant bacteria across the food chain may be increased by dietary mineral supplementation. The aim of this study is to describe metal resistance and antimicrobial resistance in *Escherichia coli* isolated from soil, water, and manure samples collected from the environment of a cow-calf ranch. This bacterium was chosen based on its almost ubiquitous presence and role as a frequent contaminant of both water and animal products with the potential to cause disease in humans and animals.

3.3 Materials and methods

3.3.1 Study site

Sampling was conducted at the W.A Ranches at the University of Calgary, northeast of Cochrane Alberta. The ranch is a 19,000-acre cow-calf operation in Southern Alberta. The ranch is part of the Beaverdam Creek watershed, which is a non-draining catchment of the Little Red Deer River Sub-watershed and the Big Hill Springs watershed, which drains into the Bow River(326). For this study, environmental samples were collected from 8 pastures on W.A Ranches: fields 2, 3, 8, 12, 13, 18, 20, and 21 (**Figure 3.1**). Each pasture has a unique management

practice, geography, and water source. Cows deliver calves in the calving pen of field 3 in late winter through spring (February through May). Cows and their calves are then split into groups based on age and are placed into the mothering pens in fields 2 and 3. As the calves grow, the cattle are moved into the more extensive pastures, 8, 12, 13, 18, and 21. Field 20 (50 acres), houses mature bulls and has a larger pasture and a smaller holding pen available for management. Some pastures are used to grow barley, barley oats, mustang oats, and peas, and allow for swath grazing and a supply of hay and silage. The soil in this area has been characterized as moderately fine texture sand clay loam, clay loam, and silty with pockets of stony loam, silt loam, and fine sandy loam(327).

The nutritional management of the cattle includes mineral supplementation with one mineral block per 100 head of cattle. Each mineral block contains: 30.0 mg/kg selenium, 15.5% calcium, 6.0% phosphorus, 6.9% sodium, 2.5% magnesium, 50 mg/kg fluorine max, 5000 mg/kg manganese, 5000 mg/kg zinc, 3000 mg/kg iron, 1500 mg/kg copper, 46 mg/kg cobalt, 200 mg/kg iodine, 500,000 iu/kg vitamin A, 50000 iu/kg vitamin D, and 2500 iu/kg vitamin E. WA Ranches has historically operated with minimal antimicrobial usage, and antimicrobials are only administered under veterinary oversight for treatment of individual disease conditions. The antimicrobial usage (AMU) in the ranch throughout 2019 included parenteral administration of florfenicol, ceftiofur, oxytetracycline, trimethoprim/sulfadoxine and tilmicosin for treatment of disease. The main diseases treated were neonatal diarrhea and undifferentiated fever associated with enzootic pneumonia.

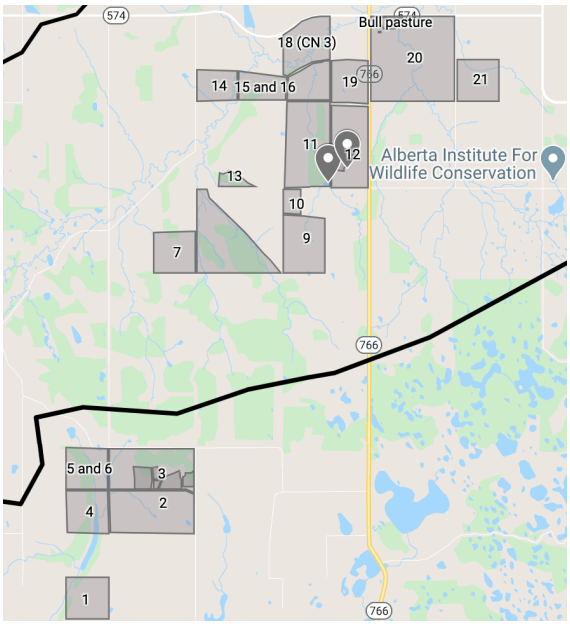

Figure 3.1 . Location of W.A Ranches with sampling sites delimited

(Google Maps, 2019).

3.3.2 Sampling procedures

3.3.2.1 Water sampling

The water samples were collected using the grab sampling method(328). Water was obtained from different sources depending on the pen (water trough, pond, pump house taps, or directly from the well). For that purpose, a previously cleaned bucket was introduced up to about 20 cm to collect water from the surface. The bucket was placed opposite the flow direction of the water. The sample was then obtained by carefully pouring the water into a 200 ml sterile sampling bottle with 0.2 ml of 10% sodium thiosulfate ($Na_2S_2O_3$) as a preservative. The samples were then chilled in a cooler on ice, and parafilm was placed around the bottle to prevent leakage.

3.3.2.2 Soil sampling

The sample sites were determined by the position of the mineral block on the field. Using a shovel, a wedge of soil about 15 cm deep was removed from the ground and set aside. From the

edge of this hole, a second wedge about 15 cm deep was taken. The sides of each sample were removed to make a "square" core, which was collected as the sample for each field, including rhizosphere and bulk soil. The rhizosphere is defined as the soil less than 2mm from the root of the plant and the bulk soil, which has been defined as the soil not bound to the roots. Enough soil was collected from a single sampling site to fill one sterile 700 ml Whirl-Pak® bag (ULINE, Edmonton, AB, CAN). The digging tool was sterilized with 70% ethanol between each sample. Gloves were worn throughout the whole procedure and were changed for each sample. Two soil samples were collected from each field, one for micromineral analysis and the other for bacterial culture. Samples were massaged for 1 minute and placed in a cooler for transport. Samples for bacterial isolation were stored at -20°C and were processed within a week of sampling.

3.3.2.3 Manure sampling

Between 1-2 g of manure were collected using a sterile cotton swab from the center of 20 different, fresh (still moist) fecal pats within a given field. Samples were collected from all the fields. The 20 fecal samples corresponding to each field were added to a sterile plastic bag to create a composite sample of 10-20 g. The collection bags were mixed and massaged by hand for 1 min prior to being placed in a cooler for transport.

3.3.3 Sample processing and bacterial isolation procedures

3.3.3.1 Water processing

The water samples were processed within 1 hour of collection and were qualitatively assessed for coliform bacterial contamination using Colilert® (IDEXX, Markham, ON, CAN) to screen for the presence of coliforms and *Escherichia coli* (*E. coli)*. A positive control sample was prepared by inoculating a loopful of *E. coli* (ATCC 25922) into 100 ml of sterilized deionized

water, and a negative control sample (blank) consisted of sterile deionized water. After 24 hours of incubation, samples were read at room conditions for coliforms and at UV 305 for *E. coli* and classified as either negative (less fluorescence than the comparator and less yellow than the comparator), positive to coliforms (equal or more yellow than the comparator) or positive (fluorescence equal or greater than the comparator) for *E. coli* . Ten ml of water from each test bottle was filtered through a 0.45 µm white membrane filter. A five-fold serial dilution was performed by adding 100 µl of the sample to 900 µl of phosphate-buffered saline (PBS) (Hardy Diagnostics, California, USA) to achieve a 10^{-5} dilution in 1000 µl from which 100 µl were spread to MacConkey agar plates (MAC) (Becton Dickinson, Sparks, MD, USA). The plates were incubated for 24 hours at 37°C, followed by morphological identification of *E. coli*. Four separate colonies were then inoculated to Trypticase Soy Agar (TSA) (Becton Dickinson, Sparks, MD, USA) and MAC and incubated for 24 hours at 37°C. From the TSA plates, colonies were obtained to perform a Spot Indole test (Becton Dickinson, Sparks, MD, USA), BBL™ Dryslide™ Oxidase test (Becton Dickinson, Sparks, MD, USA), and Gram stain. Samples with positive Spot Indole, negative Oxidase test, and negative Gram stain were then classified as presumptive *E. coli*. After this phase, colonies of presumptive *E. coli* from a 24-hour culture were inoculated to 1000 µl of BD Difco™ Lysogeny broth (LB) (Becton Dickinson, Sparks, MD, USA), and added to 300 µl of 50% glycerol in a sterile cryovial. Samples were then labeled and stored at -80°C until further use

3.3.3.2 Manure processing

Fecal samples were weighed, and 10 g was transferred to a sterile bag to which ninety ml of buffered peptone water (BPW) (Hardy Diagnostics, California, USA) were added to the bag, followed by massaging thoroughly by hand for 1 minute. The sample was allowed to sit for 16 hours at room temperature. A five-fold serial dilution was performed by adding 100 µl of the

sample to 900 µl of BPW to achieve a 10^{-5} dilution in 1000 µl from which 25 µl were spread onto MacConkey Agar plates (MAC). Plates were then incubated aerobically for 24 hours at 37°C. Morphological identification of *E. coli* was performed and followed by the isolation process discussed in Section 2.3.1.

3.3.3.3 Soil processing

The soil bags collected for Cu and Zn analysis were stored at 4°C and were transported to within 48 hours of sampling to be analyzed at AGAT labs (Calgary, AB, CAN). The samples obtained for bacterial isolation were thawed for three days at 4°C. Ten g of mixed soil was weighed out and placed in 50 ml sterile centrifuge tubes and suspended in 15 mL of sterile PBS. Centrifuge tubes were agitated on a rotary shaker (200 r/min) for 90 min at room temperature. The samples were then sonicated with a Q500 sonicator (QSonica, Newtown, CT, USA) at 20 kHz for 45 seconds (1/8 probe, amplitude 40%, pulsing 5 seconds on, 5 seconds off), and shaken on a rotary shaker (200 r/min) for 30 min at room temperature, and then allowed to settle for 30 minutes on the lab bench. From the liquid part of the sample a two-fold serial dilution was performed, and 50 µl of the 10^{-2} dilution was spread onto MAC agar plates. Plates were incubated at 37°C for 24 hours. Morphological identification of *E. coli* was performed and followed as per the isolation process discussed in the water processing section.

3.3.4 Antimicrobial susceptibility testing

The Kirby-Bauer susceptibility test was used to assess the antimicrobial susceptibility profile in presumptive *E. coli* isolates. The antimicrobials tested were cefoxitin and ceftriaxone (cephalosporins), trimethoprim-sulfamethoxazole (sulfonamides), doxycycline (tetracyclines), ampicillin, and amoxicillin-clavulanic acid (penicillins) (Becton Dickinson, Sparks, MD, USA).

The antimicrobials tested were chosen to represent both the critically important (cefoxitin, ceftriaxone, ampicillin and amoxicillin-clavulanic acid) and highly important classes (doxycycline and trimethoprim-sulfamethoxazole) based on their importance as therapeutics in human medicine(34). The Clinical and Laboratory Standards Institute (CLSI) disk diffusion protocols for direct colony suspension method and inoculum method were used to obtain a 0.5 McFarland standard before inoculating Mueller Hinton agar (MHA) plates and applying antimicrobial disks to the inoculated agar plates(329). These plates were incubated at 37°C for 18 hours. The zones of inhibition were measured for each disk to determine the antimicrobial susceptibility profile for each isolate as per CLSI guidelines. The isolates were then classified as either susceptible, intermediate, or resistant.

3.3.5 Zn and Cu resistance assay

A microdilution assay was used to assess Zn and Cu resistance levels of the *E. coli* isolates recovered from the water, manure, and soil(330)Isolates were cultured in TSA for 22 hours at 37°C, and then an inoculum of 0.5 McFarland standard was prepared in a 5ml tube of demineralized water. One hundred µl was then transferred to a 5 ml tube of cation-adjusted Mueller Hinton broth (CAMHB) (Becton Dickinson, Sparks, MD, USA), which was used to inoculate 96-well microtiter plates. Subsequently, 50µl of a previously prepared 96-well microtiter plate with concentrations ranging between 0-6.95 and 0-12.01 µmol/ml of Zn and Cu, respectively. Inoculated microtiter plates were incubated for 24 hours at 37°C. The absorbance of each plate was recorded before incubation, with the purpose of adjusting for the turbidity of the metal solutions. The plates were incubated for 24 hours at 37°C, and the absorbance was measured using a microtiter plate reader (Spectramax M2, Molecular Devices, USA) set to 600 nm. The final absorbance for each one of the wells was recorded. The minimum inhibitory concentration (MIC)

of the reference strain (*E. coli* ATCC 25922) was used as the point of comparison between all the isolates (2.78 μmol/ml Zn, 8.41 μmol/ml Cu). The absorbance units (AU) at those points were considered as the growth for each isolate.

3.3.6 Statistical analyses

Both the antimicrobial resistance and metal resistance results were used to classify the samples. First, the isolates were grouped based on antimicrobial susceptibility as negative for susceptible and positive for either resistant or intermediate to any one of the antimicrobial tested. Then, the samples were grouped as resistant or susceptible to Zn and Cu to provide a binary outcome based on the AU (0.08) observed at the MIC of the reference strain (ATCC 25922). Using the AU allows the comparison of growth of different strains at the same concentrations of Zn and Cu regardless of the MIC of each one and therefore allowing the classification of the isolates. All isolates above 0.08 AU were considered resistant to Zn or Cu and the ones below were considered susceptible. Isolates were then classified in a contingency table. Once all the isolates were classified, a Fisher's Exact Test was performed to determine if a statistical difference in resistance to Zn and Cu existed between antimicrobial resistant positive and negative isolates. A Principal Component Analysis (PCA) was conducted to visualize the distribution of isolates based on their results in the Zn and Cu resistance assay. RStudio version 1.1.463 was used to conduct all the statistical analyses (315).

3.4 Results

3.4.1 Antimicrobial susceptibility testing

Antimicrobial susceptibility assay results are summarized in **Table 3.1** and **Table 3.2**. The majority of the isolates were susceptible to all antimicrobials tested. No isolates showed resistance to trimethoprim-sulfamethoxazole. None of the isolates showed resistance to the cephalosporins cefoxitin, ceftriaxone, but resistance to the penicillin antibiotics ampicillin and amoxicillin-clavulanic acid was observed in 10% of the isolates. Furthermore, all the isolates resistant to amoxicillin-clavulanic acid were also resistant to ampicillin. The single isolate resistant to doxycycline was also resistant to amoxicillin-clavulanic acid and ampicillin. The resistance to amoxicillin-clavulanic acid or ampicillin was observed in four fecal samples, no water or soil samples showed resistance to either antimicrobial. Three isolates from soil showed intermediate phenotypes to amoxicillin-clavulanic acid, but none showed resistance to any antimicrobial.

3.4.2 Soil analyses

The levels of Cu and Zn in soil from the eight pastures were analyzed (**Table 3.3**). Copper levels ranged from 12 to 175 mg/kg. Levels of Zn ranged from 61 to 660 mg/kg. The highest concentrations of Cu and Zn were found the sample from field 2. The lowest concentrations of Cu and Zn were found in the sample from field 18.

3.4.3 Description zinc and copper resistance testing results

The isolates tested for Zn and Cu resistance were grouped based on the antimicrobial resistance phenotype obtained on the antimicrobial susceptibility testing (**Figure 3.2A, 3.2B**). Isolates with resistant or intermediate phenotypes were classified as positive and isolates that were susceptible to all antimicrobials were classified as negative. antimicrobial resistant isolates were

significantly (p value=0.013) more likely to be resistant to Cu when compared to negative isolates using Fisher's Exact test (**Table 3.4**). Antimicrobial resistant isolates were significantly (p value<0.001) more likely to be resistant to Zn than negative isolates (**Table 3.5**). The PCA plot using the metal resistance assay results showed that 6/8 isolates that were antimicrobial resistant were separated from the sensitive strains and placed within the same cluster. In other words, resistance to both the Zn and Cu were related to antimicrobial resistance and the metal resistance could be used to discriminate between antimicrobial resistant positive and negative isolates. (**Figure 3.3**)

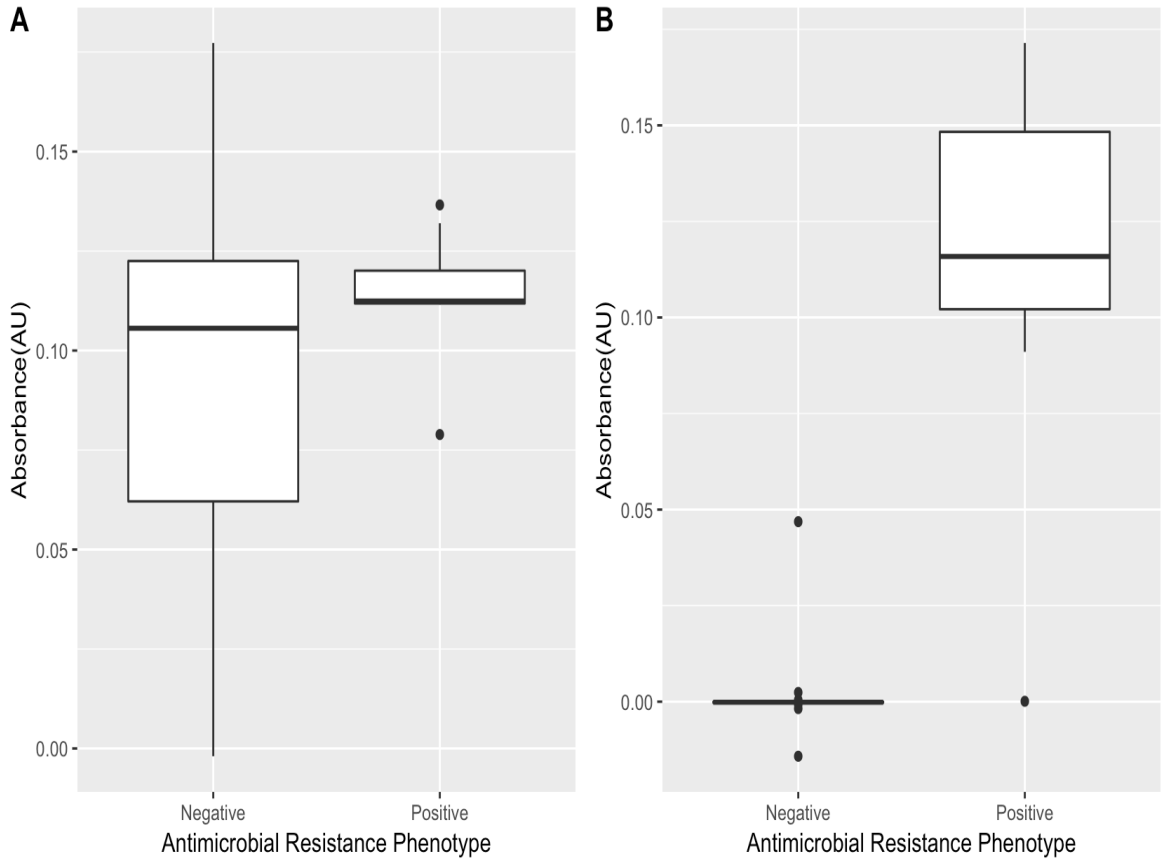

Figure 3.2 Absorbance units (AU) representing growth of the isolates at the MIC for *E. coli* ATCC 25922.

Groups classified by the antimicrobial resistance pattern independent of the antimicrobial (susceptible/negative and resistant, intermediate/positive). (A) Cu resistance assay absorbance at 8.41 µmol/ml. (B) Zn resistance assay at 2.78 µmol/ml.

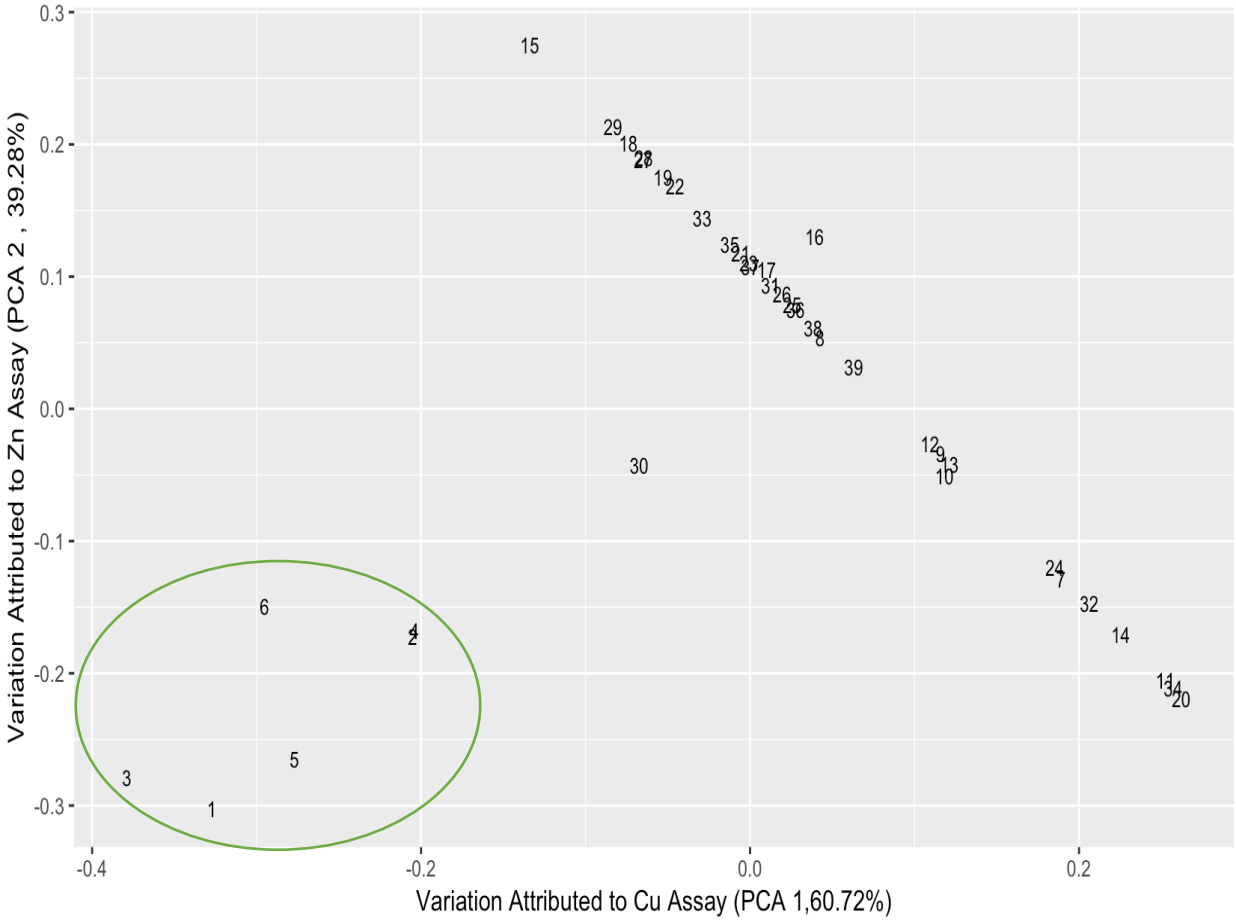

Figure 3.3. Principal Component Analysis of copper and zinc absorbance for positive and negative isolates.

Isolates were labelled by number (1-8 antimicrobial resistance positive, 9-39 antimicrobial resistance negative). The circle indicates the group of antimicrobial resistance positive isolates that cluster separately from the antimicrobial resistance negative based on the results of the Zn and Cu resistance assay.

3.5. Discussion

The key finding of the present study was the observation of the co-occurrence of antimicrobial resistance and Cu/Zn resistance in *E. coli* from a cow-calf environment. The co-occurrence of resistance might indicate the possibility of co-selection within those isolates. To the author's knowledge, the finding of possible co-selection in a cow-calf environment has not been reported in the literature and provides valuable new insights for the beef cattle industry. The

environmental sampling also adds information about the distribution of antimicrobial resistance in cow-calf production systems with all the resistant isolates obtained from manure, and three intermediate isolates obtained from soil.

Previous studies have looked at the antimicrobial resistance in *E. coli* isolated from feces from both cows and calves from cow-calf production systems(331-333). However, there are differences between the resistome in soil, water, and manure posing challenges when comparing our results with previous findings(324). Furthermore, studies have shown that *E. coli* from spring calves carry significantly higher levels of antimicrobial resistance when compared to *E. coli* from cows of the same herd(332). In our work, manure was collected from the surface of the pasture, meaning we cannot separate manure samples from calves or cows. Therefore, the results were compared with the antimicrobial resistance prevalence in *E. coli* isolates from beef cows. The relatively low prevalence of antimicrobial resistance in this study aligns with a recent study in cow-calf production systems that reported a 4% prevalence of resistance to at least one antimicrobial in *E. coli* obtained from cows during pregnancy testing, in 105 herds throughout western Canada in 2014(333). A previous study showed a 9.8% prevalence of antimicrobial resistance in *E. coli* isolates obtained from 69 herds across western Canada in 2002(332). A fundamental difference between findings is the higher prevalence of resistance for both amoxicillin-clavulanic acid (10%) and ampicillin (10%) in our *E. coli* in comparison to the low percentage encountered (below 1% isolate prevalence) in both studies mentioned above(332, 333).

Another important finding of the present study is the widespread susceptibility of the isolates to the most frequently used antimicrobials in W.A Ranches (cephalosporins and tetracyclines).No usage of penicillin was reported; still, the amoxicillin-clavulanic acid and ampicillin resistance were the most prevalent with both considered as Category 1 and Category 2

based on their importance for human medicine(334). A possible cause for the presence of resistance could be the historical usage of penicillin at the study ranch. Reports of western Canada AMU practices encompassing 2013-2014 showed that penicillin was the most used antimicrobial in beef cows during that time (21). That same study showed a reduced usage of long-acting penicillin in beef cows in comparison to a previous study reporting AMU for disease treatment during the 2002 calving season(53). The changes in AMU practices observed in W.A Ranches may also be a result of the new veterinary oversight requirements actions by Health Canada, implement in 2018, that required a prescription for all previously available over the counter MIA implemented in 2018, a list that included penicillin(143). The time required for the reversibility of antimicrobial resistance varies depending on the fitness cost of the mutation and the presence of compensatory mutations(335). Other factors, as the presence of a fitness beneficial resistance and co-selection, can also slow down the reversibility of antimicrobial resistance(336). Consequently, the estimation of the time required for the reversibility of antimicrobial resistance is a complicated task.

The resistance levels to Zn and Cu of the *E. coli* isolated show that those with resistant or intermediate antimicrobial resistance phenotypes are more likely to be resistant to metals. This is highlighted in the PCA plot where antibiotic resistant strains cluster away from those that are not resistant.

The resistant isolates seem to occur throughout different types of fields and appear to have no relationship with the levels of Zn and Cu in soil. This can be attested by the presence of intermediate resistance phenotypes in field 18, which had the lowest in both Zn and Cu levels in the soil. Additionally, a large amount of variability was observed between levels of Zn and Cu in soil between different fields. This was expected as 19,000-acre W.A Ranches has different soil

types and compositions, which also has an effect on the mineral content within them. The supplementation is the same throughout the fields, indicating that the soil levels might be independent of the management. The Zn and Cu levels in field 2 (660 and 175 mg/kg) are above the limit (250 and 63 mg/kg) established by the Alberta Tier 1 Soil and Groundwater Remediation Guidelines(337). The guidelines recommend controlling the source as a method to prevent pollution in soils and the contamination of groundwater resources.

The exposure of bacteria to high concentrations of trace minerals has associated with the co-selection of antimicrobial resistance since 2010(277, 338). The proximity of resistance determinants within the chromosome or a mobile genetic element has been shown as a mechanism for co-selection(339). In some cases, cross-resistance can cause co-selection by a single gene conferring resistance to both metals and antimicrobials(340). Experimental evidence suggests that co-selection in *E. coli* can be caused by either of the mechanisms discussed(341). The results from our study show that *E. coli* with antimicrobial-resistant or intermediate phenotypes were also more likely to tolerate higher concentrations of Zn and Cu when compared to *E. coli* pan-susceptible to antimicrobials. In addition, the co-occurrence of antimicrobial resistance and metal resistance can increase the frequency of horizontal gene transfer. A recent study showed that plasmids carrying metal resistance and antimicrobial resistance are more likely to be conjugative, increasing the potential for horizontal gene transfer(342). All of the above highlights the importance of co-selection as a mechanism for the spread and maintenance of antimicrobial resistance in cow-calf environments. As no molecular analyses were conducted yet, the possible mechanisms behind those results are yet to be determined.

On the present study, fecal isolates were the only ones found to be resistant to antimicrobials, suggesting that the direct exposure to minerals and antimicrobials might have an

effect on the phenotypes. The presence of both antimicrobial resistance and metal resistance *in vitro* does suggest the possibility of co-selection in this environment. The influence of dietary Zn and Cu as selectors of antimicrobial-resistant bacteria in cow-calf production systems is unknown and should be explored further as nutritional management varies from the one on feedlots. Experimental evidence of the effects of Zn and Cu in antimicrobial resistance has not been confirmed in beef cattle. A previous study compared the effects of supplementing 10x and 1x the National Research Council recommended the amount of Zn and Cu on antimicrobial resistance phenotypes and abundance of resistance genes in *E. coli* and *Enterococcus* spp. from beef heifers in experimental conditions(299). The results showed that the treatment caused minimal effects on both resistance phenotypes and the abundance of resistance genes throughout the 32-day experiment. However, isolates were resistant to erythromycin, clindamycin, penicillin, tiamulin, tilmicosin, and tylosin from day 0 of the experiment limiting the effect of the treatments (244). Associations between increased supplementation of Zn and increased proportions of multi-resistant isolates and resistance to beta-lactams and been observed in *E. coli* isolated from swine feces(308, 343). The inherent differences between monogastric and ruminant digestive systems complicate the extrapolation of results. Currently, *in vitro* studies like the present one, provide the most reliable evidence of the effects of Zn and Cu supplementation on antimicrobial resistance in *E. coli* from beef cattle.

The small sample size in this study and, most importantly, the participation of only one ranch poses a challenge when extrapolating the findings across this entire ranch and to other cow-calf ranches in southern Alberta. However, the results obtained from this pilot study show that the development of a larger scale study with more ranches and more sampling within ranches is justified. Another future direction to study is the implementation of more efficient and affordable

supplements for beef cattle as the cost of more efficient alternatives is prohibitive for cow-calf ranchers. Chelated minerals provide a greater absorbance rate amassing to 30%, and up to 100% increased absorbance than sulfate forms of both Cu and Zn, respectively(325). The increased cost of this form of mineral supplementation limits its use to stress situations and the high presence of inhibitors in diet such as iron and molybdenum. The enhanced absorbance of chelated minerals will help reduce the amount of fecal excretion of both Zn and Cu from diet. The effect that this particular type of mineral supplements might have on antimicrobial resistance in fecal bacteria is unclear and is a topic worth exploring. One of the clear benefits could be the reduced risk of co-selection of antimicrobial resistance and metal resistance as fecal bacteria become less exposed to minerals. Correlations between fecal levels of Zn and Cu and antimicrobial resistance have been observed in other studies and is worthwhile to explore in beef cattle production systems(229). Quantification of Zn and Cu concentrations in feces coupled with antimicrobial sensitivity testing and resistance gene quantification in feces could provide a insight into the situation in beef cattle production systems.

Table 3.1. Antimicrobial susceptibility profiles from E. coli isolates (n=39).

Antimicrobial Tested	Susceptible Isolates (%)	Intermediate Isolates (%)	Resistant Isolates (%)
Doxycycline	36 (92)	2(5)	1(3)
Cefoxitin	39(100)	0	0
Ceftriaxone	39 (100)	0	0
Trimethoprim-sulfamethoxazole (TMS)	39 (100)	0	0
Ampicillin	35 (90)	0	4 (10)
Amoxicillin-clavulanic acid	32 (82)	3(8)	4(10)
At least 2 antimicrobials	34 (87)	1(3)	4(10)
3 Antimicrobials	38(97)	0	1(3)

Table 3.2 Field location and resistance patterns of resistant or intermediate isolates

Field Location	Source of Isolate	Isolates per Field	Antimicrobial Resistance Pattern
20	Manure		Ampicillin Amoxicillin-clavulanic acid
20	Manure		Ampicillin Amoxicillin-clavulanic acid
20	Manure	3	Ampicillin Amoxicillin-clavulanic acid
13	Manure	1	Ampicillin Amoxicillin-clavulanic acid Doxycycline
2 Mothering Pen	Soil	1	Amoxicillin-clavulanic acid (Intermediate) Doxycycline (Intermediate)
18	Soil		Amoxicillin-clavulanic acid (Intermediate)
18	Soil	2	Amoxicillin-clavulanic acid (Intermediate)
21	Manure	1	Doxycycline (Intermediate)

Table 3.3. Concentrations of Zn and Cu obtained in soil analyses

Field Number	Cu (mg/kg)	Zn (mg/kg)
8	22.2	104
20	17.8	96
21	23.0	119
18	12.0	61
13	41	114
12	20.9	86
2	175	660
3	30	118

Table 3.4. Copper resistance assay results. Fisher's exact test conducted (p value=0.012)

	Positive	Negative
Above 0.08 Absorbance	7	10
Below 0.08 Absorbance	1	21

Table 3.5. Zinc resistance assay results. Fisher's exact test conducted (p value=<0.001)

	Positive	Negative
Above 0.08 Absorbance	7	0
Below 0.08 Absorbance	1	31

Chapter 4: Discussion

Overall this research project has made important contributions to our understanding of antimicrobial resistance in cow-calf herds. A method for the estimation of Zn and Cu resistance levels in *E. coli* was standardized and described. This method has been applied to an investigation of antimicrobial and metal resistance in samples collected from a cow-calf operation. Significant associations between metal resistance and antimicrobial resistance were found. Antimicrobial resistance patterns were also influenced by the origin of the isolate with no water samples showing resistance to any of the antimicrobials tested.

4.1 Antimicrobial usage in cow-calf production systems

With the successful purification of penicillin, treatment of bacterial diseases became an easier task, but this has changed as bacteria have evolved to survive antimicrobials(344). The presence of selective pressures (antimicrobials, metals) causes bacteria to acquire or develop resistance mechanisms to resist those effects(345). Antimicrobial usage in cow-calf production systems focuses on individual treatments based on the presence of clinical signs of disease. However, the dissemination of resistant bacteria can affect other animals that have not been treated previously. The excretion of resistant bacteria in cattle feces can play a role in spreading antimicrobial resistance directly or through soil and/or water. Feces from calves have a higher concentration of *E. coli* with 10^8 to 10^9 CFU/g during the first two weeks of life, when compared to 10^6 to 10^7 CFU/g between two-five months of life, and 10^4 CFU/g as adults(346). Neonatal diarrhea is the main cause of treatment in cow-calf ranches, thus, it's plausible to infer that antimicrobial resistant *E. coli* can be transferred within that environment(333). The causes of

neonatal diarrhea can bacterial, viral or protozoal, with an increased volume of feces in all cases and, as a consequence, affecting the excretion of bacteria to the environment. *E. coli* is also capable of causing mastitis and metritis in dams which highlights the consequences of an increase of resistant *E. coli* in cow-calf operations(347, 348).

The antimicrobial usage practices in feedlots are different than the ones implemented in cow-calf production systems (21, 349). Calves arrive to the feedlots after being bought in auction markets, usually recently weaned, and get transported to then commingle with calves from different origins(19). The mass medication of antimicrobials (metaphylaxis) is used to prevent outbreaks of bovine respiratory disease(BRD) in high-risk calves at arrival(120). The contact between calves causes the interaction of diverse bacterial communities, in turn, increasing the potential for horizontal gene transfer of antimicrobial resistance genes(350). Therefore, the pool of antimicrobial resistance genes during the early feeding period on feedlots can be traced, at least in part, back to cow-calf production systems. This highlights the importance of understanding the factors involved in the spread of antimicrobial resistance in cow-calf operations.

4.2 Beef cattle nutritional management in cow-calf operations

Cow-calf producers take advantage of the short, warm summers by calving in late spring so calves and cows can best utilize the green pastures prior to weaning in fall. Essential minerals are required to assure proper growth and health in beef cattle. Hence, they need to be supplemented if feed does not meet the minimum requirements. Unfortunately, the levels of essential minerals in pastures are dependent on the mineral contents in the soil and are often insufficient(351). Therefore, mineral supplementation is an important factor for beef cattle nutrition in cow-calf operations. In the case of cow-calf production systems mineral blocks are placed in each field to

give access to supplements. Excretion levels of essential minerals are an issue in those environments as the absorption rate for inorganic Zn and Cu is very poor(227). Throughout the digestion process minerals complexes degrade in the rumen and form different compounds to facilitate absorption in the small intestine(352). Once excreted, metals do not degrade and buildup in the environment. Bacteria in those environments, particularly *E. coli* become constantly exposed to high concentrations of metals which can cause the co-occurrence of metal resistance and antimicrobial resistance(353, 354). The presence of resistance determinants for both metals and antimicrobials then allow co-selection as a mechanism for proliferation of antimicrobial resistance.

4.3 Spectrophotometric microtiter-plate assay for determination of zinc and copper resistance in *Escherichia coli*

The present study focused on investigating the relationship between Zn and Cu resistance and antimicrobial resistance in a cow-calf ranch. An assay was developed to accurately assess the resistance levels to Zn and Cu in *E. coli* ATCC 25922. The development of the assay included an assessment of the culture media, temperature and method to be used for the bacterium in question. The usage of Cation-Adjusted Mueller Hinton Broth (CAMHB) provided an improvement from previous methods that use less adequate media for Zn and Cu resistance testing in *E. coli*. A spectrophotometric based microtiter plate assay was used as an efficient and cost-effective way obtain quantitative data for multiple isolates in a high throughput format. The higher resistance of *E. coli* to Cu (8.41 μmol/ml) when compared to Zn (2.78 μmol/ml) aligns with findings from previous studies(284). The standardization process included the assessment of linearity between absorbance and CFU counts with both determination coefficients (R^2) being above the acceptable threshold (0.95)(316). The precision of the assay was calculated to assess the replicability of the

assay. The results showed a relative standard deviation between 15-35 % in all concentrations of Cu and all but three concentrations of Zn. During the development of the assay different bacteria such as *Pasteurella multocida* and *Histophilus somni* were used to assess the suitability of the method. During those trials, bacteria showed decreased growth as concentrations of Zn and Cu increased. Nonetheless, the precision and linearity of the assay was affected in both cases with *H. somni* showing more variability. For this reason, it was concluded that the assay developed is suitable for *E. coli* and should be modified for usage in different bacteria. The differences in assay performance between species can be attributed to inherent differences both in growth requirements and resistance to Zn and Cu between bacteria. This is the case of *H. somni* as the growth of this semi-fastidious bacterium is enhanced by the supplementation of factors V (NAD) and X (haemin) found within red blood cells(355).

4.4 Investigation of antimicrobial resistance and Zn and Cu resistance in the environment of a cow-calf operation in Alberta

The study proceeded with the sampling of W.A Ranches a cow-calf ranch in Cochrane, Alberta. The objectives were to to first evaluate the resistance phenotypes present in *E. coli* isolated from water, soil and manure collected from the ranch. Second to evaluate the Zn and Cu resistance levels of the ranch samples using the previously developed assay. The last objective was to assess correlation of the antimicrobial resistance phenotypes with the Zn and Cu resistance levels of the samples. The antimicrobial resistance patterns were obtained by disc diffusion assay and the antimicrobial classes were chosen based on usage history and public health relevance. The results from the susceptibility testing showed a 10% resistance to ampicillin and 10% resistance to amoxicillin-clavulanic acid despite the absence of usage during the year of sampling. The Zn and

Cu resistance testing showed that isolates with antimicrobial resistance phenotypes were also more tolerant to Zn and Cu. Those results suggest the co-occurrence of Zn and Cu resistance and resistance to antimicrobials. This is an important finding as resistance determinants for Zn and Cu can be localized within the same mobile genetic elements carrying antimicrobial resistance genes(291). The fitness cost of carrying resistance determinants reduces the growth rate of resistant bacteria when compared to their susceptible counterparts(335). Therefore, the presence of selection pressure is necessary to maintain resistant populations. The time required for bacterial populations to change from resistant to susceptible once selective pressures are removed vary depending on the bacterium and resistome in the environment(356). It has become generally accepted that bacterial populations carrying resistance determinants with high fitness cost are also the fastest to reverse to susceptible phenotypes(357). Nevertheless, not all the resistance mechanisms lead to fitness loss, as it has been attested in *E. coli* carrying extend-spectrum beta-lactamase in plasmids(358). Bacteria carrying determinants with high fitness cost can maintain those genes either by compensatory evolution or co-selection in the absence of selection pressure. Based on previous studies that estimated the levels of metals required to select for resistance, the levels of Zn and Cu found in environmental samples from W.A Ranches could select for bacteria carrying metal resistance determinants which demonstrates the possibility of co-selection (290). Co-selection might also help explain the persistence of resistance to ampicillin and amoxicillin-clavulanic acid on the ranch either by co-resistance or cross-resistance mechanisms.

4.5 Limitations and future steps

Overall, the study provides a clear outlook of the current resistance phenotypes present in the ranch's environment and the potential for co-selection for metal (Zn or Cu) resistance and

antimicrobial resistance based on a physiological or genetic linkage between them. The assay developed for Zn and Cu resistance testing provided precise (RSD< 35% in all but three Zn concentrations) results for *E. coli* isolates and is a great tool to use on future studies. The lack of specificity for live/viable cells is the main limitation of the method. One of the main limitations of the cow-calf study was the inability to genotype the *E. coli* in the manure and compare them with the *E. coli* recovered from water and soil. The genotyping would also have allowed for co-selection experiments to further explain the co-occurrence of Zn and Cu resistance and antimicrobial resistance. The lack of molecular detection of resistance determinants also limits the scope of the study as the presence of genes in the environment also affects the reversion of antimicrobial resistance by horizontal gene transfer(359). A qPCR assay to quantify resistance determinants before and after exposure to fecal levels of Cu and Zn could have been possible with a molecular component within the project. The limited sample size and the single study site also poses some challenges when drawing conclusions with soil and water sources varying within the ranch and between different ranches.

As antimicrobial resistance becomes more prevalent and treatment options start to become scarce, new perspectives are required. Bacteria from food animal production systems are a common cause of resistant infections in humans(360). Therefore, there is a need to better understand the factors determining the spread and maintenance of foodborne pathogens. This pilot study provides valuable information from an often-overlooked part of the beef production cycle. Future studies shall focus on robust environmental studies, individual sampling from cattle and further experimental components such as co-selection experiments that expose bacteria to loads of Zn and Cu similar to fecal excretions. The molecular typing of the *E. coli* isolated and characterization of the resistance determinants would also add valuable information about the

resistome present in cow-calf operations. Moreover, the implementation of a longitudinal sampling scheme could provide more insight on the associations between metal resistance and antimicrobial resistance. This could be accomplished by phenotype testing and the relative abundance of genes of interest in particularly relations between metal and antimicrobial resistance gene loads. Bacteria from environmental and cattle origin should be studied in conjunction to further explain the factors causing the persistence of antimicrobial resistance in beef cattle production systems.

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