CRITICALLY IMPORTANT AND OF HIGHEST PRIORITY: THE EVOLVING STORY OF CEPHALOSPORIN USE AND ANTIMICROBIAL RESISTANCE IN U.S. DAIRY CATTLE

A Dissertation

by

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ABSTRACT

Under current United States (U.S.) regulations, a dairy cow is eligible for slaughter 13 days following the last of 2 doses of ceftiofur crystalline-free acid (CCFA) for the treatment of metritis. A matched-pair longitudinal study was employed to monitor levels of antimicrobial resistance among fecal *Escherichia coli* and *Salmonella* across 3 dairy farms in the U.S. High Plains to evaluate microbial safety pertaining to antimicrobial resistance on the first-eligible slaughter date. Environmental samples were collected from multiple areas throughout the farm prior to beginning the animal trial. Cows diagnosed with post-parturient metritis via veterinary protocol were pair-matched based on lactation number and calving date. A baseline fecal sample (day 0) was taken prior to the first administered dose of CCFA with a second CCFA dose administered 72 hours later. Additional fecal samples were taken on study days 6, 16, 28, and 56. Samples were processed for *E. coli* and *Salmonella* for both the general and third-generation cephalosporin (3GC) resistant populations. Isolates from 3GC-selective plates underwent phenotypic antimicrobial susceptibility testing and whole genome sequencing (WGS). Full-factorial multi-level mixed linear regression showed a significant difference ($P \leq 0.05$) in quantitative resistance levels among *E. coli* populations when comparing treated (metritis) and untreated (control) cattle on days 6 and 16. These resistance levels became similar on days 28 and 56. Overall, levels of *Salmonella* shedding were higher in both groups on day 0 decreasing further in treated cows on days 6 and 16 and with resistance being infrequent. Resistance differences were observed primarily by dairy farm based on phenotypic antimicrobial susceptibility testing. Resistance genes and *Salmonell*a serotypes were identified from WGS. Based on the results of this study, it is recommended to extend voluntary slaughter withholding period to 28 days following the first administration of CCFA. This is to allow populations of

resistant *E. coli* to decrease to levels equivalent to that of their untreated counterparts. Such an extended slaughter withholding will allow for a reduction of the risk of slaughter fecal contamination by resistant enteric bacterial populations.

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NOMENCLATURE

AMR	Antimicrobial Resistance
bp	Base Pairs
BGA	Brilliant Green Agar
BTS	Bacterial Test Standard
CCFA	Ceftiofur Crystalline-Free Acid
CFU	Colony-Forming Unit
CHROM-ESBL	Extended-Spectrum Beta-Lactamase CHROMAgar
CVMBS	College of Veterinary Medicine and Biomedical Science
DNA	Deoxyribonucleic Acid
ECM	Energy-Corrected Milk
ENMU	Eastern New Mexico University
ESBL	Extended-Spectrum Beta-Lactamase
FDA	Food and Drug Administration
g	Gram
GDP	Gross Domestic Product
HHMP	Herd Health Management Programs
hr(s)	Hour(s)
HUS	Hemolytic-Uremic Syndrome
MAC	Plain MacConkey Agar
MAC broth	MacConkey Broth

MACCCA	MacConkey Agar with 4 µg/mL of Ceftriaxone and 4 µg/mL of Clavulanic Acid
MACCEF	MacConkey Agar with 4 µg/mL of Ceftriaxone
MACFEP	MacConkey Agar with 16 µg/mL of Cefepime
MACFOX	Maconkey Agar with 32 µg/mL of Cefoxitin
MALDI-TOF	Matrix Assisted Laser Desorption Ionization – Time of Flight
MDR	Multidrug Resistance
MIC	Minimum Inhibitory Concentration
MIC 50	The antibiotic concentration at which 50% of isolates are inhibited
MIC 90	The antibiotic concentration at which 90% of isolates are inhibited
mL	Milliliter
NARMS	National Antimicrobial Resistance Monitoring System
NMSU	New Mexico State University
OB	Obstetrician
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulse-Field Gel Electrophoresis
qPCR	Quantitative Polymerase Chain Reaction
RFM	Retained Fetal Membranes
RNA	Ribonucleic Acid

RV	Rappaport Vassiliadis R10 Broth
TAMU	Texas A&M University
TMR	Total Mixed Ration
TSB	Tryptic Soy Broth
TTB	Tetrathionate Broth
µg	Microgram
µL	Microliter

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CHAPTER I

INTRODUCTION

1.1 DEVELOPMENT AND EXPANSION OF ANTIMICROBIAL RESISTANCE

Not long after the discovery of penicillin by Alexander Fleming in 1928, antimicrobial resistance (AMR) was first reported. In 1940, before penicillin was made available for clinical use, the AmpC β-lactamase gene encoding for penicillin resistance within *Escherichia coli* (*E. coli*) was discovered (Abraham and Chain, 1940). It has been estimated that millions of metric tons of antibiotics have been released into the biosphere in the past 50 years, leading to selection pressures for resistant bacteria (Davies and Davies, 2010; Ozawa et al., 2012). In addition to environmental pollutants, other drivers of AMR include the use, overuse, and misuse of antimicrobials in human medicine and animal agriculture (McEwen and Collignon, 2018).

The repeated introduction of novel antimicrobials* over the decades has led to multidrug-resistant pathogens, such as *Staphylococcus aureus, Enterococcus* spp., and the Enterobacteriaceae family (Davies and Davies, 2010; Centers for Disease Control and Prevention, 2013). The spread of resistance genes via vertical and horizontal gene transfer (Daniels et al., 2009) facilitates the development of what are sometimes termed "superbugs" (Bennett, 2008; Davies and Davies, 2010). Forms of gene transfer, including the horizontal methods of transformation, transduction, and transconjugation (or, simply: conjugation), allow for the transfer of genetic material among bacteria (Bennett, 2008). Resistant bacteria utilize a variety of mechanisms to avoid succumbing to antimicrobial compounds, including preventing drug uptake, enzyme production, efflux pumps, or modifying the drug target (Holmes et al.,

*An antibiotic is an organic molecule produced by a variety of microorganisms that inhibit the growth of other microbes. The term antimicrobial includes these antibiotics, as well as other inorganic and organic compounds exhibiting inhibitory effects on microbes. The two terms will be used interchangeably throughout this document.

2016). The development of resistance mechanisms can vary based on the drug and species of bacteria, in addition to dosing, route of administration, and length of administration (McEwen, 2006). Resistance mechanisms can be the result of genetic mutations in the chromosomal DNA or else through plasmid transfer. Bacterial resistance to antimicrobials can be spread from the local to international scale through human travel, animal movement (including migration of wild birds), trade in food products and via environmental factors, such as water, soil and feed (McEwen, 2006).

The use of β-lactam antibiotics is the major selector for β-lactamase producing coliform bacteria (Livermore, 1995). Plasmid-mediated β-lactamases have become increasingly more common in gram-negative bacterial populations over the past few decades, including the most common plasmid-medicated AmpC gene, $bla_{\text{CMY-2}}$ (Livermore, 1995; Jacoby, 2009; Schmid et al., 2013). The increase in plasmid-mediated beta-lactam resistance has been assisted by the emergence and expansion of the $bla_{\text{CTX-M}}$ extended-spectrum β-lactamase (ESBL) gene family. Overall, since their emergence in the 1980s, $bla_{\text{CTX-M}}$ variants have surpassed those of bla_{TEM} and bla_{SHV} as the dominant beta-lactamase gene family in Europe and throughout the world (Bonnet, 2004; Livermore, 2007; Schmid et al., 2013). While *E. coli* containing $bla_{\text{CTX-M}}$ were isolated from gram-negative bacteria in agriculture in other areas of the globe as early as 2000 (Shiraki et al., 2004), this resistance gene was not reported among *E. coli* in agricultural settings in the United States until 2010 (Wittum et al., 2010).

1.2 POLICY CHANGES TO COMBAT AMR

Due to continuing bacterial adaptations against antibiotics and the resulting medical and economic pressures, and in search of understanding and mitigating the threats allowing for such expansions, there have been research and policy efforts focused on the systems and practices aiding AMR development. The *Joint Committee on the use of Antibiotics in Animal Husbandry and Veterinary Medicine* report made recommendations in 1969 to regulate feed and therapeutic antibiotics separately (Swann, 1969). These recommendations became accepted throughout Europe, beginning with Sweden in the early 1980s (McEwen and Collignon, 2018), and removed the over-the-counter availability of several antibiotic classes used in growth promotion (Kirchhelle, 2016). The European Union, as a whole, finally ended growth promotion uses of antibiotics in 2006 (McEwen and Collignon, 2018).

In 1997, the United States began to shift its policies regarding antibiotic usage when extra-label uses of fluoroquinolones and glycopeptides became prohibited by the Food and Drug Administration (FDA) (Doyle et al., 2013). A broader ban in 2001 on fluoroquinolone use in poultry was supported by a risk assessment evaluating the human health impact of fluoroquinolone-resistant *Campylobacter* due to chicken consumption (FDA, 2001). While antimicrobial usage in animal agriculture tends to be unregulated (and likely underestimated) in many countries around the globe, there has been a push to greatly reduce antimicrobial usage across these settings (Scott, Midgley, and Loneragan, 2015). Avoiding selection pressures for resistance to certain forms of treatment has proven difficult due to the potential for co-selection by other antimicrobials (McEwen, 2006; Scott, Midgley, and Loneragan, 2015). This co-selection can occur via resistance genes found on the same plasmid, transposon, or integron; in other words, by using 1 antibiotic, resistance to another antibiotic may be unintentionally

selected. However, non-antibiotic options exist for disease prevention including biosecurity, vaccination, and herd management, among other approaches (McEwen, 2006).

1.3 CEPHALOSPORINS IN ANIMAL AGRICULTURE

Having provided historical context to the issues faced with regards to antimicrobial resistance, our focus shifts to the use of cephalosporins in animal agriculture, primarily in dairy production settings. When first approved for therapeutic use in U.S. dairy cattle in 1988 (Donaldson et al., 2006), third-generation cephalosporins, such as ceftiofur as sodium or hydrochloride and later as crystalline-free acid (CCFA) were not prohibited from extra-label usage, although prescriptions from licensed veterinarians were required (Tragesser et al., 2006). However, in 2012, the FDA banned many extra label uses of cephalosporins in animal agriculture (Food and Drug Administration, 2012). Third-generation cephalosporins (including CCFA), along with penicillin, ampicillin, and oxytetracycline have each been used as treatment for acute metritis in dairy cows (Drillich et al, 2001; Liebana et al., 2004; Haimerl and Heuwieser, 2014; Reppert, 2015). While fourth-generation cephalosporins are available for use in food and companion animal medicine in Europe (Liebana et al., 2004), ceftiofur is currently the only third- or higher-generation cephalosporin licensed for the treatment of food animals in U.S. agriculture (Food and Drug Administration, 2017). Among ceftiofur formulations, a two-dose labeled treatment of CCFA for metritis is now (since April 2012) required due to the rate at which concentrations of drug decrease within uterine tissues. The previously approved one-dose treatment regimen did not maintain drug concentrations elevated enough to achieve sufficient efficacy according to the manufacturer, who applied for a revised product label (Zoetis, 2012). A popular medication used in dairy production medicine, CCFA does not produce detectable levels

of antibiotic residues in milk and therefore does not require a milk withholding period (Zwald, 2004; Tragesser et al., 2006; Heider et al., 2009). While milk is not withheld, under section 522.313a of the Code of Federal Regulations Title 21, Chapter 1, an animal having received a two-dose treatment of CCFA is not eligible to go to slaughter until a 13-day withdrawal period has elapsed since the last dosing; specifically, to avoid antibiotic residues in meat products (21 C.F.R. § 522.313a; 2017).

1.4 AmpC VERSUS ESBL RESISTANCE PATTERNS

AmpC and ESBL forms of cephalosporin resistance have each continued to expand with the continued usage of cephalosporins (Tragesser et al., 2006; Lowrance et al., 2007; Dolejska et al., 2011; Snow et al., 2012). This is particularly concerning, as the World Health Organization (2019) considers third and higher generation cephalosporins to be of highest priority among the critically important antimicrobials for human medicine. Bacteria harboring and exhibiting AmpC forms of resistance (e.g., the *bla*$_{\text{CMY}}$ family of genes) are unaffected by penicillins and aminopenicillins, first-generation cephalosporins, cephamycins (second-generation cephalosporins) and third-generation cephalosporins (Tragesser, 2006; Jacoby, 2009; Pfeifer, Cullik, and Witte, 2010; Schmid et al., 2013) as well as these classes of antibiotics paired with a beta-lactamase inhibitor such as clavulanic acid, sulbactam, or tazobactam. Their ESBL counterparts (i.e., those with *bla*$_{\text{CTX-M}}$ genes, among others) are resistant to penicillins, aminopenicillins, and first-, third-, and fourth-generation cephalosporins (Bonnet, 2004; Drieux et al., 2008); however, their susceptibility to these classes of antibiotics is recovered once a beta-lactamase inhibitor is added. Thus, ESBL resistance mechanisms are rendered inactive with the addition of clavulanic acid, sulbactam, or tazobactam (Matsuura et al., 1980; Cormican,

Marshall, and Jones, 1996; Bonnet, 2004; Drieux et al., 2008; Schmid et al., 2013; Rossi et al., 2016). As a comparison, while AmpC and ESBL types both are resistant to third-generation cephalosporins, AmpCs are inhibited by fourth-generation cephalosporins, and unaffected by clavulanic acid (Akova, Yang, and Livermore, 1990) while ESBLs are inhibited by second-generation cephalosporins and the addition of clavulanic acid to aminopenicillins and 3rd generation cephalosporins (Thomson, 2001; Bonnet, 2004; Drieux et al., 2008). Thus, the use of an appropriately chosen array of broth or agar media, each containing these distinguishing antibiotics, provides a mechanism to phenotypically quantify and distinguish the presence or absence of the different genotypes in a genotypically mixed population.

1.5 CMY AND CTX-M BETA-LACTAMASE ENCODING GENES

These 2 broad forms of resistance, AmpC and ESBL, can further be broken down into 2 major gene groupings: 1) the cephamycinases with the *bla*$_{\text{CMY}}$ (AmpC) genes and, 2) the cefotaximases utilizing the *bla*$_{\text{CTX-M}}$ (ESBL) gene. Within these 2 classifications, numeric groupings are used for differentiation among variations of the 2 gene families. The *bla*$_{\text{CMY}}$ genes can be located chromosomally or else on a plasmid (Liebana et al., 2004), including the most common plasmid-mediated AmpC, *bla*$_{\text{CMY-2}}$ gene (Jacoby, 2009). Through treatment with higher-order potentiated aminopenicillins and cephalosporins, bacteria possessing the *bla*$_{\text{CMY-2}}$ gene have the ability to change the gut flora via amplification (Daniels et al., 2009). While *bla*$_{\text{CMY-2}}$ is the widest spread AmpC gene, the ESBL *bla*$_{CTX\text{-}M}$ gene has surpassed extended-spectrum beta-lactamase TEM and SHV variants in many European countries as the leading beta-lactamase gene (Livermore et al., 2007; Schmid et al., 2013). This gene can be found on many mobilized

genetic elements, but is mainly found on plasmids encoding for multiple resistance (Livermore et al., 2007).

1.6 ORGANISM DESCRIPTIONS

Escherichia coli is a motile, facultatively anaerobic, gram-negative bacterium. When grown on MacConkey agar, *E. coli* presents as pink, lactose-fermenting colonies of circular morphology. The organism is a rod-shaped, indole-positive bacterium, meaning it actively creates indole and pyruvate from tryptophan. *E. coli* can be either commensal or pathogenic depending on the type and location of bacteria (Kaper, Nataro, and Mobley, 2004). As such, it is highly prevalent and in large quantities in the feces of domestic mammals and birds; thus, it serves as a useful indicator organism for other less common coliforms such as *Salmonella*, *Enterobacter*, and *Citrobacter*. The pathogenic forms of *E. coli* can be categorized as enteropathogenic, enterohaemorrhagic, enterotoxigenic, enteroaggregative, enteroinvasive and diffusely adherent *E. coli*, all of which are typed and grouped via their O and H antigenic formulae (Kaper, Nataro, and Mobley, 2004).

Much like *E. coli*, *Salmonella* are facultatively anaerobic, gram-negative, motile, rod-shaped bacterium in the Enterobacteriaceae family. From a human health perspective, *Salmonella* tend to be grouped as Typhi and non-Typhi to differentiate based on human-host specificity. When grown on brilliant green agar (BGA), *Salmonella* appear red to a white-pink in coloration. O-antigen tests can be utilized to further identify an isolate as *Salmonella*. The ability to cause disease depends on the location of bacterial infection and host species susceptibility; most often, *Salmonella* cause illness in the gastrointestinal system due to the consumption of

contaminated food or water. However, infections with *Salmonella* can also be found in other extra-intestinal locations including the blood, thereafter leading to sepsis.

1.7 CLINICAL SIGNIFICANCE

Enteropathogenic *E. coli* was the first pathogenic form of *E. coli* to be described after causing large outbreaks in the United Kingdom in 1945 (Kaper, Nataro, and Mobley, 2004). Typically associated with diarrhea, pathogenic forms of *E. coli* can also result in urinary tract infections, meningitis, sepsis, and hemolytic-uremic syndrome (HUS) (Kaper, Nataro, and Mobley, 2004). While our study did explore nor type the *E. coli* found as pathogenic or commensal, *E. coli* serves as a useful indicator species for other enteric pathogens, such as *Salmonella* (Lowrance et al., 2007; Cummings, Aprea, & Altier, 2014). It has been well-established that there are inherent risks of pathogen contamination in food products (Todd, 1997; Cassin et al., 1998; Zhao et al., 2001; Schroeder et al., 2003; Johnson et al., 2005; Vogt and Dippold, 2005; World Health Organization, 2017[a]) with a particular risk of *E. coli* contaminants being introduced onto meat products due to fecal contamination at slaughter or through the handling of animal tissue (Jackson et al., 2001).

A 2011 publication estimated that 9.4 million laboratory-confirmed cases of foodborne illnesses, resulting in 55,961 hospitalizations and 1,351 deaths, occur in the United States annually (Scallan et al., 2011). Non-typhoidal *Salmonella* accounted for the second highest number of foodborne disease cases and the most hospitalizations and deaths. That equates to 1,034,000 (11%) cases, 19,587 (35%) hospitalizations, and 379 (28%) deaths from foodborne non-typhoidal *Salmonella*. These data are likely to be an under-estimation of true incidence due

to under-diagnosis and under-reporting resulting from patients not seeking treatment, no causative organism being isolated, and the illness not being reported to public health agencies (Scallan et al., 2011). Therefore, these laboratory-confirmed cases may not be a representation of all salmonellosis cases. Furthermore, estimates are based on the 2006 population of the United States at 299 million people. According to the most recent United States population estimate on July 1, 2017 (United States Census Bureau, 2017), there are over 325.7 million people in the country; meaning, foodborne disease cases, hospitalizations, and deaths have all likely increased in absolute quantity. A better understanding of the population dynamics leading to an increased risk of slaughter contamination with AMR bacterial populations can be achieved by studying AMR *E. coli* and *Salmonella* populations in animals in their pre-slaughter environment. Understanding these dynamics allows for interventions to be designed to mitigate against any such risks that might be increased following a two-dose CCFA treatment for metritis.

1.8 STUDY SIGNIFICANCE

Our hypotheses were motivated by a risk assessment framework suggested by the late H. Scott Hurd (2004, 2006) in which he and colleagues explored the risk of AMR bacteria and their determinants escaping the farm at levels higher than 'baseline' levels and causing disease consequences (such as treatment failure due to microbial resistance) in humans. By temporally evaluating the effects of CCFA on AMR enteric bacterial populations, such as *E. coli* and *Salmonella* in dairy cattle, we directly address a current knowledge gap concerning animal slaughter withholding periods following two-dose CCFA treatment; further, we will quantitatively assess the potential, if any, for increased levels of AMR bacteria at the time of first slaughter eligibility. McEwen (2006) notes there has historically been much more policy focus

regarding antibiotic residues in food products than antibiotic resistant bacterial populations. The impacts of CCFA on *E. coli* populations may then be used to model the less prevalent *Salmonella* due to similarities in serovar behaviors, genetic materials encoding resistance (e.g., genes, integrons, plasmids, and genetic dispersion (Lowrance et al., 2007; Cummings, Aprea, and Altier, 2014), thereby reducing sample size requirements (Lowrance et al., 2007). Results from this study will allow dairy producers to make better informed decisions regarding animal slaughter withholding times and potential risks posed to consumers, provide a better understanding of AMR in the dairy production system, and to improve voluntary antimicrobial stewardship strategies. By understanding how levels of AMR *Escherichia coli* and *Salmonella* fluctuate in the feces of dairy cattle following two-dose CCFA treatment for metritis, steps can be taken to better protect the public from consuming AMR organisms via meat from culled dairy cows.

1.9 OBJECTIVES

Our study evaluates the temporal dynamics of 3GC resistance within fecal *E. coli* and *Salmonella* populations of Holstein-Friesian dairy cattle over 56 days following a two-dose treatment of CCFA for metritis. Our study facilitates the evaluation of the effectiveness of the current withholding period of 13 days in allowing levels of 3GC resistant *E. coli* to return to pretreatment (baseline) levels prior to slaughter. We hypothesized that the two-dose CCFA treatment initially drives down total *E. coli* counts, while increasing the levels of 3GC resistant *E. coli*. This rebounds over time, but the proportion of resistance in the total *E. coli* populations could remain elevated above baseline at the first eligible slaughter date. Phenotypic evaluation of suspected ESBL *E. coli* resistance profiles to 14 antibiotics via microbroth dilution allows for

exploring the hypothesis that, following treatment administration, there is an increase in the number of antimicrobials and classes of antimicrobials to which isolates are resistant due to co-selection.

Examining the shedding of *Salmonella* throughout the first 3 sampling days allows us to explore similar hypotheses that the two-dose CCFA treatment drives down *Salmonella* shedding initially before the populations begin to rebound. Sequencing these isolates provides information regarding the background resistance profiles of the population. The application of micro and molecular biological techniques provides a more complete picture of Enterobacteriaceae population dynamics, 3GC resistance mechanism fluctuations, and the disruption of gut microflora ecology following a two-dose treatment of CCFA for metritis.

CHAPTER II

CRITICAL REVIEW OF THE LITERATURE

2.1 ECONOMIC IMPACTS OF AMR

Not only acting as a threat to the treatment of infectious diseases, AMR also impacts the economy. The scope of this burden depends on the perspective through which it is viewed. These focus of these impacts could be broad, such as patient, hospital, or societal perspectives (Eliopoulos, Cosgrove and Carmeli, 2003) or more specific, like physicians, patients, healthcare businesses, pharmaceuticals, and public perspectives (McGowan Jr., 2001). An argument can be made to include surveillance and prevention activities in these estimates, in addition to how resistance impacts those who receive prophylactic antibiotics for surgeries, cancer treatment, and those patients who are immunocompromised (Smith and Coast, 2013). Differing perspectives, the inability to accurately judge costs, and a need to include a variety of industry experts, along with frequent differences in study design and estimate generation, make creating accurate estimates difficult (McGowan Jr., 2001; Eliopoulos, Cosgrove and Carmeli, 2003; Smith and Coast, 2013). Because costs have not been placed on the burden of AMR, progress has been slow in the way of health policy (Smith and Coast, 2013). While agreeing with McGowan Jr. (2001), Eliopoulos, Cosgrove and Carmeli (2003), and Smith and Coast (2013) that economic impact is important in proposing new legislation, to place a large degree of blame on health economists is unfair given the scale and multidisciplinary aspects of developing an accurate estimation.

These papers (McGowan Jr., 2001; Eliopoulos, Cosgrove and Carmeli, 2003; Smith and Coast, 2013) failed to consider the perspectives of animal agriculture and veterinary medicine. Within these 2 groups lie many differing viewpoints, including how to use antibiotics

(therapeutic, metaphylactic, prophylactic or growth promotion), how to administer antibiotics (parenteral, in water, in feed), and public health, moral (animal welfare), and economic obligations (Scott, Midgley, and Loneragan, 2015). While science is playing a larger role in decision-making, debate still rages regarding less empirical standards, such as the "Precautionary Principle" (Scott, Midgley, and Loneragan, 2015).

To better understand these moral obligations, the team of Jan et al. (2010) performed a cross-sectional study using the Theory of Planned Behavior to determine what moral obligation feedlot veterinarians felt toward certain interest groups. These interest groups were divided into 5 categories consisting of veterinarians and nutritionists in the feedlot industry; feedlot owners/managers; beef packers, retailers, and consumers; professional organizations, the FDA (and other federal regulators), and licensing boards; and pharmaceutical companies. The team sent out 325 18-page surveys to feedlot veterinarians receiving a response rate similar to industry average at 32% or 103 completed surveys. The four categories of interest for antibiotic usage were for acutely sick, chronically ill, at-risk, or high-risk cattle. For each situation mentioned, it was asked if antibiotics would improve cattle health, increase profitability, or improve cattle well-being, among a host of other questions based on a theoretical framework from social psychology.

The team found subjective norms and moral obligations did play a role in veterinarian decision making regarding antimicrobial usage in each of these 5 groups (Jan et al., 2010). However, these pressures varied in usage by category. For instance, pressures from colleagues led veterinarians to favor antibiotic use in chronically ill cattle, while organizational pressure led to a favorable view of antibiotic usage in acutely ill and high-risk cattle. A decrease was noticed when pharmaceutical company pressures were applied to use antibiotics in acutely ill cattle.

Moral obligations to clients created a positive view of antibiotic use for veterinarians in 3 of the four categories, the exception being usage in high-risk cattle. Subjective norms from colleagues provided positive indications of veterinarians using antibiotics in all categories, except at-risk cattle (those without evidence of disease). Interestingly, pressure from the groups that establish these norms led to decreased frequency in antibiotic uses in acutely ill or high-risk cattle. Antibiotic usage in at-risk cattle proved to be the only category in which client pressures increased usage; that is, preventive uses and uses of sub-therapeutic antibiotics (legal at the time of the survey) were highly impacted by client expectations.

This study provides insight into the external factors veterinarians consider when administering antimicrobials; however, some of the information conflicts. Organizational pressures were found to increase antibiotic usage favorability in acutely ill cattle, but pressures from the pharmaceutical industry caused a decrease in antibiotic usage in this same group. An interesting next step would be to see which view is valued or, in the case of negative effects, not valued the most. Do the negative views of pharmaceutical company pressures suppress the positive views of organizational encouragement? Is this view ultimately impacted by each individual case and the severity of such case? Better understanding these interactions could provide further insight and also aid in tailoring antimicrobial stewardship programs to better represent all involved.

In order to adequately access potential costs associated with AMR, all of the above components need to be included. These standards are broad and not well defined, as antibiotic usage varies within each field of medical practice and with each practitioner. It depends on how the use of antibiotics is defined. Is the focus treating infection, preventing infection, or another

purpose? To what extent is resistance being considered? Through which lens is the threat of resistance viewed among those listed above?

Even with such difficulties in estimating the burden of AMR, there are groups that have attempted to address such issues. High-level AMR models from the World Bank project the global gross domestic product (GDP) of low-income countries will decrease by 5.6%, while decreasing the GDP of the world by 3.8% on average by 2050 (World Bank Group, 2017). In low-level AMR situations, the average GDP is projected to decrease by 1.1% by the same year (World Bank Group, 2017). Moreover, world trade and livestock production are projected to drop by four and ten percent, respectively (World Bank Group, 2017). Due to decreased trade and livestock production, as well as increased cost of medical care, 6.3 to 26.2 million people in low-income countries are expected to fall into extreme poverty. On the other hand, 0.6 to 2.1 million people throughout the rest of the world will become extremely impoverished because of antimicrobial resistance (World Bank Group, 2017). It is important to keep economics and drivers of prescriptions in mind when designing and generating buy-in for stewardship and intervention programs. Ultimately, animal agriculture is a business and it is vital to appeal to economic, along with idealistic, concerns.

2.2 POLICY AND REGULATORY CHANGES TO COMBAT AMR

A 2002 review of animal antimicrobial use and resistance helped provide a now historical viewpoint of policies that were in place and provides a way to measure changes over the decades (McEwen and Fedorka-Cray, 2002). The review mentions the 1988 mandate of the FDA to make new antimicrobials accessible only by prescription, during a time when extra-label uses could be prescribed by a veterinarian and there was continued discussion of banning antimicrobial usage as growth promoters. The review alluded to other areas of AMR mobilization among animals,

such as husbandry protocols, animal movement, number of animals treated, the environment, and more. Pathogen testing at slaughter facilities included in the Hazard Analysis Critical Control Point has aided in preventing meat products with pathogens from entering the food supply, but regulatory activities based on surveillance programs are difficult to enforce for a variety of reasons. The establishment of the National Antimicrobial Resistance Monitoring System by the Food and Drug Administration, the U.S. Department of Agriculture, and the Centers for Disease Control and Prevention in 1996 has made strides in evaluating trends in resistance profiles starting with *Salmonella* and since adding more bacterial targets. The program looks to provide descriptive data, identify changes in resistance level and profile, provide information to medical personnel, extend antimicrobial time of effectiveness, and identify areas of further research and understanding.

A literature review by McEwen (2006) proposed recommendations for future policy developments. Future policies regarding antimicrobial uses in animal agriculture should apply research in a One Health approach that has outcomes valuable not only for humans and the environment, but the animals afflicted by disease (McEwen, 2006). Additionally, an understanding that the efficacy of these medications is not unlimited and there needs to be movement toward reaching an appropriate balance between risk and reward, while maintaining effectiveness and consumer confidence. McEwen (2006) suggested establishing veterinary relationships, nutritional programs, and treatment directed solely for the target organism, along with developing effective and practical guidelines specific to region by engaging veterinarians and stakeholders. Another suggestion involved shifting from a focus on antibiotic residues in meat products to resistant bacterial load that can then transfer resistance genes on mobile genetic elements to other enteric bacteria within the consumer. McEwen's (2006) approach was largely

on pace with the overarching goals of this current PhD project, as various stakeholders from multiple sectors, including feedlots and dairy farms, public health regulators, veterinarians, and consumer advocates, have been engaged as a way to begin conceptualizing and building voluntary stewardship programs that aim to be beneficial and practical for all involved. Additionally, the project includes microbiological and molecular techniques to evaluate resistant bacterial populations instead of antibiotic residues. Along with animals, the environment is taken into account with endpoints of human interest in ensuring animals are not being sent to slaughter with levels of antimicrobial resistance increased above baseline.

Another stewardship study conducted in the state of Washington used a two-survey method considering calving management, disease control, AMR, biosecurity, and descriptive dairy data (Raymond et al., 2006). The first survey was sent to 589 dairy farms in 2003, with follow-up materials sent regarding a variety of dairy production practices. Dairy producers who completed the first survey received an additional survey in February of 2005. Both the 2003 and 2005 surveys had five-dollar completion incentives. Of the 589 original surveys, 381 or 65% were returned. Unfortunately, some dairy farms had removed the identification label and some had gone out of business by the time of the second survey. This left 360 dairy farms, of which 292 completed the second survey. With regards specifically to antibiotics and infectious disease, 23% of responders reported using antibiotics in prohibited or unapproved ways. Additionally, 81% of farmers reported not testing animals new to the herd for infectious diseases and 33% utilized calving and sick pens for multiple other uses.

Overall, producers seemed to be concerned with antimicrobial resistance, as 59% or greater agreed a resistant infection in one cow threatens the herd, antibiotics lose effectiveness when frequently used, and antibiotic uses in food animal production could negatively impact

human health. The additional materials sent between survey distributions appeared effective, as dairymen reported decreased antibiotic usage, increased biosecurity, and changes in vaccination protocols in between surveys. The team noted changes were not significant before versus after intervention, although changes were reported during the second survey, citing the Hawthorne Effect where behavior changes based on observation (Landsberger, 1958). Education is an important way to provide individuals with the necessary tools to make decisions; that is, one cannot simply expect a person to stop a negative behavior if they do not perceive it as such. However, as this study showed, education was not the only thing needed and many factors played a role in decision making. Another problem with a study using survey methodology, the individuals responding could be the ones most concerned about AMR and thus inflate the concern represented on Washington dairy farms. Perhaps non-respondents did not care about or not see the threat of AMR and, therefore, failed to respond. As cited by the authors, the Hawthorne Effect could play a role, although these dairy farms were not being directly monitored. The Hawthorne Effect suggests these individuals were actually changing behavior due to being observed. However, the authors did not know for certain the dairy producers were changing their behaviors; they were simply taking the producers at their word. Producers could have been reporting a change due to the distributed material and the perception they were expected to change. This would indicate social desirability bias (Edwards, 1953).

In 2012, the Society for Healthcare Epidemiology of America, Infectious Diseases Society of America, and Pediatric Infectious Diseases Society developed a variety of recommendations to address antimicrobial resistance and create stewardship techniques, which it defines as:

"…coordinated interventions designed to improve and measure the appropriate use of antimicrobial agents by promoting the selection of the optimal antimicrobial drug regimen including dosing, duration of therapy and route of admission… to achieve best clinical outcomes related to antimicrobial use while minimizing toxicity and other adverse events, thereby limiting the selective pressure on bacterial populations that drives the emergence of antimicrobial-resistant strains. (Fishman, 2012)"

Among these recommendations were an optimization of antimicrobial usage in ambulatory settings, further research on the mechanisms of antimicrobial resistance, and improved monitoring of antimicrobial usage. The organizations determined a standardization of the ways in which antimicrobials should and should not be used, patient-centered outcomes for clinical effectiveness and economic sensibility, and improved knowledge of generic and branded antimicrobial usage would be beneficial. Additional recommendations included improved diagnostic testing to avoid using antibiotics to treat individuals with viral infections.

Some of these recommendations are overdue in part due to the complexity associated with clinical medicine in that no 2 patients are exactly the same, nor do 2 treatment regimens work the exact same way each time. These issues put standardization at risk and the ability to place a monetary cost to AMR expansion. Clinical effectiveness should be a constant focus of the medical and veterinary fields. The goal should always be to improve the overall health and well-being of the patient. Having patient-centered outcomes and improved diagnostic testing would not only reduce selection pressures for AMR bacteria, but improve patient response to treatment. Much like the studies included in the economics section, the focus of these organizations is human patients, but antimicrobials are not only used in human medicine, nor

should they be. To make impactful improvements in antimicrobial usage and AMR prevention, veterinary medicine and animal agriculture must be included in the discussion.

From May to August 2015 meetings and workshops were held involving a group in the United Kingdom to develop voluntary policies to curb antimicrobial usage, as instigated by 48 conventional and 25 organic dairy herds (van Dijk et al., 2017). Involving 70 producers and 27 veterinarians, the discussion revolved around infection control, proper treatment protocol, removing the use of antibiotics as a preventative measure, and ensuring proper data collection. Discussions took place in small groups prior to larger group discussion. Suggestions were then ranked by the complete group on ease of implementation. An overwhelming majority agreed that antimicrobial resistance is not a "passing fad" and antibiotics are used too often in United Kingdom animal agriculture. An additional majority agreed antimicrobial usage could decrease further and still maintain current health and production levels. However, responses regarding the estimation of antimicrobials used and AMR organisms on dairy farms varied. Both producers and veterinarians viewed veterinarians as being more important than producers in addressing these topics. The group developed 5 principles consisting of reducing and eliminating antibiotic therapies where possible, using veterinarians to address disease, supervising staff in using antimicrobials, reducing antimicrobial usage in prevention protocols, and using data as a comparative measure between dairy farms.

These policy developments hit a snag, as producers struggled to develop a set of common terms (Dijk et al., 2017). Producers felt confident in training regular staff on antimicrobial usage, but were concerned about temporary workers and a high turnover rate. The United Kingdom requires data collection on antimicrobial usage, but in practice collection efforts struggle due to limited tools and methodologies. Ultimately, it was determined dairy producers need a better

understanding of antimicrobials and their administration, along with developing ways to connect dairy and veterinary information. Those goals set the stage for the first year. This study highlights the struggles of developing voluntary policy standards. Even when producers agreed antimicrobial usage and AMR was an important issue and a broad set of standards were established, it became difficult to decide on details and implementation due to a high volume of turnover and a lack of tools. Importantly, the combined group identified areas of improvement necessary prior to the establishment of a new system. By identifying those areas, producers showed a commitment toward developing a better method of practice. These discussions were a starting point and it is yet to be seen if measurable improvements stemmed from these meetings in the first year. Follow-up studies tracking progress will ultimately determine the level of success and commitment of producers and veterinarians.

2.3 CEFTIOFUR EFFECTIVENESS

In 2000, a study was conducted across 8 dairy farms in 5 states (California, New York, Florida, Michigan, and Texas) to evaluate the efficacy of ceftiofur hydrochloride for the treatment of metritis in dairy cows (Chenault et al., 2004). Holstein cattle were enrolled in the after passing a variety of exclusion criteria, including not having other treatments or caesarian section. The rectal temperatures of cows were checked for 14 days following birth and animals were examined for vaginal discharge, dehydration, heart rate, and rumen contractions. Cows were blocked in groups to receive saline (0.9% NaCl) at 2 mL per 45.4 kg, ceftiofur hydrochloride at 1.1 mg per kg, or ceftiofur hydrochloride at 2.2 mg per kg. Eligible cattle were evaluated on study days 6, 10, and 14, and enrolled cattle on days 1, 5 and 9 after treatment. A clinical cure was documented based on a rectal temperature below 39.5°C and absence of a fetid

vaginal discharge. Animals that needed additional treatment were removed from the study and noted as a failure to cure. In total, 60.6% of the 406 enrolled cows were heifers. Of the 406, 30 were removed from statistical analysis due to variation from the protocol.

Cure rates were found to be higher among the group receiving a 2.2 mg per kg ceftiofur treatment, but no statistical difference was observed between the 1.1 mg/kg ceftiofur group and the saline group (Chenault et al., 2004). No significant difference was observed for any of the treatment groups on study days 6 and 10. Through the first 5 days, the rectal temperatures of all ceftiofur treated cows decreased at similar rates and were significantly different from those in the control group. Upon enrollment, all cows had a vaginal discharge score of 4, but by day 14 an almost linear decrease was observed and the average score was 2.5. Based on the cure rates, both ceftiofur treatments (2.2 mg/kg and 1.1 mg/kg) had cure rates significantly higher than the control group (P=0.004 and P=0.021, respectively). The study enrolled a large number of cows across the country and had strict inclusion criteria to accurately attribute success or failure to the treatment group in which the cow was enrolled. Furthermore, it addressed metritis from a deeper perspective than temperature. Other variables, such as dehydration and heart rate were not mentioned outside of the methods section, so it is likely that those variables were only for physical examination purposes and not evaluated for the study.

As with any pharmaceutical approved by the FDA, CCFA has undergone testing to ensure its efficacy for its label indications. McLaughlin et al. (2012) performed a randomized study on 15 dairy farms across the United States from June through October of 2006 to establish the efficacy of a two-dose CCFA suspension (CCFA, 6.6 mg/kg) treatment of metritis in dairy cattle. Thirty animals from each location were chosen to comprise each of 2 treatment groups: the CCFA group and a group receiving saline as a placebo (1.5 mL/45.4 kg). Eligible cows had

to have calved within the past 10 days and have temperatures evaluated as exhibiting a fever to determine eligibility. Cattle were excluded if they received any pharmaceutical treatment other than a topical antibiotic since calving; they were given an antibiotic other than topical within 14 days prior to calving; caesarian section, fetotomy or uterine prolapse occurred during calving; or retained fetal membranes (RFM) occurred requiring intrauterine or parenteral antibiotic administration. A sample of vaginal discharge was collected and scored on a scale of 0 to 4. Post-injection measurements, rectal temperature, physical exams, and injection site evaluations were all regularly performed. It was found a two-dose treatment with CCFA at the base of the ear 72 hours apart increased the cure rate of metritis by clinical standards 19% above the saline control group. Because this study utilized a large number of dairy farms across the country and enrolled a number of cows sufficient to provide statistical power, these results can likely be more broadly applied to U.S. dairy cattle populations. Additionally, the team used a variety of metrics to evaluate metritis improvement and was careful to avoid any cattle that could confound results or leave doubt as to if effective treatment was due to CCFA, a previously administered medication, or a combination thereof. Oddly, the previous one-dose CCFA treatment for metritis was not included in the treatment protocol for this study. It would have made a useful comparison group to see the difference between treatment with a one and two-dose treatment.

Another study on a large Florida dairy farm tested the efficacy of ampicillin trihydrate and ceftiofur hydrochloride for treatment of metritis in dairy cows (Lima et al., 2014). A total of 528 animals were enrolled in the study, evenly split between heifers and cows. An additional 268 animals without metritis were enrolled on the twelfth day of the study. Control animals were selected based on calving date and parity. Vaginal discharge was scored on a five-point scale and temperature was considered elevated when recorded as higher than 39.5°C. Temperature was

recorded and documented daily. Cows were blocked in groups of 3 in which 1 received ampicillin, 1 ceftiofur, and the other, a control, did not have metritis. Treatments consisted of ampicillin at 11 mg/kg and ceftiofur at 2.2 mg/kg daily for 5 days. Metritis diagnosis was recorded as study day 1 and animals were assigned body condition scores upon enrollment. Any enrollees with difficult or assisted births were noted as having a calving-related disorder. Once diagnosed with metritis, animals had temperature data collected from days 1 through 7 and again on day 12. Vaginal discharge scores were reassigned on days 5, 7, and 12. Cure was evaluated based solely on vaginal discharge, again with vaginal discharge and rectal temperature, and whether or not additional treatment was required.

The average temperature of cows enrolled in the ampicillin group was higher than the ceftiofur group, although differences were not observed upon the study end (Lima et al., 2014). Metritic cows exhibited a decrease in temperature after the start of antimicrobial treatments. Throughout the study, ampicillin performed as well or better than ceftiofur in cure rates. Those in the ampicillin treatment group had significantly higher cure rates on day 7 when evaluating vaginal discharge alone or in combination with rectal temperature when compared to the ceftiofur group. These differences were not observed at the end of the study. This study provides significant data showing treatment with ampicillin can be an effective form of treatment for metritis. While ampicillin outperformed ceftiofur in many of the early study day data collection metrics, the findings do not indicate ceftiofur was not effective in comparison to the control group. Unlike many other metritis studies, this 1 seemed to focus more on vaginal discharge than rectal temperature. The only time temperature was evaluated was in combination with vaginal discharge, which is interesting. Similar to the criticisms of other publications, this study was performed on only 1 dairy in 1 region. For increased confidence in the results, replication of this

study in multiple study sites, if not regions/states, is needed to determine if consistent results are observed regardless of location.

A literature review of treatment for puerperal metritis found ceftiofur was the most investigated treatment form and, while 7 studies reported clinical improvements, reproductive performance was never improved in comparison to control cattle (Haimerl and Heuwieser, 2014). This review found less than half of the studies used bacteriological exams and 3 or less evaluated AMR, antibiotic applications, or antibiotic usage guidelines. Haimerl and Heuwieser (2014) searched with the keywords "bovine metritis AND antibiotics" and had strict exclusion criteria, including descriptive and *in vitro* studies, conference proceedings, review articles and more.

However, a literature evaluation by Reppert (2015) of CCFA effectiveness was proven difficult due to small sample sizes, lack of control groups, inconsistent dosing and administration protocols, and variance among the case definition of metritis. To find studies, the keywords "dairy cattle", "metritis", and "ceftiofur" were used. Studies had to be controlled, prospective, and experimental studies meeting a metritis definition. The definition had to include a temperature of 39.5℃, fetid red-brown discharge, and diagnosis occurred within 21 days after calving. Additionally, case cows could only be treated with ceftiofur with a variety of measures indicating clinical improvement routinely taken. In total, the review included 34 studies, but was unable to perform robust analysis due to a lack of consistency among study protocols. The main finding of this review was the inability to do a proper review based on available information. This review used some additional keywords than the previously mentioned review above, but was more selective in the words used and was very selective in papers evaluated. The reasoning behind finding limited options is relatable. The most common complaint of studies reviewed in

this critical review is a lack of sample size and an inconsistency in dosing and administration in the literature as a whole. Some studies utilize multiple administration techniques to determine effectiveness, but these techniques are largely variable throughout.

2.4 METRITIS

In seeking to provide a solution to the variety of metritis definitions, Sheldon et al. (2006) published with the intent of describing postpartum uterine diseases. The team defined puerperal metritis as consisting of an enlarged uterus with a red-brown discharge, fever greater than 39.5℃, and signs of systemic illness occurring within 21 days of calving (Sheldon et al., 2006). Clinical endometritis was described as having purulent or mucopurulent discharge in the vagina 21 and 26 days after calving, respectively (Sheldon et al., 2006). These definitions have proven useful for other publications (McLaughlin et al., 2012; Reppert, 2015), but have not completely solved the problem (Krogh and Enevoldsen, 2014) and helped form the definition used for our study. A variety of calving difficulties are risk factors for the development of metritis, including dystocia, twins, retained placenta, and stillbirths, in addition to ketosis (Sandals et al., 1979; Dohoo and Martin, 1984; Gröhn et al., 1989; Rajala and Gröhn, 1998; Benzaquen et al., 2006; Giuliodori et al., 2013).

The causes of metritis have been the focus of research for decades. Data from 1973 to 1976 evaluated calving, heat and breeding dates, disease occurrence, and treatment of 293 Holstein-Friesian cows and 652 calvings in the Guelph area of Ontario (Sandals et al., 1979). For the study, retained placenta was defined as an inability for the cow to expel the placenta within 24 hours of calving. Farmers monitored the cow's health and were instructed to administer a

two-dose treatment of penicillin-streptomycin once the rectal temperature became higher than 39℃. Case cows were placed in 1 of 3 groups: retained placenta, retained placenta and metritis complex, or just metritis complex. Control cows were typically matched within a year of age of their case counterparts. Throughout the study, 33 cases fell ill in the first grouping, 40 in the second, and 13 in the last. Cows birthing multiple calves were 4.6 times more likely to have a retained placenta than their counterparts birthing 1 calf. Most cases happened in the October to December calving months, but the authors note this may be due to the increase in calving events during those months. While not statistically significant, cows with retained placentas took approximately 30 more days to get pregnant than control cows. However, cows with metritis took longer to conceive at a statistically significant level ($P < 0.05$) of approximately 51 days longer than control cows. The study continued for an extended period of time and had a robust sample size. Their findings are consistent with expectations in that animals suffering from complications or infections from the previous calving would require a longer period of time to achieve pregnancy again due to an extended recovery period.

At the same university, Dohoo and Martin (1984) utilized a case-control study design consisting of 1,844 lactations within southern Ontario with disease cases having the disease of interest and control cows being disease free. Diseases were wide-ranging, including milk fever, ketosis, mastitis, respiratory disease, and others, along with calving difficulties. The team used every cow with the disease of interest on a farm as a case. As many control cows as possible were enrolled. Control groups were evenly split to match with each case cow. Three models were evaluated consisting of 490 lactations from heifers, 751 mature cows with data from the previous lactation, and 1,315 lactations, regardless of cow age, while using age as an external factor. The team determined the presence of 1 disease increased the likelihood of the cow having another

disease. Of the 136 possible disease pairings based on diseases of interest, the team found 32 different pairs of disease associations in their study. All of these pairs had odds ratios above 1. The mature cow model showed age was linked to 6 diseases, while the complete model indicated a relationship with 8 of the 19 internal variables. Having the disease in a previous lactation was found to be a risk factor for future disease development in 9 of the 17 disease states of interest. The only association with metritis was retained placenta. A cross-tabulated model showed more significant relationships, but the authors believed it to be due to the cross-tabulation recognizing the disease states going from A to B and B to A, statistical changes in sample size and distribution when using a four-fold table, and disease intensity for each case. Additionally, the study revealed high milk yields did not make a cow more likely to have a certain disease in the future. This study provides insight into how disease states in dairy cattle impact the occurrence of future disease states, providing farmers with information to better manage herds and reduce production loss. The large sample size and multiple groupings of cases and controls, in addition to the cross-tabulated and case-control methodologies, make this study very robust and provides multiple platforms for evaluation.

A research team used retrospective data from the Danish Cattle Database to perform a longitudinal study evaluating risk factors for metritis (Bruun, Ersbøll, and Alban, 2002). Of the 2,391 contacted farmers, 2,148 agreed to be interviewed by students trained to administer questionnaires. Cows included in the study calved between July 1, 1993 and June 30, 1994 with disease states recorded both 30 days prior to and after calving. Metritic cattle were recorded in the Danish Cattle Database as veterinary treatment. Heifers were not included in the study. With these criteria, 102,060 cows were included in the study. Dry-cow mastitis, reproductive disease, and breed were all variables used in analysis. Confounding was considered a change in test

statistic greater than 20% and interactions were evaluated using a variety of variables including: RFM, breed, parity, dystocia, grazing, and calving season. In total, 733 cases of metritis were included with cumulative lactation incidence ranging from 1 to 21% and 391 herds reporting at least 1 case. A variety of variables came back as risk factors. Parity, grazing, calving season, dystocia, reproductive disease, ketosis, and an interaction between RFM and breed were all implicated as risk factors.

Upon further breakdown, the team noted a "u-shaped association" between parity and metritis suggesting young cows and cows of parity four or higher were more likely to develop metritis. Risk of metritis was lower among cows eligible to graze. Calving from November through April elevated metritis risk, along with dystocia and reproductive illness due to the reduced health of cattle in the winter and traumas of difficult calving or illness. The team noted the interaction of breed and RFM was the first of its kind since most studies only enroll 1 breed of cow. They did, however, mention a multitude of studies identifying RFM as a metritic risk factor. Due to the large number of variables considered, this study shows the breadth of risk factors associated with metritis. With that said, there was a very large sample size which could lead to some variables testing as more significant than they would otherwise. Additionally, as was the case with ketosis, there was some conflict among how results were expressed in the paper. Some p-values were displayed in the article, but there was not a breakdown of these p-values within the category. For instance, parity was listed as significant with a p-value of <0.001. Further breakdown would be beneficial in providing a more complete picture of the variables, especially as some levels were significant and others are not.

A 2007 study evaluated rectal temperature and calving issues in relation to puerperal metritis on a 1,000 head dairy herd in northeast Florida from August of 2002 through April of

2003 (Benzaquen et al., 2007). Dystocia, twinning, and RFM were noted with RFM being defined as membranes not being expelled within 24 hours. RFM was rated on a five-point scale. After waiting 60 days, cows began undergoing artificial insemination again and were checked for pregnancy by rectal palpation. Cows with cesarean section were not included in the study. Cows were monitored 3 to 13 days after giving birth for rectal temperature and attitude. Puerperal metritis occurred in 21% of study animals and of those 94 cows, 55 did not have an increased rectal temperature. Heifers were more likely to have puerperal metritis in the summer than the winter, but cows did not exhibit a seasonal difference. There was a significant interaction between day and puerperal metritis. Rectal temperature was significantly higher 3 days prior to diagnosis, and maintained as elevated four days after diagnosis. These temperatures began to decrease a day after treatment began. Those animals without an increased temperature did not see a significant decrease in temperature. The statistical hazard of animals becoming pregnant at first conception was impacted by season and parity. The time between last calving and conceiving again in 150-day intervals was impacted by season. The team determined calving difficulties was a risk factor for puerperal metritis, in addition to calving in the winter for heifers. The team also recommended including attitude and uterine examination into the diagnosis of puerperal metritis. These findings shed some light on metritis development. While performed on a large dairy, it would be beneficial to see if these findings are similar on other dairy farms in other climates since rectal temperature still seems to be a large player in metritis diagnosis, even though the study shows metritis can still develop without a fever. Like Dohoo and Martin (1984), a seasonal difference was found, but Dohoo and Martin (1984) had mentioned more calvings occurred in winter, which may impact results. Benzaquen et al. (2007) did not mention an increase in calvings in the winter or summer. This information would be useful in determining

how much of a role seasonality plays or if it is by happenstance due to a difference in the number of calvings occurring in each season.

A study to evaluate if cows at risk for metritis could be identified by their pre-calving behavior and dry matter consumption began at the University of British Columbia's Dairy Education Research Center in August of 2005 and extended into March of 2006 enrolling 32 heifers and 69 cows (Huzzey et al., 2007). Feed and water consumption was measured electronically using a transponder attached to the animal's ear tag. As the cow approached the feed gate, it opened and the time and bin weight were recorded. Once the cow left the bin, the gate closed and time and bin weight were again recorded. The number of times a cow was moved away from the feed or water bin due to another cow or vice versa was recorded daily as a behavior indicator. Cattle body condition score and weight were monitored both before and after the calving. Retained placenta followed the same definition of Sandals et al. (1979), Dohoo and Martin (1984), and Benzaquen et al. (2007). Mastitis exams were conducted every 3 days after calving for 21 days. Time of calving, feed, and water consumption were observed via video to ensure the day 0 feed and water consumption data was measured from the time the calving occurred. Study animals were evaluated for vaginal discharge at the same intervals as mastitis exams. Vaginal discharge and odor were measured on a scale from 0 to 4. Miscellaneous health issues and mastitis removed a combined total of 23 animals from the study.

Of those with metritis, 7 with severe metritis had a retained placenta and 2 had twins. Mild metritis cases consisted of 2 animals with retained placenta. Those animals with mild and severe metritis had decreases in milk production by 5.7 and 8.3 kg of milk per day for the first 3 weeks of production, respectively. An assisted calving, indicating dystocia, was associated with a 15.8 times higher risk of developing severe metritis. Decreases in dietary consumption and days

of gestation were also indicators of severe metritis. The number of calvings or pre-calving body weights or conditions were not associated with a risk of developing severe metritis. Animals eventually developing severe metritis were found to move other animals from feed or water less frequently and also tended to consume less feed and water beginning approximately a week prior to calving. The research group concluded these findings were indicators of a heifer or cow developing severe metritis; however, it is important to remember correlation does not mean causation. Furthermore, lack of energy and appetite is common among most disease states, so these metrics might not exclusively indicate metritis, but a more general state of disease. The study provides additional insight into potential risk factors of metritis development, but further research into the mechanisms for metritis development resulting from decreased aggressive behavior, feed intake, and water consumption would provide further support to how closely linked these findings are to metritis development.

An Argentinian study utilized a 1,600 head Holstein herd to enroll 303 autumn calving cows to evaluate risk factors and calving performance associated with metritis (Giuliodori et al., 2013). The study ran from May through August. The dairy had a 40% cumulative incidence of metritis in the 3 years preceding the start of the study. A three-point scale was used for dystocia rating and retained placenta utilized the same definition, inability to expel the placenta within 24 hours, as Sandals et al. (1979), Dohoo and Martin (1984), Benzaquen et al. (2007) and Huzzey et al. (2007). Dystocia, retained placenta, and stillbirths were all documented throughout the study. Metritis definitions were followed as described by Sheldon et al. (2006). Metritis cows were randomly assigned to the treatment or control group of ceftiofur hydrochloride in which treated cows received 2.2 mg/kg of body weight for 3 days.

Risk factors for metritis, as determined by the study and interpreted by the team, were heifers, abnormal calvings, and poor energy levels prior to calving, which provides additional support for the findings of Huzzey et al. (2007). Metritis increased the time-to-conception with the most impact being with puerperal metritis and both clinical and puerperal metritis decreased milk production early in lactation. The team did not find an increased cure rate or milk production with the metritis regimen, but did increase the risk of pregnancy with artificial insemination and reduced the risk of being culled for reproductive issues. This study's findings remain consistent with others found in the literature regarding decreased milk yield and increasing time-to-conception. The authors mention an increased cure rate was not observed with ceftiofur and noted most studies only utilized temperature as an indicator of improvement. However, both Haimerl and Heuwieser (2014) and Reppert (2015) reported improvements in their literature reviews. Chenault et al. (2004) and Lima et al. (2014) each utilized vaginal discharge, in addition to temperature, in their investigations into the efficacy of ceftiofur for the treatment of metritis and came to the conclusion ceftiofur is an effective form of treatment for metritis in cattle, although the data of Lima et al. (2014) suggests ampicillin may be a more effective form of treatment. McLaughlin et al. (2012) also utilized routine physical examination in their measurements.

2.5 PRODUCTION COSTS OF METRITIS

Metritis is a disease associated with large production costs on dairy farms. A literature review estimated that the prevention and treatment of metritis costs producers on average of $4.70 per dairy cow in inventory annually, although estimates vary by state (Bellows, Ott, and Bellows, 2002). A 1998 publication evaluating the impact of dystocia, retained placenta, and

metritis on milk production utilized 37,776 Finnish Ayrshire dairy cows from 2,337 herds that calved during 1993 (Rajala and Gröhn, 1998). Metritis was broken down by early and late metritis, depending on how many days diagnosis followed calving. Milk yields were tested every 30 days and the lactation period was separated into 17 stages with milk records taken every 10 days for the first 60 of lactation, every 20 days for lactation days 61-180, and every 30 days from lactation days 181-330. Lactations were grouped into categories of 1, 2, 3, and 4 or more lactations for curves. Rajala and Gröhn (1998) found early metritis was associated with a reduced milk yield, seasonality had significant effects, and cows calving in the winter produced more milk than those calving in the summer. For parity 1 cows, retained placenta was significant for decreased milk yield and early metritis had an association with decreased milk yield, although not statistically significant. Milk production among high producing parity 2 cows with early metritis was again associated with a non-significant decrease in milk yield. Dystocia dropped milk yields of parity 2 cows significantly and retained placenta negatively affected the milk production of high yielding animals. High yielding parity 3 cows had a decrease in milk production before diagnosis with early metritis. This decrease was more noticeable when dystocia or retained placenta were not included in the model, which would show those variables are confounders of early metritis should the difference have a minimum change of 10%. Parity 4 cows did not indicate a statistically significant decrease in milk yield for any of the 3 conditions of interest, but decreases in production were observed.

When 305-day milk production was evaluated, none of the diseases resulted in a decrease in milk production, rather a higher yield (Rajala and Gröhn, 1998). Only late metritis had a significant impact on 305-day production. Each disease showed higher levels of impact when modeled alone than when involving the other 2 options. This lead the authors to suggest other

studies evaluating just 1 option could overestimate the impact of the individual disease or condition being evaluating. Some conditions and diseases were associated with higher milk yields, which the authors noted could lead to false conclusions if multiple and frequent production measurements not been taken. It was also noted that treatments are only administered by veterinarians in Finland and retained placenta is rarely treated unless systemic disease is beginning to show. Because study animals required a veterinary diagnosis, findings could be elevated due to the progressed disease state warranting veterinary examination and treatment. This study offers strengths in its sample size and multiple points of measurement. It provides good insight into milk production and how various diseases affect milk yield throughout lactation. It would be interesting to see the results of a study of similar design in an area where farmers could treat animals themselves or animal health is more closely monitored to see if these same trends are observed in animals with mild forms of disease.

A study performed across 5 Israeli commercial dairy herds determined cows and heifers impacted by RFM or clinical metritis can result in 300-500 kilograms of reduced milk production in comparison to cattle that did not experience clinical metritis or RFM (Goshen and Shpigel, 2006). Cattle that did receive treatment for clinical metritis observed a 305 day corrected lactation of 654 kg more milk than the untreated groups with no statistical difference with treatment simply for RFM absent metritis. The study ran from April of 2000 until December of 2001 and focused on cows that calved in that time period. Collected data included clinical, reproduction, production, and management data. Odd numbered cattle with clinical metritis or retained fetal membranes were randomly placed into a treatment group receiving 5 grams of chlortetracycline twice a week for 2 weeks or no treatment at all. The study contained a total of 1,416 cows and 804 heifers. Within these populations, 967 cows and 489 heifers did not

experience clinical metritis or RFM. Additionally, cows with clinical metritis had a more difficult time conceiving than cows without a metritis diagnosis. This impacts milk production indirectly, as the cows need to calve to produce milk. Any delay in conception delays milk production in the future. This study provides a numerical impact on the issues of metritis. A decrease of 300 to 500 kg of milk production per cow with metritis is a large economic impact for producers. With different countries potentially employing varying management practices, for instance, a two-dose CCFA treatment for metritis in the United States, it would be worth performing similar studies in different countries to see if the economic impact is the same.

Even within the structured protocols and science of dairy production and metritis management, there is still a large degree of variation among producers. A 2012 study was designed to evaluate dairy interventions, including herd health management programs (HHMP), by evaluating data from 121 herds consisting of 76,953 lactations over 15 years in Denmark (Krogh and Enevoldsen, 2014). Cattle were routinely examined by veterinarians and vaginal discharge rated on a scale of 0 to 9 was employed to determine the degree of metritis. Measurement, examination, and treatment varied by veterinarian. Because HHMP is unclear, Krogh and Enevoldsen (2014) focused on milk production as a resulting factor of vaginal discharge, specifically the relationship between metritis treatment and milk yield. A baseline loss of energy-corrected milk (ECM) due to metritis was considered at 192 kg, but became 69 kilograms after enrollment in an HHMP. Parity 2 cows had a decrease of 91 kg of ECM due to metritis disease and treatment if the calf was healthy regardless of HHMP enrollment, although the loss was 348 kg if the calf was dead. In cattle with parity greater than 2, a decrease of 247 kg in ECM was observed with metritis if the calf was born healthy, regardless of HHMP enrollment, but the loss was only 192 kg of ECM with a dead calf. Cows enrolled in HHMP saw 17% less

305 day ECM production loss; however, this was based on examination and metritis detection, in addition to treatment protocols. Based on study results, HHMPs provided a positive result in reducing ECM production loss. A consistent problem remained variation among dairy protocols and management practices; perhaps this study showed any HHMP proves beneficial, but a more standardized, voluntary version amongst producers utilizing best practices could increase production, as not all dairy producers have available capital to make vast improvements at any given time.

Understanding the reasons for CCFA usage instead of other metritis treatments as well as the risk factors for and production costs of metritis are critical for our study. Additionally, it is important to prevent and treat metritis effectively for the economic well-being of the farmer, and the health and welfare of the cows. The lack of a milk withholding period with this treatment decreases the economic loss associated with other treatments. Therefore, this is among the most commonly used drugs for metritis treatment. Furthermore, this knowledge of metritis highlights the scope of the problem caused by the disease. By understanding the risks for metritis and mitigating those risks, antibiotic stewardship can be improved by preventing the disease before a treatment is necessary.

2.6 THIRD-GENERATION CEPHALOSPORIN RESISTANCE OF FECAL *E. coli* IN DAIRY CATTLE POPULATIONS

AMR in dairy cattle has been studied from a variety of designs depending on the organism, mechanisms of interest, stage of production, and antimicrobial of interest. Zwald et al. (2004) evaluated antimicrobial usage and management procedures on conventional (n=32) and organic (n=99) dairy farms in 4 states consisting of Michigan, Minnesota, New York, and

Wisconsin from May of 2000 through March of 2001. Dairy farms were contacted via letter to determine interest in the study and further eligibility. After study dairy farms were selected, a 64-question survey was sent inquiring about herd size, herd expansion, housing, feed and water protocols, calf management, manure management, and antimicrobial usage. Conventional dairy farms tended to be larger and produce more milk than organic dairy farms. Differences in management practices were largely attributed to differences in herd size, but it was noted organic dairy farms were less likely to use feed from off-site and mainly fed soybean-based diets. It was discovered ceftiofur was the most commonly used antibiotic on each dairy type, since in the U.S. organic dairy farms can use antibiotics in ill cattle so long as milk from the animal is no longer sold as organic and the cow leaves the farm after recovering. This is another example of the difference between dairy production styles; however, it would have been better to have some more empirical measurements, as surveys can be prone to bias. Producers could be answering questions as they feel is socially acceptable or could be incorrectly remembering antibiotic usage. These biases, while sometimes subconscious or unintentional, can create misleading results and incorrect conclusions. A follow-up study that more closely follows these categories of interest for a period of time would be beneficial in providing more information toward the accuracy of these results.

In Ohio, a study consisting of 18 dairy farms and 1,266 dairy cows evaluated ceftiofur usage and reduced susceptibility to ceftriaxone (Tragesser et al., 2006). Information regarding dairy antimicrobial usage protocols and individual cattle antimicrobial usage from the previous 6 months was obtained. Fecal samples were enriched and plated to antibiotic specific media to screen for reduced susceptibility to ceftriaxone before performing PCR to check for the AmpC β-lactamase gene, $bla_{\text{CMY-2}}$. Prevalence of reduced susceptibility was found to be between 0 and

97% with 83% of the reduced susceptibility isolates containing the bla_{CMY-2} gene. Further susceptibility testing was performed on 81 isolates containing the gene. These isolates were chosen to represent all herds involved in the study. This additional testing indicated those isolates with reduced susceptibility to ceftiofur contained resistance to additional antibiotics. The team determined dairy farms using CCFA were 25 times more likely to have *E. coli* with reduced susceptibility to ceftriaxone than their non-CCFA counterparts, which makes sense given both medications are third-generation cephalosporins. A linear relationship between reduced susceptibility and percentage of treated cows was not observed, leading to their suggestion that any interventions should be applied at herd, instead of individual, levels. This recommendation was made due to the herd-level association without the individual association; that is, the authors believed herd-level interventions may be more impactful in curbing the spread of AMR genes. Because the goal was for screening, plates were not colony counted or prepared in a way to allow for it. Those counts could have aided in determining if there was a difference among individual cows and farms beyond the positive/negative designations given to each sample. Plate counts could have allowed for better decision making regarding how individual animals are impacted and further supported or, perhaps, provided evidence against a herd – level intervention.

The effects of ceftiofur on *E. coli* populations within dairy cattle were further tested on a central Pennsylvanian dairy farm (Donaldson et al., 2006). On this dairy farm, 96 calves were sampled over 5 months. Ceftiofur was used to treat respiratory infections and scours, but documentation was not kept on dosage or number of times administered. The study ran from April through August of 2003, but it was noted that the dairy traditionally used a milk-replacer medicated with tetracycline and neomycin before switching to non-medicated replacer in May of the study period. Calves were weaned after 8 to 9 weeks. Donaldson et al. (2006) found 88.5% of

calves in their study were positive for shedding ceftiofur resistant gram-negative enteric bacteria and, based on count data, suggested commensal *E. coli* can be important in spreading multidrug resistance on dairy farms, as MDR was found in many of the collected isolates. Caution should be used when applying the results of a study using only 1 dairy to a broader population. A broader study should be employed in order to comfortably extrapolate the findings to a larger population. The team attributes their findings to the use of ceftiofur, but the usage of an antibiotic milk-replacer to begin the study complicates whether these findings can be directly attributed to the use of ceftiofur or to what extent the tetracycline-neomycin milk-replacer played a role in co-selection. Additionally, it would be beneficial to know the dosage or administration frequency of ceftiofur to gain a better understanding of the role it may have played.

In 2007, Lowrance et al. performed a prospective cohort study with 61 steers distributed into four cohorts of varying CCFA dosage protocols to evaluate the levels of antimicrobial resistance among fecal *E. coli* populations in feedlot cattle. The cohorts consisted of 10 steers with a single dose of CCFA (6.6 mg/kg) on day 0, 10 steers given a single dose of CCFA (4.4 mg/kg; note, this is an extra-label usage now banned) on day 0, 10 steers given a three-dose treatment with CCFA (6.6 mg/kg; note, this is an extra-label usage now banned) administered on days 0, 6, and 13, and a final control cohort of 31 steers not given CCFA. The team determined, while ceftiofur susceptible isolates tended to be resistant to 1 antimicrobial and ceftiofur-resistant *E. coli* were inclined to contain resistance to 7 antimicrobials, levels of population resistance tended to recede to baseline within 2 weeks of final administration. This led to the hypothesis that susceptible variants were better equipped for survival in the absence of the ceftiofur selection pressure than their ceftiofur-resistant counterparts. Based on the reduced resistance population over a relatively brief period of time, the hypothesis of resistance genes removing a

level of fitness under non-selective pressures makes sense. It is interesting this occurred with all treatment groups regardless of dosage administered or number of treatments given. These results are in agreement with Tragesser et al. (2006) and Donaldson et al. (2006) linking ceftiofur use to increased levels of resistance, albeit at the individual animal level.

A study performed in the Czech Republic aimed to determine ESBL *E. coli* prevalence on a traditional dairy that utilized cephalosporins versus an organic dairy farm without cephalosporins (Dolejska et al., 2011). The traditional dairy farm primarily used ceftiofur, although other cephalosporins were used (4^{th} generation cephalosporins are approved for use in animal agriculture in Europe), with a varying number of administrations. The organic farm used a small number of antibiotics (European organic farms are able to treat sick cows), primarily tetracycline, but no cephalosporins were used. A total of 309 rectal swabs were taken from the traditional dairy farm, with 154 cattle and 46 calves having sample swabs taken from the organic dairy. It was found that a dairy farm utilizing third- and fourth-generation cephalosporins was more likely to isolate ESBL-producing *E. coli*, all containing the $bla_{\text{CTX-M-1}}$ gene, as 119 swabs tested positive for ESBL *E. coli* on the traditional dairy farm with only 1 swab from the organic dairy testing positive (Dolejska et al., 2011). Each sample testing positive for the $bla_{CTX\text{-}M\text{-}1}$ gene on the traditional dairy was transferred through conjugation to both *E. coli* and *Salmonella*, in addition to being associated with the IncN plasmid group. This study is interesting in comparing traditional and organic dairy farms to evaluate ESBL resistance prevalence between the 2. The small number of locations in these studies makes it difficult to make broad generalizations.

While commonly used to treat metritis, CCFA can treat other gram-negative bacterial infections (Wittum, et al., 1996; Haimerl and Heuwieser, 2014). A matched-pair cohort study involving cattle with *Leptospira borgpetersenii* serovar Hardjo-bovis requiring a treatment of 2.2

mg/kg of ceftiofur (no formulation specified), intramuscularly, daily for 5 days was performed on a dairy farm milking 150 cows in central Illinois (Singer, Patterson, & Wallace, 2008). All cows were housed in a single barn. Five cows were selected as cases and 5 as controls. Lactation number was a determining factor in matching. Samples were collected before, during, and after treatment. Collected samples were colony counted for *E. coli*. Selected isolates were tested for antimicrobial sensitivity and the $bla_{\text{CMY-2}}$ gene via PCR.

The team found that usage led to a significant difference in total *E. coli* load and antimicrobial resistance index on study days 4, 5, and 6 with no evidence of $bla_{\text{CMY-2}}$ gene transfer (Singer, Patterson, & Wallace, 2008). The $bla_{\text{CMY-2}}$ gene was found in 12 of 203 isolates from case cattle, but never found in control group isolates. Similar to Lowrance et al. (2007), general *E. coli* populations decreased upon treatment, but eventually rebounded without an expansion of the resistant bacterial population. As stated repeatedly, studies at only 1 location, especially with such a small sample size, are hard to generalize from in a broader sense. This study has similar results to other studies mentioned throughout the document, but sample size and population are important in the establishment and utilization of epidemiology studies. A study with only 5 case and 5 control cattle leaves much to be desired in terms of what can be learned and applied.

A cross-sectional study collected cattle fecal samples across 50 Ohio dairy farms from the summer of 2004 through the spring of 2006 with herd owners administered surveys regarding antimicrobial usage and dairy demographics (Heider et al., 2009). Herds with less than 100 milking cows had samples collected from every cow, while herds with greater than 100 milking cows had up to 100 samples collected totaling 3,840 samples. Of the 50 sample locations, 88% used ceftiofur. Based on data collected from MacConkey agar plates made with ceftriaxone

regarding bacterial growth, 92%of the herds tested positive for ceftriaxone-resistant *E. coli*. In total, nearly 61% of samples tested positive for ceftriaxone-resistant *E. coli*. Alternatively, 44% of dairy farms tested positive for *Salmonella* and nearly 10% of the total samples tested positive. All *Salmonella* isolates had ceftriaxone MICs of less than 8 µg/mL. *E. coli* with reduced susceptibility were found more often on farms positive for *Salmonella* and increased by 62% with every 454 kg increase in average milk production on farm, although an association was not found between reduced susceptibility and ceftiofur usage. A subset of *E. coli* with reduced susceptibility to ceftiofur were selected to run PCR for the bla_{CMY-2} gene and all tested positive. The team hypothesized dairy farms with *Salmonella* were more likely to use ceftiofur, but their data did not support this. Therefore, they further hypothesized there were other herd-level factors promoting *Salmonella* growth and *E. coli* with reduced susceptibility to ceftriaxone.

A 2012 cross-sectional study consisting of 25 Ohio dairy farms collected 30 fresh samples from the housing floor that appeared to be from only 1 animal with mechanisms in place to refrain from sampling the same animal twice (Mollenkopf et al., 2012). Samples were plated on MacConkey agar with cefepime and MacConkey agar with cefoxitin in search of bla_{CTX-M} and bla_{CMY-2} *E. coli* phenotypes. Bacteria susceptibility was determined with a semi-automated broth microdilution system and further characterized using PFGE, PCR, and southern blot hybridization. The team found 5 herds tested positive for the CTX-M gene with a variance of 1-30 positive samples per positive herd. Just under 95% of total samples tested positive for the CMY-2 gene comprising all study dairy farms. Of the 9.4% of samples containing the CTX-M gene, a subset of 30 showed a single strain of *E. coli* containing both IncI 1 and IncF plasmids. The team did not find a link between farmer reported ceftiofur usage and the CTX-M gene, but all dairy farms, with the exception of an organic dairy farm, listed ceftiofur as a treatment option.

PFGE analysis of isolates containing *bla*$_{CTX-M}$ showed a predominant *E. coli* strain throughout 3 of the dairy farms. Many herds had homogeny among CTX-M isolates, but 1 dairy contained 6 varied isolates. The study utilized many techniques to determine the diversity of CTX-M isolates across a large number of dairy farms, which provides a more complete picture. However, a more effective monitoring of ceftiofur usage beyond dairy producer reporting would be beneficial. Self-reporting can lead to underreporting, recall bias, and social desirability bias. These biases, intended or not, can impact results and any association that could exist between ceftiofur usage and CTX-M development.

A study in northwest England and northern Wales evaluated 65 dairy farms positive for *bla*$_{CTX-M}$ *E. coli* for risk factors associated with bacteria with this resistance gene to better understand the dissemination of the gene (Snow et al., 2012). Dairy farms with links to *bla*$_{CTX-M}$ positive farms served as case farms and controls were selected from a similar geographic location, but without a *bla*$_{CTX-M}$ link. Samples were collected from weaned calves and various environmental locations around the dairy. Of the 65 dairy farms, 48 were controls. Farms with a *bla*$_{CTX-M}$ ESBL *E. coli* link had a prevalence of 58.8%, as 10 of the 17 farms tested positive. The prevalence on control farms was 35.4% and 41.5% of the total dairy farms tested positive for ESBL *E.coli*. These differences were not statistically significant. The research group hypothesized that due to the large number of control dairy farms testing positive, either the *bla*$_{CTX-M}$ gene was already widely disseminated, some cattle on control farms came from positive farms not identified as such, or else the cow was positive for ESBL *E. coli* independent of farm, since individual cattle status at time of movement was not determined. Developed models determined dairy farms with closed cattle policies had reduced risks of being ESBL *E. coli* positive. It was found that the usage of third- and fourth-generation cephalosporins made a farm

four times as likely to have *E. coli* possessing the $bla_{CTX\text{-}M}$ gene, but amoxicillin/clavulanate usage did not increase risk (Snow et al., 2012). This study utilized a much larger number of locations making its results more applicable to broader populations. Their results are consistent with those of other studies showing third-generation cephalosporin usage increases ESBL *E. coli* prevalence; but interestingly, illustrated that having linkage to an ESBL *E. coli* positive farm does not lead to a statistically significant increase in ESBL prevalence over control farms, even with looking for an assorted number of $bla_{CTX\text{-}M}$ genes. Of studies explored herein to this point, Snow et al. (2012) had the largest number of study sites and evaluated more than 1 $bla_{CTX\text{-}M}$ gene.

Another study performed in the United Kingdom sought to evaluate the epidemiology of ESBL *E. coli* containing the $bla_{CTX\text{-}M\text{-}15}$ gene on a dairy farm (Watson et al., 2012). The team selected a commercial dairy farm positive for the gene of interest comprised primarily of Holstein cattle and observed for two years, all while collecting fresh, fecal pat samples, isolating *E. coli*, and testing them for the $bla_{CTX\text{-}M\text{-}15}$ gene via PCR. These 55 samples were collected bimonthly from bulling heifers, dry cows, and low- and high-yield lactating cattle. Within the study, 24 cattle were selected randomly to sample at pre-calving throughout post-calving to test for $bla_{CTX\text{-}M}$. Additionally, calves were randomly tested for 21 days. It was discovered that cows and heifers on a farm already positive for *E. coli* containing the $bla_{CTX\text{-}M\text{-}15}$ gene were 8 times more likely to test positive for such isolates in the 10 days post-calving than pre-calving. While this study involved a wide variety of sampling times and cattle in various stages of life and production, some caution should be exercised when applying this study to broader dairy populations, as it was performed on only 1 dairy farm. A follow-up study would be advised to include more dairy farms in order to see if the results are consistent across study locations. It would be beneficial to have comparison dairy farms that had not tested positive for a $bla_{\text{CTX-M}}$

gene to see if there were similar trends with other resistance genes or if there were differences in the protocol of handling these animals. Perhaps there were differences in levels of CCFA usage, differences in the usage of other antibiotics, or other areas of husbandry that allowed for ESBL development.

A retrospective study in the United States analyzed 3,373 samples sent to the Cornell University Animal Health Diagnostic Center collected from what was assumed to be primarily calves in the northeastern United States from 2004-2011 (Cummings, Aprea, and Altier, 2014). Verified *E. coli* isolates were primarily from fecal samples, although isolates were also collected from the gastrointestinal tract and other anatomical locations. Isolates were subjected to antimicrobial susceptibility testing with the lowest percentage of resistance being found for enrofloxacin at 2.7% and the highest level of resistance found for oxytetracycline at 91.3%. Slightly over 70% of isolates tested were resistant to at least 2 antimicrobials on the NARMS panel. However, less than 1 percent had resistance to ceftiofur (ceftriaxone was not used in susceptibility testing). Over the time span of the study, only enrofloxacin was shown to significantly increase in the proportion of resistant isolates.

Admittedly, the authors mention having background information on the number, type of, and frequency of antimicrobial usage in these populations would be beneficial in making better conclusions. Additionally, it would be valuable to know if these samples were overwhelmingly collected from calves, as the authors assumed, and from what type of cattle they were collected. The study just mentions they are bovine, but were they from beef cattle or dairy? Given the large number of dairy farms in New York State, 1 could assume they are primarily dairy cattle samples, but it is best to not make assumptions when performing scientific work. Having mature animals in the study would provide a more well-rounded scope, as it would provide insight into

whether or not these patterns extend into adulthood. It could also provide additional insight or research questions into how resistance levels change throughout the life cycle of the bovine.

A similar 2015 study evaluated the prevalence of antimicrobial resistance in Australian beef, dairy, and veal cattle (Barlow et al., 2015). Target sample collections were 900, 300, and 300, respectively, with those samples coming from slaughterhouses that represented 85% of beef exports. Fecal samples were randomly collected from rectal end of the intestine during slaughter between February and March of 2013 and August and September of the same year. *E. coli* were isolated and micro-broth dilution was used to evaluate phenotypic levels of resistance. In total, 1,500 fecal samples were collected from 910 beef cattle, 290 dairy cattle, and 300 veal calves. Of those samples collected, 92.3% of them tested positive for *E. coli.* Veal had the highest percentage of positive samples followed by dairy and beef cattle. The team discovered *E. coli* resistant to antibiotics, other than tetracycline, remained low at 5 percent or less.

The results of Barlow et al. (2015) conflict with those of the Cummings, Aprea, and Altier (2014) study, suggesting differences in production practices or antimicrobial usage in Australia and the northeastern United States; however, there are some key differences in the studies. The results of Cummings, Aprea, and Altier (2014) assessed resistance retrospectively prior to further changes in American regulations regarding antimicrobial usage, evaluated across an 8 year period, and consisted of samples assumed to be primarily from calves of unidentified production. Barlow et al. (2015) evaluated 1 year with samples only collected for a quarter of the year. The study also consisted of adult beef and dairy cows, in addition to veal calves. While these studies have conflicting results, it is important to understand the differences in study design, sample population, and methodology before drawing any conclusions between levels of AMR in Australia and the northeastern United States.

A Pennsylvanian study further surveyed 23 herds from central and south-central Pennsylvania for antimicrobial resistant gram-negative enteric bacteria in healthy cattle feces in 2001 and 2002 (Sawant et al., 2007). Types of antibiotics used and reasoning for administration was kept in records, but dosage, frequency of administration, animals treated, and completion of treatment were not kept in farm records. Therefore, this information was not available to researchers. Ampicillin, florfenicol, spectinomycin, and tetracycline were all used on the farms and samples from 213 lactating cows were collected. Study cows were randomly selected while exiting the milking parlor. Samples were plated to MacConkey agar and 1 of 5 additional MacConkey agars each containing a different antibiotic (ampicillin, tetracycline, spectinomycin, enrofloxacin, or florfenicol) at 2 times the MIC breakpoint values for resistance. Of the samples processed, 258 isolates (223 of which were *E. coli* and the others general gram-negative Enterobacteriaceae) were selected from MacConkey agar to represent the 23 farms and 213 sampled cattle. Isolates were then subjected to microbroth dilution to determine levels of resistance. PCR, pulsed-field gel electrophoresis (PFGE), and southern blot hybridization assays were used to explore isolates for tetracycline resistance genes. The highest prevalence of resistance in samples and per farm was tetracycline followed by ampicillin, florfenicol, spectinomycin, and enrofloxacin. Multidrug resistance was found in 90 of the isolates and 8 percent of isolates were resistant to all screened antimicrobials. The isolates contained 21 different resistance profiles. Of those isolates resistant to tetracycline, 105 possessed the *tet*(B) gene and 8 the *tet*(A) gene. The group found the most common bacteria present in samples were *E. coli* and postulated lactating cattle were a reservoir for tetracycline and ampicillin resistance since those held the highest prevalence. Ceftiofur resistance was found at very low levels in 2001, similar to that reported in NARMS reports at that time.

This study could have been made more complete by having the missing antimicrobial usage data. The team determined lactating cattle were reservoirs of tetracycline and ampicillin resistance, but it would have been nice to know how extensive the selection pressure was for these bacteria to obtain and maintain these resistances. Were these resistance levels naturally found in the intestines of lactating cattle or were these the 2 drugs of choice on study farms for lactating cattle, thus promoting a resistance profile found among a majority of these cows on the farms? It is uncertain why the team used antibiotics in their agar plates at double the MIC. By using this method, they were likely to be missing clinically relevant and resistant isolates. Since ceftiofur was not listed as being used on study farms, it is not a surprise these levels of resistance remained low.. Furthermore, the results of this study are consistent with those of Cummings, Aprea, and Altier (2014) and Barlow et al. (2015) in that tetracycline was the antibiotic to which most *E. coli* were resistant. The team also used multiple antibiotic plates to determine resistance.

These prevalence-based studies have the ability to provide a great perspective of the distribution of a disease or disease-causing organism. It is beneficial to understand how resistant populations and *Salmonella* in general are distributed in a geographic area to then understand the risk to public health and ways to mitigate spread. However, the team seemed to make a wrong turn by suggesting farms testing positive for reduced susceptibility *E. coli* correlated with *Salmonella* positive farms due to increased ceftiofur usage when they did not find a correlation between reduced susceptibility *E. coli* and ceftiofur usage. Other studies included in this critical analysis have found connections between ceftiofur usage and 3GC resistant *E. coli* (Tragesser et al., 2006; Lowrance et al., 2007; Dolejska et al., 2011; Snow et al., 2012). As mentioned throughout this review, there is inherent skepticism with using survey data. The fact that these survey data do not show an association between ceftiofur usage and reduced susceptibility *E.*

coli, while others that monitored ceftiofur usage did, furthers such skepticism. Furthermore, the team did not produce bacterial counts. While there might not have been an association between ceftiofur usage and the presence of *E. coli* with reduced susceptibility to ceftriaxone, there could be a measurable effect between ceftiofur and the number of colonies exhibiting reduced susceptibility.

Our study design will allow us to evaluate 3GC resistance among *E. coli* from animals with known CCFA treatment status, along with known historical CCFA usage on each farm. This will allow us to fill in gaps remaining from previous studies. Furthermore, the use of various techniques to evaluate ESBL *E. coli* prevalence will be helpful in phenotypically and genotypically determining the extent of ESBL *E. coli* prevalence across time and by dairy farm, and also the gene variants at play. Additionally, by using count data across time, we will be able to evaluate previous hypotheses regarding the time at which 3GC resistance decreases in the *E. coli* population. Lastly, many of the studies analyzed were from Europe or the Midwest or Northeast United States. With study sites in the panhandle of Texas and northeastern New Mexico, our study provides information regarding very large dairy farms from a different geographic locations and climates.

2.7 FECAL *SALMONELLA* SHEDDING AND THIRD-GENERATION CEPHALOSPORIN RESISTANCE IN DAIRY CATTLE POPULATIONS

Conducted in 20 states comprising 83% of United States dairy cows, the National Animal Health Monitoring System enrolled 3,700 dairy producers to evaluate fecal *Salmonella* shedding among dairy cattle on farm and at slaughter from 1994 to 1996 (Wells et al., 2001). A total of

100 dairy farms were selected, including 50 small (less than 100 animals) and 50 large (greater than 100 animals) dairy farms, proportional to the number of each herd type per state. Small dairy farms were visited once and large dairy farms were visited 3 times while collecting fecal samples via rectal palpation. The same number of markets as dairy farms were visited with samples collected in a similar manner. There was 10% fecal shedding across all samples, but 18.1% for animals to be culled in the next week. Furthermore, the team determined larger dairy farms had more samples with *Salmonella* shedding. Prevalence was elevated in May, June, and July. Some markets required the collection of floor samples. Those samples had a higher prevalence of *Salmonella*, but only accounted for 16% of the total samples taken. Milking cows in the South had the highest herd-level shedding at 45%, although the authors noted caution due to the varying seasons of sample collection. However, market prevalences were higher in the South and Midwest at 76% and 79%, respectively. Dairy farm samples tested positive for multiple serotypes at 12.1% and 40% of market samples tested positive for multiple serotypes. Levels of antimicrobial resistance remained low throughout the isolates. In total, 91 herds and 97 markets were included across 19 states. The authors note the sample size and geographic diversity, in addition to healthy populations, as strengths of this study. Sample size and geography are strengths of the study given the large percentage of milk production for which those states account. However, these populations were not necessarily healthy. Animals get sick on dairy farms every day, animals tend to mask symptoms, and animals do not have to be completely healthy to go to slaughter. Even so, as stated by the authors, many studies are performed using ill populations. This study used what one would imagine as a mostly healthy population providing insight into what resistance and fecal shedding levels consist of in everyday dairy production.

A unique study conducted on a southwestern dairy farm with 3,000 Holstein cows collected samples from 60 lactating and 60 non-lactating cows at 7 o'clock in the morning and 5 o'clock in the evening with a replicate trial conducted 2 weeks later (Fitzgerald et al., 2003). These samples were then processed for isolation of *E. coli* O157:H7 and *Salmonella* to evaluate the effects of heat stress on the fecal shedding of these organisms. Fecal shedding was similar at both times (between 30% and 35%), but the percentage of cows shedding *E. coli* O157:H7 decreased by 5 percent and *Salmonella* increased by 3 percent between the morning and evening sampling times. The difference in *E. coli* shedding was statistically significant for non-lactating cows, but not those lactating. *Salmonella* shedding was significant for both groups, but in different directions. Lactating cows shed more *Salmonella* in the morning, while non-lactating cows shed more in the evening. Through antibiotic susceptibility testing, the team found 79% of *Salmonella* was resistant to at least 1 antimicrobial, while neither *Salmonella* nor *E. coli* showed resistance to ceftiofur at an MIC of 8 µg/mL. The group noted temperatures were uncharacteristically consistent throughout the sampling days.

Only 120 cows were enrolled from a 3,000 head herd, which seems low for the dairy size (Fitzgerald et al., 2003). The prevalence portion provided some interesting results, as the peak *Salmonella* shedding times seems to be opposite for lactating and non-lactating cattle and the peak shedding times for *E. coli* and *Salmonella* tend to be opposite too. These results were only evaluated as positive/negative and not for counts, but counts would have been beneficial in determining if cows also shed more *E. coli* or *Salmonella* during their peak times or if there were simply more cows shedding those organisms. While sampling lactating and non-lactating cows, one might imagine the team had healthy and ill cattle at the proportion at which they occurred on

the farm. It would be beneficial to know how many sampled animals, if any, were ill and how that impacted fecal shedding and antimicrobial resistance profiles.

From October of 1998 until February of 2000, 65 dairy farms in New York having recently tested positive for *Salmonella enterica* subgroup *enterica* serogroup B by Cornell University were enrolled in a study in which fecal samples were collected to evaluate how antimicrobial treatments impacted *Salmonella* shedding in feces (Warnick et al., 2003). Priority was given to animals recently diagnosed with salmonellosis, sick calves and cows, and recently calved cows. The median percentage of samples testing positive for *Salmonella* per herd was 2.5% and the median herd size was 240 cows, which was larger than the median dairy herd in New York at the time. In total, there were 2,726 samples collected from 2,381 cows. Nearly 10 percent of those samples tested positive for *Salmonella*, but 60% of herds tested positive. Among the antimicrobials administered to enrolled cows, ceftiofur was administered to the highest number of animals at 9 percent. Positive samples were found to be increased based on antimicrobial treatment, with a significant interaction of antimicrobial treatment and age group. Calves not receiving antimicrobials were found to be *Salmonella* positive more than animals at other stages of life not receiving antimicrobials. The team hypothesized antimicrobial treatment led to more *Salmonella*-positive samples due to lengthening the infection duration, increasing clinical *Salmonella* infection incidence, or an interaction of treatment with disease severity. At first, these hypotheses seem to be counter-intuitive. However, it is important to remember the sample population did not only included animals with recent salmonellosis, merely dairy farms that had a recent case. Therefore, the additionally enrolled ill cattle could be colonized by *Salmonella* due to the removal or reduction of species previously inhabiting the gut. When interpreting the results, it is important to remember the *Salmonella* status of these dairy farms

and the results of this study might not be generalized to all dairy farms or differing climates. With greater resources and collaboration, enrolling dairy farms of varying statuses and climates could have provided a more complete picture of how antimicrobial treatments increased or decreased the number of samples positive for the shedding of *Salmonella*.

Using isolates from Warnick et al. (2003), Alcaine et al. (2005) looked into antimicrobial resistance profiles and utilized PCR, sequencing, and multi-locus sequence typing to determine how ceftiofur-resistant *Salmonella* isolates acquired their resistance genes. The team determined the bla_{CMY-2} gene was commonly acquired because all isolates carried the gene on a similar allele. However, differences in multi-locus sequence types indicated the acquisition of genes was based on geographic location. Furthermore, they were able to determine the relationship between the bla_{CMY-2} gene and third-generation cephalosporin resistance, along with the geographic spread of the gene in New York. Based on other studies cited throughout this review (Tragesser, 2006; Heider et al, 2009; Jacoby, 2009; Pfeifer, Cullik, and Witte, 2010; Schmid et al., 2013), the linkages between the bla_{CMY-2} gene and third-generation cephalosporin resistance is readily understood and the gene is found around the globe, although this study was among the first to uncover these findings. It also stands to reason the allelic basis of the gene in 1 part of New York would differentiate from those in other areas of the state, country, or globe; that is, unless the sale of animals brings a cow carrying resistance from 1 area of the state to another region.

Using the same dairy farms as Zwald et al. (2004), Ray et al. (2006) collected fecal and environmental samples every 2 months from August of 2000 through October of 2001 to determine the differences in antimicrobial susceptibility among conventional and organic dairy farms. Samples were collected from healthy cows at various stages of production, cows to be culled, and sick cows to test susceptibility via microdilution. Samples from 120 farms tested

positive for *Salmonella*; however, 11 conventional dairy farms were excluded from analysis, due to being much larger than their organic counterparts, leaving the total samples with isolated *Salmonella* at 1,246. Conventional dairy farms were associated with *Salmonella* resistant to 5 or more antimicrobials, although management style was only associated with higher levels of resistance to streptomycin and sulfamethoxazole. None of the farms testing positive for *Salmonella* exhibited resistance to ceftriaxone at the, then, breakpoint of 64 µg/mL, but 13.0% would be considered resistant by the current breakpoint of 4 µg/mL. It is interesting that conventional dairy farms were more likely to have resistance to 5 or more antimicrobials, but management style only accounted for increased levels of resistance to streptomycin and sulfamethoxazole. For conventional dairy farms to have resistances to that many antimicrobials, but significance only with 2 antimicrobials, there has to be a variation of what comprises those 5 or more antimicrobial groups for each isolate. A breakdown of those results by state or region within state could show regional variability for the differing resistance profiles. Instead of broadly reporting the data, a more in-depth analysis could help explain some of these findings.

The same team published a very similar paper a year later with the same or very similar data that seemed to focus more on state-level variances than differences in organic and conventional dairy practices (Ray et al., 2007). Wisconsin dairy herds were positive for *Salmonella* resistant to at least 1 antibiotic at a prevalence of 96.8%. Michigan had the second highest prevalence at 89.7% with Minnesota and New York both having a prevalence of 83.3%. The group reported 81.2% of all *Salmonella* tested as pan-susceptible, but some of the more common resistance patterns involved resistance to 5, 9, and 10 antimicrobials. Of those isolates testing as resistant to 5 or more antimicrobials, 25.3% were from calves, 13.3% were from ill cows, and 4.2% from healthy cows, showing calves were at higher risk for harboring multi-drug

resistance than their ill or healthy adult counterparts. Sixty-four percent of the isolates were resistant to 9 or more antimicrobial agents on 1 Wisconsin dairy farm. This follow-up paper touches on some of the remaining questions mentioned in the above analysis, as there was more focus on the state from which the isolate came and not the type of dairy farm. Wisconsin had the highest prevalence of isolates resistant to 1 antimicrobial while 1 particular Wisconsin dairy herd contained over half of the isolates resistant to 9 or more antimicrobials. One might then wonder how many of the remaining isolates with such levels of resistance were found in Wisconsin or the distribution of isolates with resistance to 5 or more antimicrobials spread across the states. These results are consistent with Barlow et al. (2015) in which calves tended to test positive for AMR bacteria, or those with higher levels of resistance, pointing to an area in which intervention could have very beneficial results.

A study conducted by Frye and Fedorka-Cray (2007) utilized NARMS *Salmonella enterica* (*S. enterica*) collected from various sources of production and clinical evaluation, along with resistance profiles and molecular techniques, from 1999 – 2003 for characterization. *Salmonella* serovars Kentucky, Heidelberg, and Typhimurium were among the most reported serovars of the study with serovar Newport (n = 1928) comprising the greatest percentage (36.2%) of samples containing resistance genes. Of the 34,411 isolates evaluated, almost 11% had resistance to ceftiofur. However, by year, this percentage increased annually from 4.0% in 1999 to 18.8% in 2003. Interestingly, ceftriaxone resistance was below 1 percent across all years, although the breakpoint has since shifted from 64 µg/mL to 4 µg/mL. All ceftiofur resistant isolates were from animal agriculture with general cattle isolates having ceftiofur resistance at levels of 17.6% and dairy cattle having the highest level at 28.3%. When only evaluating clinical samples, ceftiofur resistance jumped to 18.5% and isolates from dairy cattle were 11% higher

than beef cattle at 35.8%. PCR was performed on 125 isolates. The *bla*$_{CMY-2}$ gene was found in 102 isolates with southern blot showing location on a large plasmid. ESBL genes were also found on select isolates. It is not any surprise that ceftiofur resistance grew over the four year period with the highest percentage of resistance found in dairy cattle, as AMR was and is an increasing threat to public health and ceftiofur is frequently utilized in dairy production. Now, further removed from 2003, another similar study would show if the rate of resistance is still increasing or if some of the stewardship programs and regulations now in place are being effective. Given ceftiofur and ceftriaxone are the same generation of cephalosporin, it is surprising that as ceftiofur resistance grew by year, ceftriaxone resistance remained low. One would expect these resistant rates to be very similar.

These studies, both within the United States and internationally, have shown links between CCFA usage and increased levels or prevalence of ceftiofur and ceftriaxone-resistant enteric bacteria. These results were shown even when comparing conventional and organic dairy farms. While the results were consistent throughout, sample sizes and locations tended to be small and documentation regarding antimicrobial types, dosage and administration were often non-existent or from unreliable sources. More controlled studies are required to truly strengthen the understanding and knowledge of how these levels of resistance develop and fluctuate within dairy cattle populations. Our study will fill the gaps regarding antimicrobial dosage, and administration impact on *Salmonella* shedding. Further, it will provide a current evaluation of these impacts and a look into the resistance mechanisms and *Salmonella* serotypes found on study dairy farms.

2.8 BACTERIAL CONTAMINATION OF RETAIL MEATS

Common pathogens of food are *Campylobacter* spp. (Zhao et al., 2001), *Salmonella* serovars (Zhao et al., 2001; White et al, 2001; Chen et al., 2004; Zhao et al., 2009; Glenn et al., 2013), and *E. coli* (Todd, 1997; Cassin et al., 1998; Zhao et al., 2001; Schroeder et al., 2002; Johnson et al., 2005; Vogt and Dippold, 2005). Contamination can be found in vegetables (Todd, 1997) and meat products, including poultry (Zhao et al., 2001; Schroeder et al., 2003; Johnson et al., 2005), pork (Zhao et al., 2001; Schroeder et al., 2002; Johnson et al., 2005), and beef (Zhao et al., 2001; Schroeder et al., 2002; Johnson et al., 2005; Vogt and Dippold, 2005).

A study performed at Iowa State University's Veterinary Diagnostic Microbiology Laboratory evaluated 377 bovine and porcine *E. coli* isolates collected between November of 1998 and December of 1999 with an additional 1,017 *E. coli* isolates from humans collected between November 1998 and March of 2000 to determine the transfer of plasmids containing the bla_{CMY-2} gene from food animals and humans (Winokur et al., 2001[a]). All samples were collected around the state of Iowa. Of the collected isolates, 59 food animal isolates were cephalosporin resistant and 6 human isolates were resistant to cephamycins, monobactams, and extended-spectrum cephalosporins. Isolates were all subjected to PFGE as well as molecular, plasmid, and integron analysis. The team identified homology between bla_{CMY-2} genes in animals and humans with plasmid similarity to determine a transmission of resistance from food animals to people. Of the over 1,000 human isolates, only 6 were of benefit in this study. In order to make broader generalizations, having more isolates would be necessary. Even so, this earlier study demonstrated how important it is to send animals to slaughter with as few AMR organisms as possible. The current study will aid in better understanding the temporal dynamics of AMR enteric bacterial populations in dairy cattle. An additional study with a more extensive

environmental focus would also be beneficial in determining how readily these resistance genes can be transferred to humans.

Microbial contaminants in retail meats are not restricted to *E. coli*, as over a million cases of foodborne Salmonellosis occur in the United States annually. A survey of 2 hundred meat samples consisting of an equal number of chicken, beef, turkey, and pork was collected from 3 markets in Washington D. C. between June and September of 1998 (White et al., 2001). Samples were processed to isolate *Salmonella*. Antimicrobial susceptibility testing was done for each *Salmonella* isolate. Isolates with resistance to ceftiofur and ceftriaxone were tested for the bla_{CMY-2} gene (the only gene encoding 3GC resistance in the U.S. at that time). Chicken samples had the highest prevalence of *Salmonella* followed by turkey, pork, and beef samples. In total, 20% of samples tested positive for *Salmonella*. Of those isolates, 84% were resistant to 1 antibiotic and 53% were resistant to 3 or more antibiotics. Isolates with ceftiofur resistance were found in all meat sources except pork. With meat samples coming from 3 different supermarkets, it was evident the issue of contamination lies within the meat itself and not the grocery. The study design was relatively basic, but provided further support for the risks of retail meat containing pathogenic or potentially pathogenic bacteria and the ability of those bacteria to spread from animal to human populations via food sources.

A 2001 study evaluated 825 retail meat samples across four supermarket chains (59 total stores) in the Greater Washington D.C. area for the presence of *E. coli* and *Salmonella* (Zhao et al., 2001). Meat samples consisted of chicken carcasses, turkey breasts, beef steaks, and pork chops. Samples were collected on alternating Mondays from June of 1999 to July of 2000. Of the meats included in the study, chicken was found to contain more *E. coli* than beef, pork, or turkey products (Zhao et al., 2001). Additionally, only 3 of 179 PCRs tested *E. coli* isolates were

positive for toxin production: 1 for heat-labile enterotoxin, 2 for heat-stable enterotoxins, and none for Shiga toxins. This led the team to determine most contaminants were due to commensal *E. coli* flora. An effect due to seasonality was not observed. In those same samples, *Salmonella* spp. were found in 3.0% of total samples. The study provided a good evaluation of the prevalence of microbial contamination among retail meats in the Greater Washington D.C. area by sampling from a variety of groceries, a variety of meat sources, and a large sample size. The PCR approach provided important insight into the pathogenicity of contaminating *E. coli* populations, although looking for virulence factor genes, other than toxins, and AMR genes could have provided further insight. These populations can still be harmful if containing AMR genes by spreading them to the enteric bacterial populations of the consumer in the case of underprepared meats or by bacterial uptake of those in the gut from those bacteria succumbing to high temperatures during cooking.

Schroeder et al. (2003) performed a study in the same laboratory and Greater Washington D.C. area as Zhao et al. (2001). The same 825 retail meats consisting of the same samples and collection times were used from the Zhao et al. (2001) study; however, 200 ground meat samples collected from June and September 1998 consisting of beef, chicken, pork, and turkey also were included. Antimicrobial susceptibility was determined by broth micro dilution. The team found the prevalence of *E. coli* in contaminated meats was higher in ground meats than whole meat cuts and isolates tended to be resistant to tetracycline, streptomycin, sulfamethoxazole, cephalothin, and ampicillin (in that order) with other resistance forms existing at lower levels. This study helped to address the AMR portion left unevaluated by the Zhao et al. (2001) and provided some additional perspective on the risk of AMR *E. coli* in retail meats. Furthermore, the distinction between ground and whole meats is important and, unsurprisingly, ground meats

tended to be more contaminated. This might be due to the additional processing and grinding presenting a greater opportunity for contamination with enteric bacteria. In addition, the grinding of a whole cut of meat increases the surface area. Finally, ground meat often includes trim from less desirable cuts of meats located in regions of the carcass more likely to be contaminated during slaughter such as the flank and umbilicus. These findings are important in the current dissertation project because a majority of culled dairy cattle become ground beef. This form of ground meat has the tendency to be more contaminated and, therefore, increases the risk of transmitting AMR *E. coli* to the consumer should levels of resistance not recede to baseline levels prior to culling after treatment with CCFA.

Further support for this risk pathway was a publication from Chen et al. (2004) testing antimicrobial susceptibility of *Salmonella* found in retail meats from Washington D. C. and the People's Republic of China. The isolate total consisted of 89 collected from Washington D. C. from June through September of 1998 (White et al., 2001) and from August of 1999 until August of 2000 with 44 isolates collected from the meat products in 10 Chinese provinces from October of 1999 until December of 2000. Susceptibility testing and PCR was performed to determine resistance profiles and the genetic components leading to such resistance. Isolates from those samples collected in Washington D. C. were resistant to at least 1 antimicrobial 82% of the time and showed resistance to various classes of antimicrobial, including beta-lactams. Of isolates from the People's Republic of China, 64% of isolates were resistant to at least 1 antimicrobial, none of which had beta-lactam resistance. However, isolates from China had greater resistance to quinolones and reduced susceptibility to ciprofloxacin was 30 times higher than isolates from American products. American isolates were found to contain bla_{CMY-2} and bla_{TEM-l} genes, whereas isolates from the People's Republic of China were found to only have bla_{TEM-l} genes. The team

noted American isolates contained more resistance than those from China and most genes were contained on integrons located within a plasmid. However, they noted a larger sample size was needed. The only information provided on sample size was with regards to the number of isolates, but not the number of food sources tested outside of what was documented in White et al. (2001). Such information would be beneficial in knowing exactly what the sample size was and to what it might need to be expanded. Additionally, samples were taken from 10 provinces in the People's Republic of China, but only 1 city in America. To adequately compare multiple places at the country level, the team should have considered sampling from additional areas around the United States. The study did provide insight into how differing policies concerning the use of antimicrobials in animal agriculture settings might create varying selection pressures for differing resistance profiles; however, further studies should be performed to allow stronger conclusions.

By utilizing a prospective study design involving 10 retail stores and 1,648 food samples consisting of meat, fruit, vegetables, and miscellaneous items in the Minneapolis and St. Paul area from 2001-2003, Johnson et al. (2005) were able to determine that, among beef and pork products, there was a greater risk of *E. coli* among these products when ground. Pork products tended to have higher levels of resistance to ampicillin, tetracycline, and sulfisoxazole. These findings are consistent with Schroeder et al. (2003). Antimicrobial-resistant organisms were found in poultry, beef, and pork items in addition to pre-cooked meals with the highest resistance prevalence being found in meats versus fruits, vegetables, and miscellaneous items (Johnson et al., 2005). The research group found many differences in the number of *E. coli* and levels of antimicrobial resistance based on a multitude of factors. The type of meat, storage procedure, season, and year all yielded statistically significant differences (Johnson et al., 2005).

Furthermore, chicken samples were contaminated with *E. coli* at a higher rate than any other sample type at 92%, which is consistent with Zhao et al. (2001). Given the higher prevalence among chicken, it is not a surprise that isolates from chicken samples also tested higher for virulence traits. Aspects of the Johnson et al. (2005) study complimented or filled in gaps from the Zhao et al. (2001) and Schroeder et al. (2003) studies. However, Johnson et al. (2005) found seasonal differences in prevalence whereas Zhao et al. (2001) did not. Additionally, the Zhao team determined prevalent *E. coli* populations were mainly commensal varieties and the Johnson team found many virulence factors, though the later team looked beyond Shiga toxins. The team noted a small number of markets from which the samples were taken and transmission from foods to consumers was not evaluated. The differences noted among these 3 studies could be due to geographic location, plant processing procedures, or other elements. To gain a better perspective, larger or and more studies from other geographic locations around the United States would be invaluable.

A study by Zhao et al. (2009) provided an interesting variant on studying antimicrobial resistance among *Salmonella* found in retail meats, by accessing *Salmonella* isolates known to have ampicillin resistance from NARMS. Isolates were from the sample years of 2002 through 2006. While there was not a large difference in the number of isolates from each year, a majority of the 344 isolates were from 2004 and 2005 and 92.9% of the isolates were from poultry or turkey. Antimicrobial susceptibility testing revealed 66.9% of isolates were resistant to 5 or more antimicrobials and 4.9% were resistant to 10 or more. While all isolates were susceptible to cefepime, 55.5% were resistant to amoxicillin with clavulanic acid, 50% were resistant to ceftiofur with the same percentage resistant to cefoxitin, and 24.7% were resistant to ceftazidime. Multi-drug resistant AmpC phenotypes were observed in 7 percent of the isolates. Ultimately, 19

isolates tested positive for bla_{CMY-2} and 13 were able to transfer the gene to a recipient in conjugation experiments. The team noted it was hard to draw conclusions regarding beef and pork products due to the low sample size in their study, but none of the beef isolates displayed ceftiofur resistance. *Salmonella* tended to be associated with poultry products at higher rates than other products. While ceftiofur resistance was not observed in *Salmonella* isolates from beef samples in this study, it is not implausible to consider *Salmonella* isolates in beef products from dairy cows likely to contain resistance to ceftiofur or other cephalosporins given the rate at which ceftiofur is used in dairy production for the treatment of metritis. Creating a study using only beef samples could provide greater insight into the forms of resistant *Salmonella* these food products harbor; that said, differentiating retail beef from fed beef versus dairy cows is difficult, especially for ground product.

Another study used *Salmonella enterica* isolates selected from various agencies to provide a sample population of *Salmonella* from animals, humans, and retail meat products from the United States and Canada (Glenn et al., 2013). Isolates with resistance to the greatest number of antimicrobials were selected for use in the study. Of the 56 isolates in the study, 32 contained the bla_{TEM} gene and 30 had the bla_{CMY-2} gene. Other resistance genes also were found within the isolates. Two plasmid replicon groups dominated the *Salmonella* isolates, as 27 isolates fit into group A with 52-180 IncA/C plasmid genes with 5-12 core regions and 27 isolates fit into group B with 52 or less IncA/C genes and 1 or fewer core regions. Interestingly, 14 of the 17 human isolates and all of the cattle isolates were contained in group A, while isolates from retail meats and other animal sources were mainly found in group B. The team noted that the small sample size limited the application of findings and, due to total DNA being utilized, it remains unknown as to whether the genes were contained on the plasmid or chromosome. The authors were correct

in their small sample size statements. The total number of isolates used was small, and became even smaller when broken down into groupings of human, animal, and retail meat categories or when doing analysis on resistance comparing countries. However, the results from the cluster analysis are intriguing and performing a larger study could be beneficial. With human and cattle isolates falling within the same group at larger sample populations, one could begin looking at similarities in pathogenesis compared to group B and begin determining if isolates from cattle pose an increased risk to human health over isolates from pork and turkey products.

As these studies sought to evaluate risks and a prevalence of *E. coli* and *Salmonella* in retail meats, a proper risk assessment involves evaluating the processing and grinding, storage, cooking, consumption, and dose-response (Cassin et al., 1998). While those are broad categories, subheadings were used to provide a more detailed outline of variables taken into account. Additional variables playing a role in the transition from 1 section into another, such as when prevalence in ground beef, probability of exposure, and ingested dose were included. Outbreaks of foodborne pathogens are not unique to the United States. This model could better identify any risks based on the original region from which the animal came, animal shipping, and contamination from the hide. AMR elements could be added, too, in order to evaluate the probability of becoming ill with a resistant pathogen. These outbreaks occur around the globe affecting every continent, with exception of uninhabited Antarctica, and show the need for better and updated surveillance systems (Todd, 1997).

Many elements of our project are highlighted throughout this critical analysis. There are many factors at play when establishing the economic burden of and regulatory/policy options to combat AMR. Due to the number of factors, stakeholders, and areas of professional expertise needed to address such topics, progress has been slow in collective, collaborative steps to address

this public health threat. Even within animal agriculture, there are differing antimicrobial regulations by country due to a variety of factors including, production systems, main animal of production, and size of production. Within dairy production, metritis is an important disease affecting the health and production of farm animals, but is difficult to prevent due to the number of host-related risk factors associated with the disease. To reduce production costs, CCFA is a popular medication to treat metritis resulting, at least partially, from a lack of need to discard milk after treatment. However, because CCFA is in the same class of drug as ceftriaxone, utilization of this drug provides a selection pressure for resistance to critically important and highest priority antimicrobials used in human medicine (WHO, 2019). Furthermore, multiple studies have illustrated the contamination of retail meats with *E. coli* and *Salmonella* bacteria, many of which carry antimicrobial resistance genes. Exploring these topics is important for a deeper understanding of the reasoning for our study and the knowledge gaps it seeks to fill.

CHAPTER III

MATERIALS AND METHODS

3.1 STUDY POPULATION

A pair-matched longitudinal study was conducted. We enrolled 3 dairy farms in west Texas and eastern New Mexico (Figure 1). Dairy cows with postpartum metritis were diagnosed by a licensed veterinarian or else the dairy herdsman using a veterinarian established protocol and thereafter prescribed a two-dose treatment with CCFA (Excede®, Zoetis Animal Health, Florham Park, NJ). The first treatment was given subcutaneously at the base of 1 ear upon diagnosis and the second was given 72 hours later in the opposite ear. Doses were administered per label at 6.6 mg per kg of body weight. Treated cattle were pair-matched with healthy, untreated control counterparts on the day of diagnosis. Control cattle were of a similar lactation number (i.e., production age) and calving date when compared to the treated, metritis-diagnosed cows, but not diagnosed with metritis and not given CCFA. Additional metrics, such as other ailments, calving difficulties, cow age, enrollment period, and time lapse from calving to enrollment were recorded. Each dairy farm had 15 pairs of cattle enrolled in a spring season replicate (April to June). A second larger replicate was performed in the fall season (September to April) with 25 pairs of cattle enrolled per dairy farm. Approval for the animal experiments was granted by the Texas A&M University Institutional Animal Care and Use Committee (Protocol No. 2016-0183).

Study Geographic Area

Figure 1: The geographic area in which the 3 enrolled dairy farms were selected is depicted by the blue circle (map adapted from Southard, 2016).

Through utilization of this study design, we were able to evaluate how the treatment protocol impacts third-generation cephalosporin (3GC) resistance among *E. coli* and *Salmonella* against what was observed among untreated populations. Additionally, by pairing cattle comprising the treated and untreated groups, we controlled for 2 potential confounding factors: age (as lactation number) and days to diagnosis post-calving. This approach improved confidence that any inference regarding differences observed in 3GC resistance result from the treatment regimen, not extraneous factors.

3.2 ENVIRONMENTAL SAMPLE COLLECTION

Prior to beginning the trial, environmental samples were collected from each dairy farm to test for the quantity of *E. coli* (total and 3GC-resistant) and determine the AMR phenotypic and genotypic profiles of 3GC-resistant isolates. The data resulting from these samples aided in understanding background levels of resistance within each dairy farm environment. Having such data assisted in our ability to assess each farm and consider ways through which antimicrobial stewardship can be improved. Furthermore, it contributed in explaining patterns observed within and among the animals of each farm, such as baseline levels of 3GC resistance, when compared to that of the other farms.

Surface manure samples were collected from milking, hospital, maternity and fresh-cow pens, in addition to the compost area. Samples were collected using an obstetric (OB) sleeve to pick up and composite approximately 5 g of feces from 12 locations along a transect line in each area of interest. Once collected, the OB sleeves were turned inside out and tied off. The dairy farm, sample location, and date were noted on the sleeve. Water samples also were collected throughout the farm. These samples, consisting of 5 mL each, were collected from 8 water locations via sterile, plastic spoons, and placed into a 50 mL conical tube (Falcon®, Corning, NY). Using the same procedure, 2.5 mL were removed from each of the same 8 water sources and placed into a 50 mL conical tube containing 20 mL of sterile, 50% glycerol (Fisher Chemical, Thermo Fisher Scientific, Waltham, MA) and thoroughly mixed. Samples were transported in a cooler on ice packs to the Agricultural Science Center (ASC) of New Mexico State University (NMSU) located in Clovis, New Mexico.

Once at the Center, the samples underwent further processing. Sleeves containing the manure samples had a hole cut in 1 of the fingers and 40 g of sample was squeezed into a 50 mL

conical tube without glycerol and 20 g was placed in a 50 mL conical tube containing 20 mL of sterile, 50% glycerol. Sample tubes were labeled the same as the OB sleeves, zip lock bags, and water tubes with a "G" written on the tube to denote those containing glycerol. All tubes containing manure and water samples were stored at 4°C before being transported on coolers with ice packs to Eastern New Mexico University (ENMU) in Portales, New Mexico and stored at -80°C. The samples were later shipped overnight on dry ice to the laboratory of Dr. H. M. Scott at Texas A&M University (TAMU) in College Station, Texas.

3.3 CATTLE FECAL SAMPLE COLLECTION

The samples from individual animals provided data for evaluation of the temporal dynamics of fecal shedding of total and 3GC resistant Enterobacteriaceae; specifically, *E. coli* and *Salmonella enterica* subspecies *enterica*. The first 3 sampling days (0, 6, and 16) are particularly important in showing population dynamics relating to disease and treatment. The day 0 sample served as the baseline, as the sample was taken prior to the administration of CCFA. The day 6 sample provided insight into how these populations initially change in the immediate aftermath of both doses of CCFA in treated versus control cows. Finally, day 16 represents the first eligible slaughter date after the two-dose treatment. Data from this day shed light on whether the current slaughter withholding is effective in allowing 3GC resistant Enterobacteriaceae to return to baseline levels. Later days provided additional information regarding potential return to baseline prevalence.

Once a cow was diagnosed with metritis, a fecal sample was taken *per rectum* with a shoulder-length OB sleeve prior to first dosing with CCFA. The OB sleeve was not contaminated

with soap or other materials prior to sampling. Once a sample was taken, the sleeve was turned inside out and tied into a knot. The farm identifier, whether the cow was treated or control, the cow ID, sample day and the date was written on the sleeve. On the same day, a control cow was selected and a fecal sample taken via the same process. The sleeves were then placed into a cooler with ice packs for storage during transportation to the laboratory for further processing. These first samples from treated and untreated cows served as the day 0 samples. After the collection of the baseline sample, treated cows were given their first dose of CCFA. The second dose was administered 72 hours later. Subsequent fecal samples were taken on study days 6, 16, 28, and 56 for both treated and control cows (Figure 2). These samples were processed in the same manner as the environmental samples. Upon arrival in College Station, the environmental and *per rectum* fecal samples were organized by dairy farm identifier and sample collection day. They were then placed into a -80˚C freezer until the time at which they were microbiologically processed.

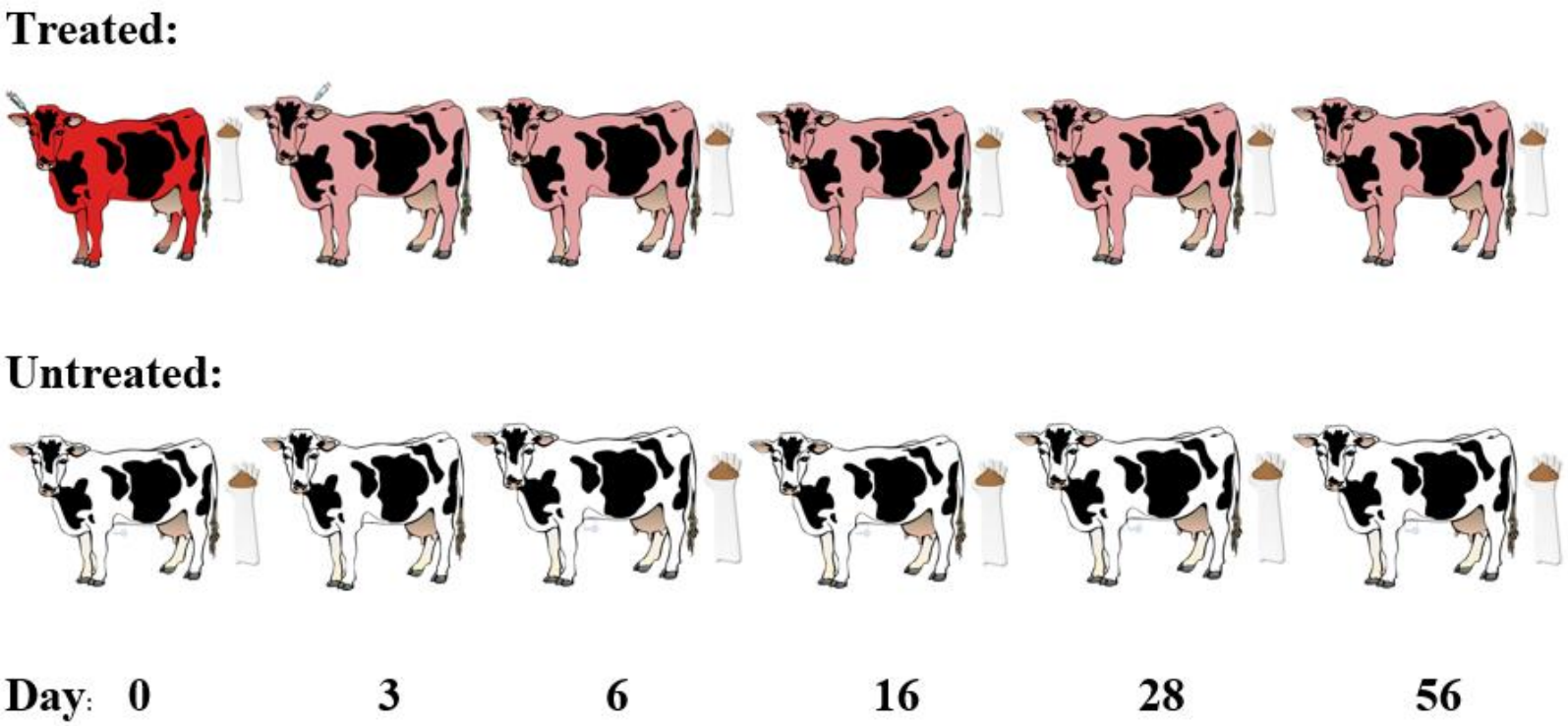

Figure 2: Study design is displayed above. The red cow is indicative of metritis diagnosis, pink cows represent the cow having been treated with CCFA, and white cows show a non-metritis state. The syringe by the ear of the first 2 cows in the treated group represent the administration of CCFA. Gloves with fecal samples represent days at which samples were collected. Day 0 samples were collected prior to CCFA administration.

3.4 *E. coli* ISOLATION FROM FECAL SAMPLES

Samples cultured for *E. coli* were spiral plated onto plain MacConkey agar (MAC) (BD Difco™, Franklin Lakes, NJ) and MacConkey agar containing ceftriaxone (Sigma-Aldrich, St. Louis, MO) at 4 µg/mL (MACCEF) in addition to CHROMAgar™ Orientation (CHROMagar™, France) with Tween 80 (Hardy Diagnostics Santa Maria, CA) and CHROMAgar™ ESBL supplement (570 µg/mL) (CHROMagar™, France) (CHROM-ESBL). Growth on MAC displays both 3GC susceptible and resistant populations. MACCEF growth displayed only those coliforms with 3GC resistance. By counting those plates for *E. coli*, we were able to determine fluctuations within populations and compare the proportion of 3GC resistant to total *E. coli* population over time. Samples with growth on CHROM-ESBL agar display an ESBL resistance profile; however, previous experience in our laboratory suggests that AmpC genotypes also are isolated using this medium. Since we enrich these samples in a manner to promote the growth of bacteria containing an ESBL resistance profile, these plates were noted only for *E. coli* growth or lack thereof. Isolates selected from the CHROM-ESBL plates were subjected to further molecular and phenotypic analysis. This allowed for a further understanding of prevalence of such resistance profiles and changes temporally. Laboratory work involving *E. coli* and *Salmonella* was conducted under Texas A&M University Institutional Biosafety Committee protocol number 2017-049.

To begin *E. coli* microbiological processing, 1 g of each environmental manure or cow fecal sample in glycerol was placed into 9 mL of 1x phosphate buffered saline (PBS) (Gibco®, Thermo Fisher Scientific, Gaithersburg, MD) in a 15 mL conical tube (Falcon®, Corning, NY) yielding a final 1:20 dilution (accounting for glycerol). After vortexing, 1 mL was pipetted into a 5 mL cup and a 50µL aliquot was spiral plated onto plain MAC and MACCEF using the Eddy

Jet 2® instrument (Neutec Group Inc., Farmingdale, NY). The 4 µg/mL ceftriaxone concentration in MACCEF agar represented the MIC for the human clinical resistance breakpoint for *E. coli* established by the Clinical Laboratory Standards Institute (CLSI, 2019), Additionally, 1 mL from the PBS solution was pipetted into 9 mL of MacConkey broth (BD Difco™, Franklin Lakes, NJ) with 2 µg/mL of ceftriaxone. At this time, 4 g of each glycerol and non-glycerol sample was transferred into a 5 mL polypropylene tube for further storage at -80˚C. Following 18 hours of incubation at 37˚C, MAC and MACCEF plates were counted for defined, pink, lactose-fermenting colonies using the Flash & Go® instrument (Neutec Group Inc., Farmingdale, NY).

After the MACCEF broth (2 µg/mL of ceftriaxone) was incubated for 18 hrs at 37˚C, the tubes were vortexed and 1 mL was pipetted into a spiral plating cup. Using the Eddy Jet 2®, 50 µL were spiral plated onto CHROM-ESBL. After incubating for 18 hrs at 37˚C, these plates were removed from the incubator and checked for pink, lactose fermenting colonies to evaluate *E. coli* growth. Two pink, isolated colonies suspected to be *E. coli* were selected with a 1 µL loop from each CHROM-ESBL agar plate and then streaked onto tryptic soy agar (TSA) containing 5% sheep blood (Remel™, Lenexa, KS) and incubated at 37˚C for 18 hrs.

Following this incubation period, isolates were indole tested using James Reagent (bioMérieux, Marcy-l'Étoile, France); in addition, a Matrix Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker, Billerica, MA) plate was prepared for organism confirmation/identification. One colony selected from the pure culture blood agar plate was rubbed onto a filter containing James Reagent. A color of pink on the area in which the colony was rubbed indicated an indole-positive colony. MALDI-TOF MS plates were prepared by placing Bacterial Test Standard (BTS) (Bruker, Billerica, MA) as a positive control in the first 2 wells of a 96 well target plate. A different sample isolate went into each of

the remaining 92 wells by being applied to the well with a sterile toothpick. A template was used to keep track of which sample was in each well. The last 2 wells contained matrix (Bruker, Billerica, MA) as a negative control. Matrix was then applied to all wells and allowed to dry. The plate was read using mass spectrometry for organism identification. Those isolates testing indole positive and identified as *E. coli* by MALDI-TOF MS were placed on cryo-beads (Scientific Device Laboratory, Des Plaines, IL) for storage at -80˚C. Water samples were processed in the same manner as environmental manure and cow fecal samples.

3.5 AmpC AND ESBL DIFFERENTIATION

A subset of 30 samples across the 5 different spring sample collection days (i.e., 0, 6, 16, 28, 56), the 3 enrolled dairy farms, and 2 treatment groups (treated and control) that tested positive on both MACCEF and ESBL agars were selected to be spiral plated onto MAC and MACCEF, along with MacConkey agar infused with cefoxitin (MACFOX) (32 µg/mL; Sigma-Aldrich, St. Louis, MO), MacConkey agar with cefepime (MACFEP) (16 µg/mL; Sigma-Aldrich, St. Louis, MO) and MacConkey agar infused with ceftriaxone (4 µg/mL) and clavulanic acid (MACCCA) (4 µg/mL; Sigma-Aldrich, St. Louis, MO). Once the sample was placed into 1x PBS for a 1:20 dilution, 50 µL was spiral plated onto each of these plates and incubated at 37˚C for 18 hrs. One mL was extracted from the 1x PBS solution and placed into a 15 mL conical tube with 9 mL MACCEF broth (1 µg/mL) for incubation at 37˚C for 18 hrs. Another mL was removed from the PBS solution and placed into 9 mL MACCEF broth (2 µg/mL) for incubation at 37˚C for 3 hrs. After the broths were incubated for their allotted times, the tubes were removed from the incubator, vortexed, and 50 µL each was spiral plated onto MAC, MACCEF, MACFOX, MACFEP, and MACCCA plates. After spiral plating, the plates were

incubated at 37˚C for 18 hrs. Since the enrichment was applied throughout the plates, a relative count could be made, even among the enriched samples. After the incubation period of each of the plates, they were removed and colony counted for pink, defined, lactose-fermenting CFUs.

The goal was to get an overall and relative count of the susceptible and resistant *E. coli* population on each of the MAC plate types (Thomson, 2001). The 30 samples were selected based upon samples that tested positive on both MACCEF and ESBL agar plates so we knew they have a CFU count for AmpC/ESBL at least as high as the limit of quantification. By doing so, we could further evaluate the effectiveness of this highly intensive differentiation method for consideration in usage with a larger number of samples. The MACCEF plate gave a combination of those bacteria displaying AmpC and ESBL forms of resistance, as those forms both allow for resistance to third-generation cephalosporins (Tragesser, 2006; Jacoby, 2009; Pfeifer, Cullik, and Witte, 2010; Schmid et al., 2013). Since cefoxitin is a second-generation cephalosporin, the MACFOX plate only grew *E. coli* with AmpC resistance, as ESBL bacteria do not have resistance to second-generation cephalosporins (Bonnet, 2004; Drieux et al., 2008). With cefepime being a fourth-generation cephalosporin, that plate would only grow ESBL coliforms because AmpC resistance profiles do not allow for resistance to fourth-generation cephalosporins (Akova, Yang, and Livermore, 1990). The MACCCA plates only grow *E. coli* with AmpC resistance profiles because the addition of clavulanic acid removes the ability of the ESBL genotype to remain resistant to third-generation cephalosporins (Matsuura et al., 1980; Cormican, Marshall, and Jones, 1996; Bonnet, 2004; Drieux et al., 2008; Schmid et al., 2013; Rossi et al., 2016). These counts allowed us to evaluate which proportion of 3GC resistant *E. coli* had an AmpC or ESBL resistance profile, as the MACCEF plates yielded both resistance forms, MACFOX and MACCCA yielded bacteria with an AmpC resistance profile, and MACFEP

produced bacteria with an ESBL resistance profile. After the plates were counted, 6 MACCEF isolates and 3 isolates from each of MACFOX, MACFEP, and MACCCA were streaked to blood agar, incubated for 18 hrs at 37°C, indole tested, MALDI-TOF MS tested, and saved to cryo-beads, if positively identified as *E. coli*. Should a plate not have the number of colonies indicated, all colonies from that plate were saved to cryo-beads.

3.6 AGAR PREPARATION

All agars and broth were prepared per manufacturers' protocols and autoclaved as recommended. Antibiotics were added to the agar after first being dissolved in 1 mL of water and the media was appropriately cooled and mixed. Once the antibiotic was suspended in water, it was added to the MAC agar or broth and allowed to further mix before the agar or broth was dispensed. Similar methods were used to prepare brilliant green agar (BGA) (Difco, Becton Dickinson, Franklin Lakes, NJ), which is a medium used in isolating *Salmonella*. Antibiotic media was consistently maintained in darkness. CHROMAgar™ Orientation was prepared using the manufacturer's protocol and Tween 80. The media were autoclaved prior to the addition of CHROMAgar™ ESBL supplement. The supplement was added in the same manner as described previously for MAC and BGA media.

3.7 BROTH MICRODILUTION

Broth microdilution is an effective way of quickly assessing phenotypic levels of susceptibility or resistance within any bacterial isolate to an array of 14 antimicrobials across 9 antimicrobial classes. These plates provide many levels of evaluation from the basic level

regarding the concentration (minimum inhibitory concentration: MIC) at which isolates fail to grow, whether they are susceptible, intermediate, or resistant to an antimicrobial based on interpretive criteria, the number of cumulative classes or antimicrobials to which an isolate is resistant (intermediate, or susceptible), and analyses to determine the concentration of antibiotic at which 50% and 90% of isolates are inhibited by a certain antimicrobial. Consequently, co-selection could be observed when combinations of phenotypes are observed to move in concert with extrinsic factors such as antibiotic treatments applied to cows. This provided insight into potential families of resistance genes within the isolate that would not have been revealed using the selective agar plating technique we utilized. These analyses provided an in-depth phenotypic evaluation of the selected 3GC isolates.

One *E. coli* isolate from each of the positive CHROM-ESBL agar plates was subjected to phenotypic antibiotic susceptibility testing using the broth microdilution Sensititre™ National Antimicrobial Resistance Monitoring System (NARMS) Gram Negative CMV3AGNF plate (TREK, Thermo Fisher Microbiology, Oakwood Village, OH). Each isolate was recovered from cryo-beads in storage at -80°C and streaked onto blood agar. Blood agar plates were incubated for 18 hrs at 37°C. One colony from each blood agar plate was selected and placed into 4 mL of sterilized water and standardized to a McFarland standard of 0.5. Afterwards, 50 µL of suspension was transferred into 11 mL of Mueller-Hinton broth before 50 µL of that suspension was added to each well on the NARMS plate via the Sensititre automated delivery system (TREK). Additionally, *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853 (American Type Culture Collection, Manassas, VA) were used as control strains whenever a new batch of plates was used. Plates were incubated for 18 hrs at

37°C before being read via Sensititre OptiRead™ (TREK, Thermo Scientific Microbiology). Clinical Laboratory Standards Institute (2019) guidelines were used in interpreting isolates as susceptible, intermediate, or resistant using SWIN software (TREK, Thermo Scientific) (National Antimicrobial Resistance Monitoring System, 2019). Isolates recorded as intermediate were reclassified as susceptible for statistical analyses of binary response variables. The resistance cutoffs are shown in Table 1. The National Antimicrobial Resistance Monitoring System generates human breakpoints when CLSI does not. Unfortunately, the plate does not have concentrations high enough to determine resistance to azithromycin or sulfisoxazole.

Table 1: Minimum Inhibitory Concentration Breakpoints for Resistance

Antibiotic	Resistance Cut-off Value
Amoxicillin/Clavulanic Acid Combination	≥32 µg/16 µg/mL
Ampicillin	≥32 µg/mL
Azithromycin	> 16 µg/mL
Cefoxitin	≥32 µg/mL
Ceftiofur	≥8 µg/mL
Ceftriaxone	≥4 µg/mL
Chloramphenicol	≥32 µg/mL
Ciprofloxacin	≥1 µg/mL
Gentamicin	≥16 µg/mL
Naladixic Acid	≥32 µg/mL
Streptomycin	≥32 µg/mL
Sulfisoxazole	> 256 µg/mL
Tetracycline	≥16 µg/mL
Trimthoprim-Sulfamethoxazole Combination	4 µg/76 µg/mL

The MIC for a resistant designation is shown for each antibiotic. Any MIC recorded below the given breakpoint was considered susceptible to that antibiotic.

3.8 *SALMONELLA* ISOLATION FROM CATTLE FECAL SAMPLES

The following protocol was utilized in isolating *Salmonella* from cattle fecal samples. Through enriching the samples, we were attempting to get the most accurate results regarding the animal-level prevalence of *Salmonella* fecal shedding from the 2 treatment groups and over time. Enrichment supported the growth of *Salmonella* in the sample, even if found in low numbers. The samples were only identified as positive or negative because the enrichment process inflated the number of colonies found in the sample. Furthermore, we were able to better understand the dynamics of any 3GC resistant *Salmonella* shedding throughout the study in addition to the shedding of total *Salmonella*.

Salmonella was cultured from all samples collected on study days 0, 6, and 16 of both trials by following the protocols published in Ohta et al. (2017). After thawing on ice, 0.5 g of fecal sample was placed into 5 mL of tryptic soy broth (TSB) (Difco, Becton Dickinson, Franklin Lakes, NJ) and left to incubate for 2 hrs at room temperature. After such time, the TSB tubes were placed in a 42°C incubator for 6 hrs. After the 6 hr incubation period, the tubes were placed in a 4°C refrigerator overnight. The tubes were then removed from the refrigerator and vortexed before 1 mL of TSB was pipetted into 9 mL of tetrathionate broth (TTB) (Difco, Becton Dickinson, Franklin Lakes, NJ) with 200 µL of iodine and then further incubated for 18 hours at 37°C. After incubation, the tubes were vortexed and 100 µL was pipetted from the TTB solution and into 10 mL of Rappaport Vassiliadis R10 broth (RV) (Difco, Becton Dickinson, Franklin Lakes, NJ) and incubated for 18 hrs at 42°C. After the incubation period, the tubes were vortexed before 50 µL of RV solution was spiral plated onto BGA (Difco, Becton Dickinson, Franklin Lakes, NJ) and incubated for 18 hrs at 37°C. The following day, the plates were checked and growth, or lack thereof, was noted. Pink colonies were selected as suspected

Salmonella. One isolate from the plain BGA plate and 3 isolates from the BGA plate containing 4 µg/mL of ceftriaxone were tested via MALDI-TOF MS and those identified as *Salmonella* were saved to cryo-beads. Beads were stored at -80°C. The same previously described protocols for MALDI-TOF MS and preservation on cyro-beads for *E. coli* were followed for *Salmonella*.

3.9 WHOLE GENOME SEQUENCING

An *E. coli* isolate recovered from each MACFEP (16 µg/mL) agar plate and *Salmonella* isolate recovered from BGA and BGACEF (4 µg/mL) agar plates were whole genome sequenced. Additionally, 15 *E. coli* isolates were selected based upon their ciprofloxacin MIC values or unique AmpC or ESBL profiles. These isolates were selected, along with the elevated ciprofloxacin MICs, to explore the genetic mechanisms behind their unique phenotypes. Sequencing these isolates provided information regarding the serogroup, serotype, and sequence type, along with resistance genes and plasmids contained in the general population (*Salmonella*) and those with known resistance to 3GCs (*Salmonella* and *E. coli*). We evaluated which serotypes predominated in the sample pre-treatment and observed changes post-treatment. The resistance genes encountered provided insight into what was observed in the general *Salmonella* population and are selected via CCFA treatment.

Each isolate (1 from each positive BGA and BGACEF sample plate) was placed into a 15 mL conical tube containing 6 mL of TSB and incubated for 18 hrs at 37°C. After incubation, DNA was extracted using the QIAcube HT via the QIAamp 96 DNA QIAcube HT Kit (Qiagen, Valencia, CA). To do so, 1 mL of the incubated broth solution was transferred into and subsequently centrifuged via Centrifuge 5424 (Eppendorf, Hamburg, Germany) for 15 minutes at

4,000 rpm in a 1.2 mL micro-collection tube. The supernatant was removed and ATL buffer (Qiagen) and DX reagent was used to resuspend the bacterial pellet. Furthermore, after adding small pathogen lysis beads (Qiagen) to the suspension, the Qiagen TissueLyser system (Qiagen) was used to agitate the solution for 5 minutes at 25 Hz. Once the tubes were centrifuged, 40 µL of Proteinase K was added to each tube. The tubes were then placed into a ThermoMixer (Eppendorf, Hauppauge, NY) for 1 hr at a temperature of 56°C while mixing at 900 rpm. A 10 minute heat shock at 95°C followed. After cooling to room temperature, 4 mL of RNAse A was added to each tube and DNA from the samples was extracted using the recommended Qiagen protocol on the QIAcube HT.

The extracted DNA was then evaluated for quality and quantity via fluorescence using the Qubit 3 Fluorometer (Invitrogen, CA) and absorbance utilizing the Fluostar Omega Plate reader (BMG LABTECH, Cary, NC). Once the DNA is determined to be of acceptable quality with a ratio of absorbance at 260 nanometers to 280 nanometers between 1.8-2.0, tagmentation steps were followed to tag the DNA with adapter sequences via bead-linked transposome. Libraries were prepared using the Illumina Nextera XT kit (Illumina, Inc., San Diego, CA). A post-tagmentation cleanup was performed to remove the adapter-tagged DNA before amplifying the tagmented DNA. The libraries were then cleaned via double-sided beads to purify the libraries. The quality of the prepared libraries was evaluated using the Fragment Analyzer (Advanced Analytical Technologies, Inc., Santa Clara, CA) as a final quality control prior to pooling and normalizing the libraries and to begin sequencing the isolates via MiSeq (Illumina, San Diego, CA). Samples were sequenced using a MiSeq v2 reagent kit with 500 cycles with 2x250 paired end reads (Illumina, San Diego, CA). Once the sequences were assembled, the web-based tool SeqSero (http://www.denglab.info/SeqSero) was used to determine *Salmonella*

serotypes using raw fastq files generated from forward and reverse reads, while making serotype decisions based on H1 and H2 antigens, along with O-antigen gene clusters (Zhang et al., 2015). *E. coli* sequence types and resistance genes for all sequenced isolates were evaluated using the online platform ResFinder (Zankari et al., 2012) from the Center for Genomic Epidemiology (Danish Technical University, Lyngby, Denmark).

3.10 STATISTICAL METHODS

Broad descriptive statistics were performed to gain a better understanding of data distribution. Furthermore, cross tabulations were performed based on replicate, dairy farm, animal group, and study day. We generated descriptive statistics regarding mean, standard errors, 95% confidence interval, and median for our quantitative CFU count data. Colony count data will be $\log_{10}$ transformed. Due to the detection limit in our colony counting methodology being ~2.5 $\log_{10}$ bacterial growth and the low levels of 3GC resistant bacteria that tended to exist in the feces, we had a high number of MACCEF agar plates displaying levels of growth below the level of detection. While these plates appeared to be negative for *E. coli* growth, based on herd mate status and shared environments, we hypothesized these animals were likely to be shedding the bacteria below the level of detection for our methodology. We utilized a multiple imputation method to distribute those plates displaying no growth across the levels below detection by establishing a lower limit of 0 $\log_{10}$ CFU and an upper limit of 2.5 $\log_{10}$ (STATA® 15, STATA Corp., College Station, TX). This method used the other count data and regression model factors to distribute the counts below detection in order to not violate the assumptions of the linear regression model.

Mixed effect linear regression on $\log_{10}$ transformed CFU count data was performed to account for fixed and random effects, while featuring nested of clusters to explore sources of variation by using the command "mixed". Random effects consisted of the season or replicate number, dairy farm, and animal. Fixed effects consisted of whether a cow was treated for metritis, sample collection day, cow lactation, and the interaction of treatment with sample day. Historical CCFA usage levels also were explored as fixed effects in the model. When evaluating differences in *E. coli* dynamics by dairy farm, farm was included in the model as a fixed effect. Additionally, ordinal variables indicating historical ceftiofur usage were evaluated as fixed effects within the models. Multivariate regression analysis with an interaction between treatment and sample day was utilized to evaluate the difference in $\log_{10}$ growth of *E. coli* across the agar plates used in AmpC and ESBL differentiation from total counts.

Logistic regression was used to explore the phenotypic ESBL *E. coli* profile based on the breakpoint interpretation of isolates selected from CHROM-ESBL agar produced using the microbroth dilution method while keeping the interaction of treatment and sample day. Survival analysis via Kaplan-Meier plots utilizing the MIC values was performed to evaluate the antimicrobial concentration at which 50% and 90% of the isolates experience inhibited growth. The role of treatment, dairy farm, and sample day were all independently evaluated for effect on MIC regarding each of the antibiotic compounds via the Mantel-Haenszel rate ratio. These analyses provided insight into the forms of resistance the isolates forming the 3GC population possessed as indicated at each time point and by treatment under the aforementioned selection criteria. BGA plates were not colony counted and were only evaluated as positive or negative for *Salmonella*; therefore, mixed-effects logistic regression was utilized to evaluate the data

(STATA® 15, STATA Corp., College Station, TX) using similar modeling approaches as outlined above.

CHAPTER IV

RESULTS

4.1 FECAL *E. coli* DATA ANALYSES

4.1.1 Descriptive Statistics

In total, 9 pen-floor samples were collected from each of fresh cow, hospital, maternity, and milking pens, along with the compost area from each dairy farm. Samples were analyzed independently and CFU means were calculated from the growth data of each sample; that is, environmental samples were not pooled outside of their farm/pen location. Total *E. coli* CFU counts were similar across all 3 dairy farms; however, Dairy Farm 3 had mean quantifiable levels of 3GC resistant *E. coli* approximately 1.5 $\log_{10}$ CFU greater than the other 2 farms (Dairy Farm 1 95% CI: 0.575-3.116; Dairy Farm 2 95% CI: 0.286-2.939; Dairy Farm 3 95% CI: 1.765-4.575; Table 2). This led to differences between total and 3GC growth of approximately 1 $\log_{10}$ CFU less than the other 2 dairy farms (Table 2). All water sources sampled across the 3 dairy farms tested negative for detectable levels of *E. coli*.

Table 2: Quantitative *E. coli* Data Descriptive Statistics from Environmental Samples

	Growth Metric											
	Log_{10} Total *E. coli* (MAC)				Log_{10} 3GC Resistant *E. coli* (MACCEF)				Log_{10} Growth Arithmetic Difference (Total – 3GC)			
Dairy Farm	Mean	Standard Error	95% CI	Median	Mean	Standard Error	95% CI	Median	Mean	Standard Error	95% CI	Median
Dairy Farm 1 (n=9)	5.241	0.183	4.864-5.618	5.220	1.846	0.618	0.575-3.116	2.603	3.396	0.637	2.085-4.706	3.593
Dairy Farm 2 (n=9)	4.917	0.315	4.270-5.563	5.328	1.613	0.645	0.286-2.939	0	3.304	0.561	2.152-4.456	3.080
Dairy Farm 3 (n=9)	5.564	0.259	5.032-6.097	5.858	3.170	0.683	1.765-4.575	3.556	2.395	0.550	1.263-3.526	2.620

Descriptive data concerning the distribution of environmental *E. coli* growth across each dairy farm. MAC, plain MacConkey agar; 3GC, third-generation cephalosporin; MACCEF, MacConkey agar with 4 µg/mL of ceftriaxone.

The mean cow lactation number was 1.7 and cows were, on average, 9.8 days in milk at the time of enrollment. The average age of cows enrolled in the study was 34.3 months and 60% of enrolled cows were primiparous (first-calf heifers). The estimated weight of first lactation animals was 545 kg, 615 kg for second lactation, and 665 kg for third and higher lactation animals on the dairy farms. Based upon the age and lactation of enrolled animals, along with the estimated weights of each lactation number on the farms, it is estimated the average weight of animals enrolled in the study was 601 kg. The treatment ratio of cows and heifer lactation days freshened in the prior year with any ceftiofur formulation was 9.0% on Dairy Farm 1, 8.4% on Dairy Farm 2, and 121.0% on Dairy Farm 3. These numbers were generated by dividing the number of ceftiofur dosages by the number of cows at risk. Of those ratios, 0% from Dairy Farm 1, 23.6% from Dairy Farm 2, and 8.6% from Dairy Farm 3 were intra-mammary formulations and not systemic therapy given via injection. This means animals on Dairy Farm 1 averaged 0.090 treatments per cow-lactation, with 0.084 treatments on Dairy Farm 2, and 1.210 treatments per fresh cow or heifer on Dairy Farm 3. Since ceftiofur formulations have been prohibited for off-label usage in the United States since April 2012, we have assumed that each dosage of CCFA administered was at 6.6 mg per kg of body weight and the other ceftiofur formulations also were at labeled dose.

Summary statistics (mean, standard error, 95% confidence intervals and the median) by treatment group, day, dairy, and season are presented in Table 3. Treated animals had lower mean and median values of growth on MAC, but higher values of growth on MACCEF; meanwhile, the arithmetic difference between those 2 outcomes decreased substantially among treated animals in comparison to the untreated group (Table 3). Similar trends were observed regarding these metrics across sample day relating to the time from drug administration. There

was a mean decrease in the total *E. coli* population on the first sampling day following treatment with an increase in mean 3GC resistant *E. coli* population on the same day. Thereafter, the total *E. coli* population increased and 3GC resistant *E. coli* population decreased as time progressed from treatment administration. Descriptive statistics illustrating these phenomena are shown in Table 4. Samples from Dairy Farm 3 exhibited higher mean and median *E. coli* growth on both MAC and MACCEF agars, with a smaller arithmetic difference between the 2 when compared to Dairy Farms 1 and 2 (Table 3). Values remained steady across plate type and arithmetic difference with regards to the metrics of mean, median, and standard error for the factor of season. Sample number varied across these metrics, as some animals were culled prior to study completion or else samples were missed during the collection period (Table 3).

Table 3: Quantitative *E. coli* Data Descriptive Statistics from Cattle Samples

Growth Metric	Descriptive Statistic	Treatment		Day					Dairy Farm			Season	
		Treated (n=595)	Untreated (n=596)	0 (n=246)	6 (n=239)	16 (n=239)	28 (n=237)	56 (n=230)	1 (n=408)	2 (n=388)	3 (n=395)	Spring/ Summer (n=440)	Fall/Winter (n=751)
Log_{10} Total *E. coli* (MAC)	Mean	4.343	4.752	4.641	3.927	4.584	4.759	4.838	4.245	4.445	4.963	4.686	4.467
	Standard Error	0.068	0.047	0.079	0.126	0.090	0.078	0.075	0.082	0.072	0.057	0.055	0.058
	95% CI	4.209-4.477	4.660-4.845	4.486-4.796	3.680-4.173	4.408-4.761	4.606-4.912	4.690-4.986	4.084-4.405	4.304-4.585	4.850-5.075	4.578-4.793	4.353-4.581
	Median	4.623	4.798	4.690	4.435	4.784	4.781	5.010	4.510	4.589	5.182	4.729	4.722
Log_{10} 3GC Resistant *E. coli* (MACCEF)	Mean	0.884	0.446	0.522	1.071	0.772	0.475	0.478	0.426	0.281	1.288	0.707	0.640
	Standard Error	0.067	0.048	0.078	0.117	0.102	0.078	0.082	0.057	0.052	0.0915	0.069	0.053
	95% CI	0.751-1.016	0.352-0.540	0.370-0.675	0.843-1.301	0.573-0.972	0.322-0.697	0.318-0.638	0.315-0.538	0.179-0.383	1.109-1.470	0.571-0.843	0.537-0.743
	75^{th} Percentile	0	0	0	2.603	0	0	0	0	0	2.904	0	0
Log_{10} Growth Arithmetic Difference (Total – 3GC)	Mean	3.459	4.306	4.119	2.855	3.812	4.285	4.360	3.819	4.164	3.674	3.978	3.827
	Standard Error	0.084	0.057	0.095	0.148	0.121	0.090	0.093	0.090	0.080	0.100	0.788	0.069
	95% CI	3.294-3.625	4.193-4.419	3.931-4.301	2.565-3.145	3.575-4.049	4.108-4.461	4.177-4.543	3.643-3.994	4.007-4.321	3.478-3.870	3.824-4.133	3.691-3.963
	Median	3.964	4.515	4.309	3.301	4.401	4.526	4.589	4.265	4.425	4.274	4.356	4.330

Descriptive data concerning the distribution of *E. coli* growth within the factors of treatment, day, dairy farm and season. Univariate summary statistics are unadjusted for clustering by farm, pen, and animal. MAC, plain MacConkey agar; MACCEF, MacConkey agar with 4 µg/mL of ceftriaxone.

Table 4: Descriptive Data on the Distribution of *E. coli* Growth across Treatment and Sampling Day.

Growth Metric	Treatment	Descriptive Statistics	Sample Day				
			0	6	16	28	56
Log_{10} Total *E. coli* (MAC)	Treated	Sample Size	123	121	118	120	113
		Mean	4.701	3.000	4.388	4.758	4.903
		Standard Error	0.113	0.190	0.145	0.115	0.115
		95% CI	4.481-4.922	2.627-3.374	4.103-4.673	4.533-4.983	4.678-5.128
		Median	4.757	3.681	4.663	4.864	5.105
	Untreated	Sample Size	123	118	121	117	117
		Mean	4.581	4.876	4.776	4.761	4.775
		Standard Error	0.111	0.108	0.105	0.106	0.098
		95% CI	4.364-4.799	4.665-5.088	4.570-4.982	4.552-4.969	4.583-4.967
		Median	4.662	4.870	5.000	4.723	4.869
Log_{10} 3GC Resistant *E. coli* (MACCEF)	Treated	Sample Size	123	121	118	120	113
		Mean	0.635	1.564	1.095	0.554	0.554
		Standard Error	0.121	0.188	0.168	0.117	0.124
		95% CI	0.398-0.873	1.195-1.933	0.766-1.424	0.325-0.784	0.311-0.798
		75^{th} Percentile	0.000	3.556	2.603	0.000	0.000
	Untreated	Sample Size	123	118	121	117	117
		Mean	0.410	0.567	0.458	0.393	0.404
		Standard Error	0.097	0.121	0.111	0.102	0.107
		95% CI	0.219-0.600	0.331-0.804	0.241-0.675	0.193-0.592	0.194-0.615
		75^{th} Percentile	0.000	0.000	0.000	0.000	0.000
Log_{10} Growth Arithmetic Difference (Total – 3GC)	Treated	Sample Size	123	121	118	120	113
		Mean	4.066	1.436	3.292	4.204	4.349
		Standard Error	0.138	0.183	0.188	0.137	0.148
		95% CI	3.795-4.337	1.077-1.796	2.924-3.662	3.934-4.473	4.059-4.639
		Median	4.292	0.176	3.623	4.511	4.5798
	Untreated	Sample Size	123	118	121	117	117
		Mean	4.172	4.309	4.318	4.368	4.371
		Standard Error	0.133	0.138	0.138	0.116	0.115
		95% CI	3.911-4.432	4.038-4.580	4.047-4.590	4.141-4.595	4.144-4.597
		Median	4.326	4.538	4.722	4.526	4.593

Descriptive data regarding *E. coli* growth. MAC, plain MacConkey agar; 3GC, third generation cephalosporin; MACCEF, MacConkey agar with 4 µg/mL of ceftriaxone

The distribution of *E. coli* CFU counts grown on MAC agar is displayed in Figure 3. Samples with quantifiable 3GC resistant *E. coli* CFU count growth were most prevalent on day 6 of the study (Table 4). Dairy Farm 3 had the largest number of samples with 3GC resistant *E. coli* both in total and on each sample day (Table 5). The distribution of CFUs on MACCEF reflected a large number (n= 966) of plates with no detectable growth (Figure 4). It is unlikely these counts were truly 0. Because of this zero-inflation, modeling the count data using linear regression was not ideal due to inappropriate residuals, non-normal error distribution, heightened instability, and enlarged coefficients. By utilizing multiple imputed data of 3GC growth below the limits of detection, model estimates increased in stability due to the more normalized data distribution (Figure 4). Furthermore, the general distribution regarding the difference between the total and 3GC resistant *E. coli* populations is displayed in Figure 5. There were a few data points that displayed more 3GC resistant *E. coli* than total *E. coli*. This is an artefact of limitations of our counting system and its inherent imprecision and also due to some plates displaying small levels of growth. Therefore, a difference of 1 3GC resistant isolate compared to no growth on MAC portrays a large difference in the 2 populations. Of the isolates presumed to be *E. coli* and selected for further testing (n=1603), 100% tested as indole positive and 98.6% of these were later confirmed via MALDI - TOF MS as *E. coli* providing confidence in our ability to phenotypically identify and include only *E. coli* in the colony counting procedure.

Table 5: Frequency of Samples with Third-Generation Cephalosporin Resistant *E. coli* Growth by Dairy Farm and Sampling Day

Growth on MacConkey Agar with Ceftriaxone (4 µg/mL)	Dairy Number Dairy Farm 1	Dairy Farm 2	Dairy Farm 3	Total
Sample Day 0				
Growth	4	9	29	42
No Growth	78	75	51	204
Total	82	84	80	246
Sample Day 6				
Growth	18	8	41	67
No Growth	66	68	38	172
Total	84	76	79	239
Sample Day 16				
Growth	9	6	35	50
No Growth	74	71	44	189
Total	83	77	79	239
Sample Day 28				
Growth	11	6	17	34
No Growth	71	70	62	203
Total	82	76	79	237
Sample Day 56				
Growth	10	1	21	32
No Growth	67	74	57	198
Total	77	75	78	230

Ceftriaxone resistance with frequency of samples testing positive shown across dairy farm by day. Samples are considered positive if confirmed *E. coli* colonies were isolated from MacConkey agar containing ceftriaxone at 4 µg/mL.

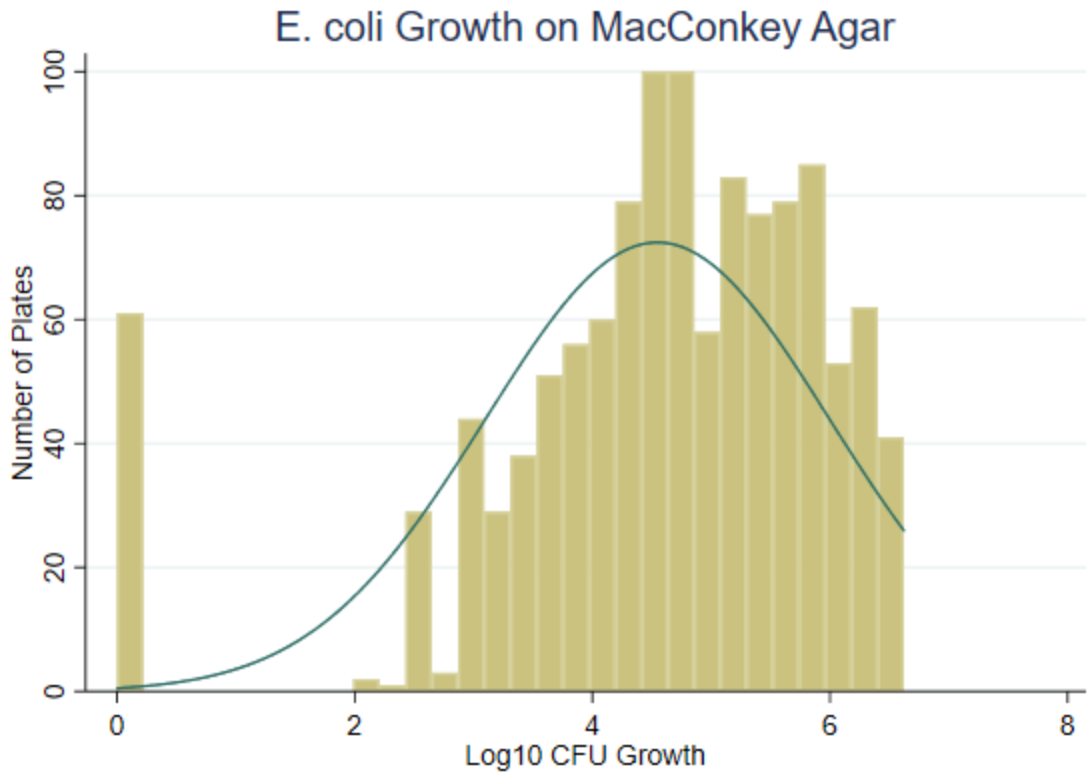

Figure 3: The distribution of total *E. coli* growth on a $\log_{10}$ scale is displayed with a line showing the distribution. CFU, colony-forming unit.

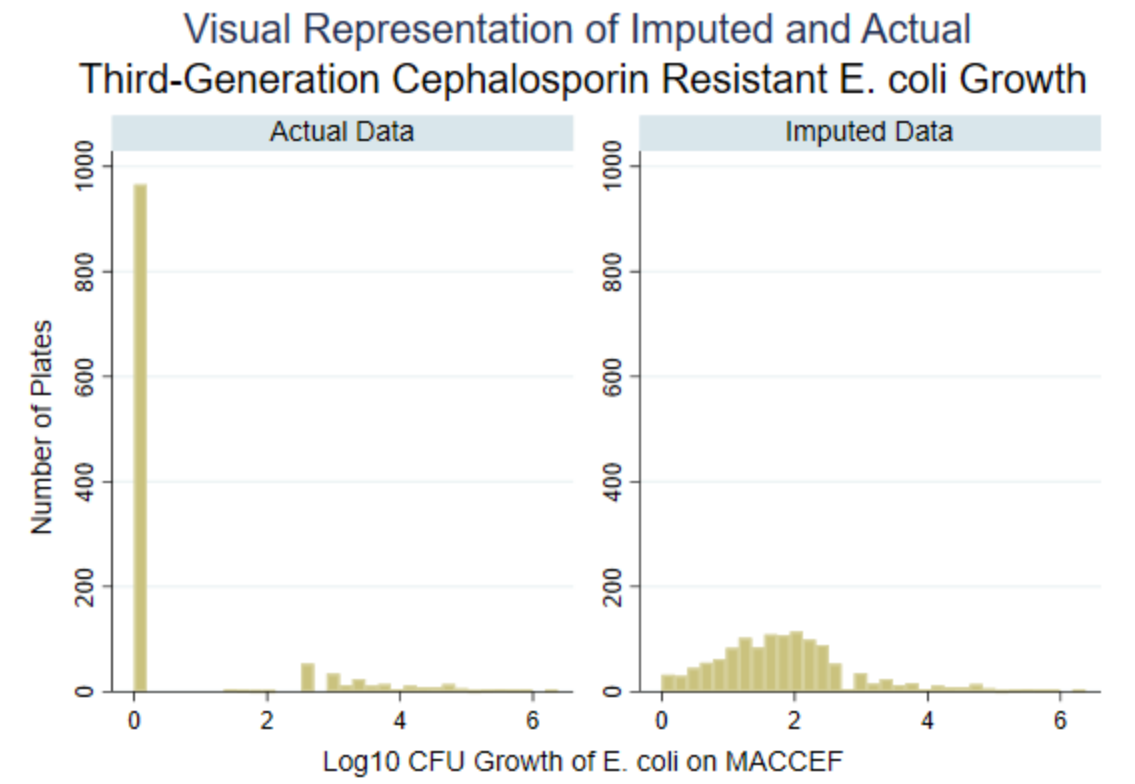

Figure 4: Left: Distribution of actual ceftriaxone-resistant *E. coli* growth data on a $\log_{10}$ scale. Right: Distribution of imputed 0 count ceftriaxone-resistant *E. coli* growth data on a $\log_{10}$ scale. CFU, colony-forming unit.

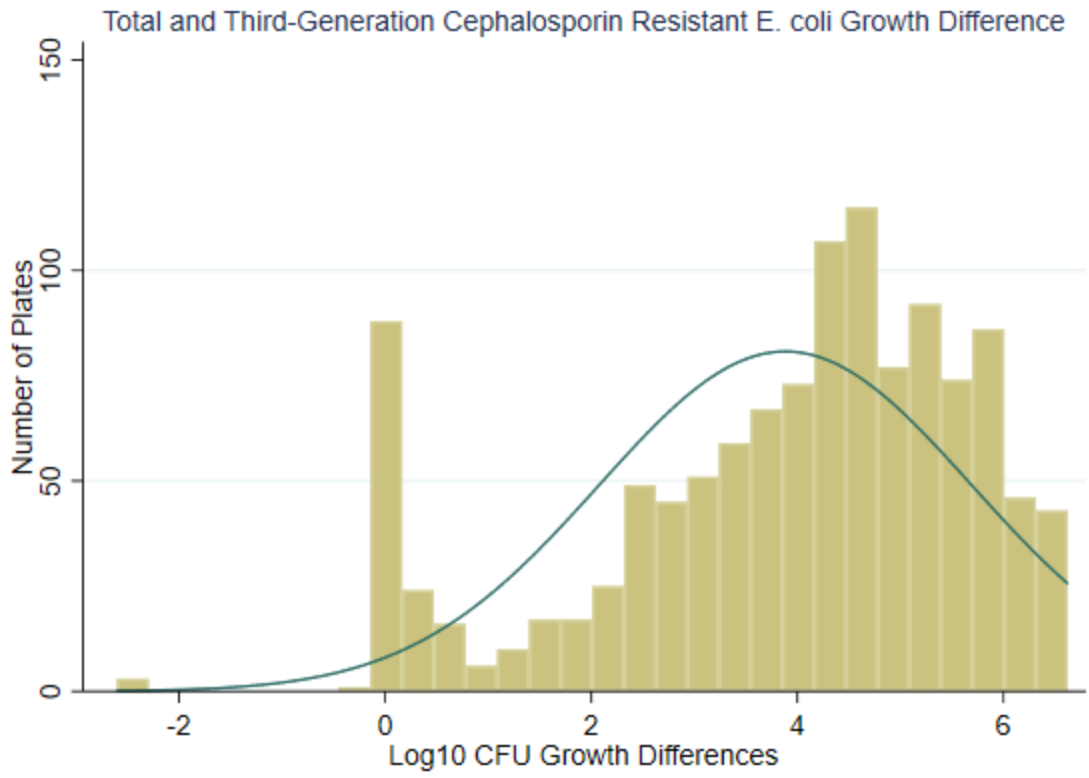

Figure 5: Distribution of difference in total and ceftriaxone-resistant *E. coli* growth on a log_{10} scale. CFU, colony-forming unit.

4.1.2 Mixed Model of Total E. coli CFU Counts

Mixed-effect linear regression was utilized to evaluate *E. coli* counts on a log_{10} scale. Figure 6 represents modeled marginal mean *E. coli* log_{10} CFU estimates with 95% confidence intervals on plain MAC agar by treatment group and across study days for all 3 farms. Both treated and untreated groups had similar quantities of enteric *E. coli* pre-treatment at the start of the trial (Day 0: P = 0.927; Treated: log_{10} CFU: 4.723; 95% CI: 4.301-5.145; Untreated: log_{10} CFU: 4.616; 95% CI: 4.194-5.037). However, following

sequential doses of CCFA, the modeled count of *E. coli* in the treated group decreased by nearly 2 $\log_{10}$ CFU below that of the untreated group ($P < 0.0001$: Treated 95% CI: 2.600-3.445; Untreated 95% CI: 4.497-5.345). At day 16, the first eligible date for slaughter after a two-dose treatment regimen with CCFA, the *E. coli* population of the treated group rebounded but remained somewhat different ($P = 0.053$; Treated 95% CI: 3.999-4.847; Untreated 95% CI: 4.396-5.242) from the control group. By study days 28 and 56, the *E. coli* populations of the treated group showed levels similar to that of the untreated group. Historical usage of ceftiofur formulations did not result in significantly different total *E. coli* growth counts with Dairy Farm 2 as referent category (Dairy Farm 1: $P = 0.854$, 95% CI = -0.685 – 0.828; Dairy Farm 3: $P = 0.144$, 95% CI = -0.192 – 1.321). Random effects attributed to dairy farm accounted for 11.4% of the variance components in the model with 9.7% attributed to animal.

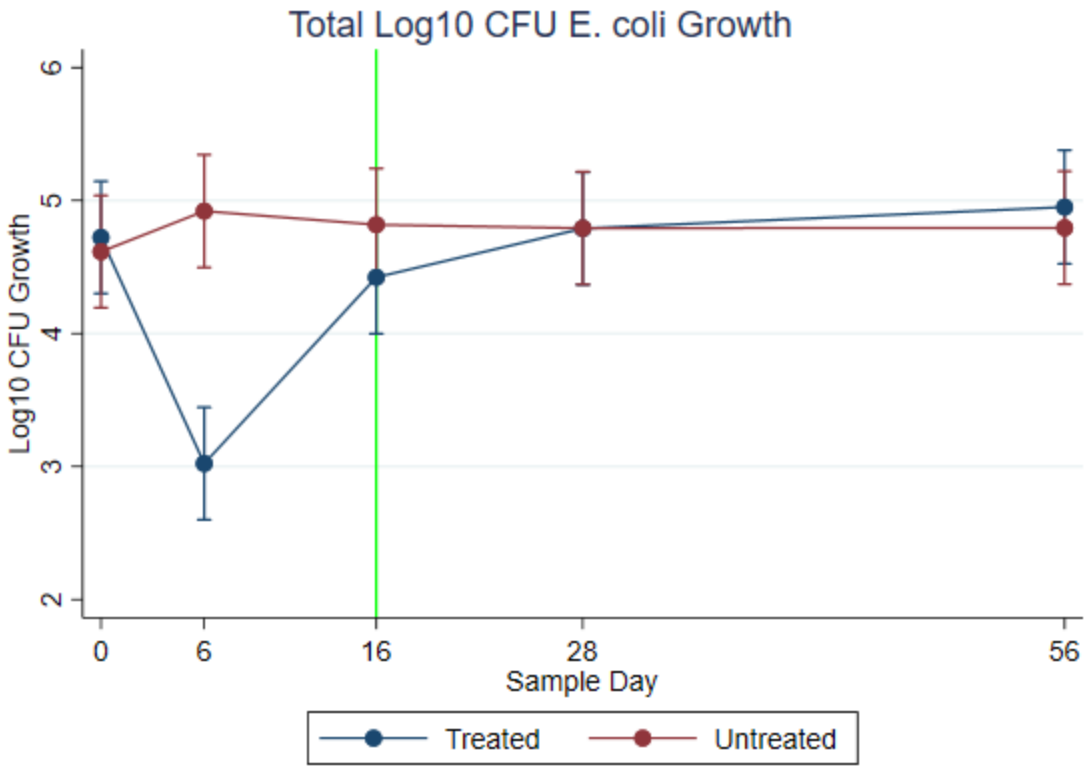

Figure 6: Total *E. coli* CFU counts grown on MacConkey agar on a log_{10} scale across study day are displayed with marginal means and 95% confidence intervals. Treated cows are shown in blue and untreated in red. The vertical green line represents the first-eligible slaughter date. CFU, colony-forming unit.

Figure 7 displays the fluctuation of the total *E. coli* population by each dairy farm across time. The treatment group from Dairy Farm 1 experienced a significant 2.416 log_{10} decrease in the total population ($P < 0.0001$, 95% CI = -3.190 – (-1.643)), but returns to similar population levels as the untreated group at day 16, as the population was 0.653 log_{10} units lower ($P = 0.099$, 95% CI = (-1.429) – 0.123). This was also observed on Dairy Farms 2 and 3. Dairy Farm 2 observed a drop in total *E. coli* population in the treated group of 1.482 log_{10} units on day 6 ($P <$

0.0001, 95% CI = -2.271 – (-0.693), but the population rebounded to levels similar to those of the untreated group by day 16 with a lower total population of 0.430 $\log_{10}$ units (P = 0.284, 95% CI = -1.216 – 0.357). The total *E. coli* drop of the treated group on Dairy Farm 3 at day 6 was -2.158 $\log_{10}$ (P < 0.0001, 95% CI = -2.959 – (-1.357)). This population rebounded on day 16 to levels similar to the untreated group, much like that of the other 2 dairy farms, at -0.425 $\log_{10}$ units lower (P = 0.298, 95% CI = 1.226 – 0.376).

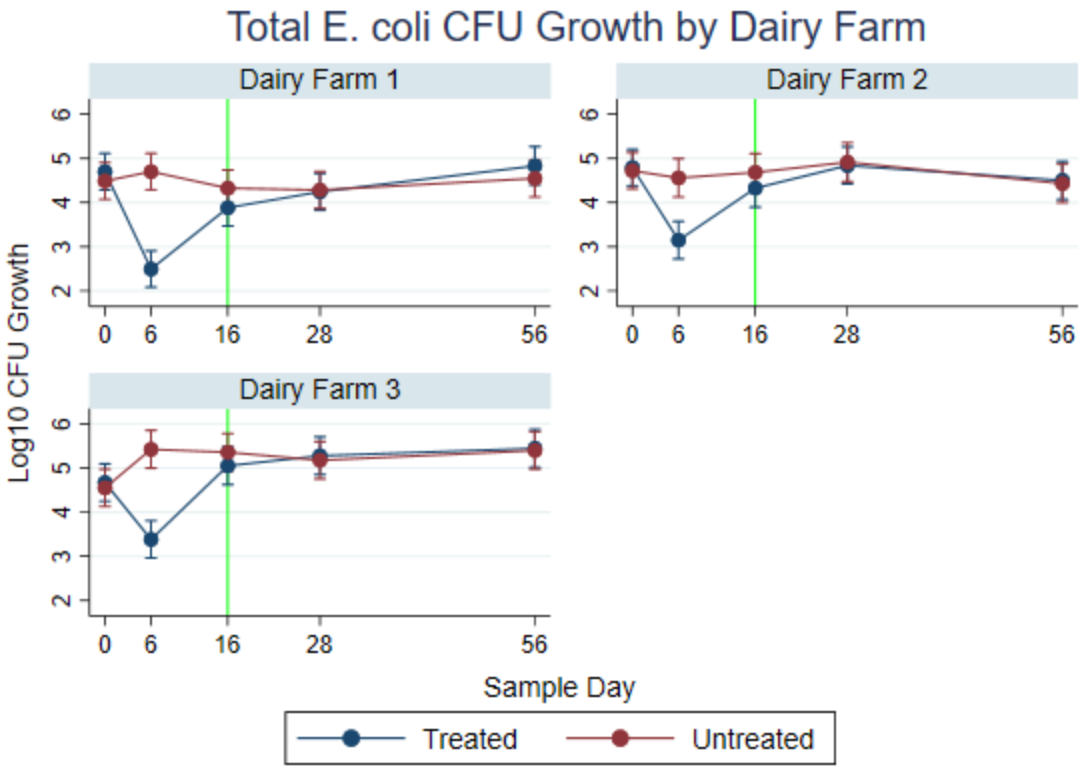

Figure 7: Total *E. coli* population growth on MacConkey agar is shown on a $\log_{10}$ with individual graphs by dairy farm for better comparison of groups within farm. 95% marginal means are displayed with treated animal values displayed in blue and untreated in red. The vertical green line represents the first-eligible slaughter data. CFU, colony-forming unit.

4.1.3 Mixed Model of 3GC Resistant E. coli CFU Counts

Figure 8 depicts the quantity of enteric *E. coli* resistant to ceftriaxone at an MIC ≥ 4 µg/mL by treatment and study day modeled with mixed effects linear regression. Following the two-dose treatment with CCFA, the 3GC resistant *E. coli* population of the treated group

increased to 1.5 $\log_{10}$ CFU above the untreated group by study day 6 ($P < 0.0001$; Treated 95% CI: 2.383-2.938; Untreated 95% CI: 1.496-2.052). On study day 16, the population of 3GC resistant bacteria in the treatment group had been reduced by 0.5 $\log_{10}$ CFU, remaining higher, though not significantly different, than the untreated group (P=0.134; Treated 95% CI: 2.031-2.587; Untreated 95% CI: 1.501-2.057). As time progressed to days 28 and 56, the population of 3GC resistant *E. coli* further decreased towards pre-treatment levels. Cows with a lactation number greater than 3 had higher relative quantities of 3GC resistant *E. coli* than first lactation animals ($P = 0.002$; 95% CI: $\log_{10}$ CFU difference of 0.114-0.501). Historical ceftiofur usage with Dairy Farm 2 as referent did not yield a statistically significant difference regarding 3GC resistant *E. coli* growth when compared to Dairy Farm 1 ($P = 0.341$, 95% CI: -0.199 – 0.576), but was substantively different when compared to Dairy Farm 3 ($P < 0.0001$, 95% CI: 0.645 – 1.405). Following imputation and incorporating all fixed and random effects, 7.63% of the variance in the model was attributed to the dairy farm with 9.8% attributed to animal.

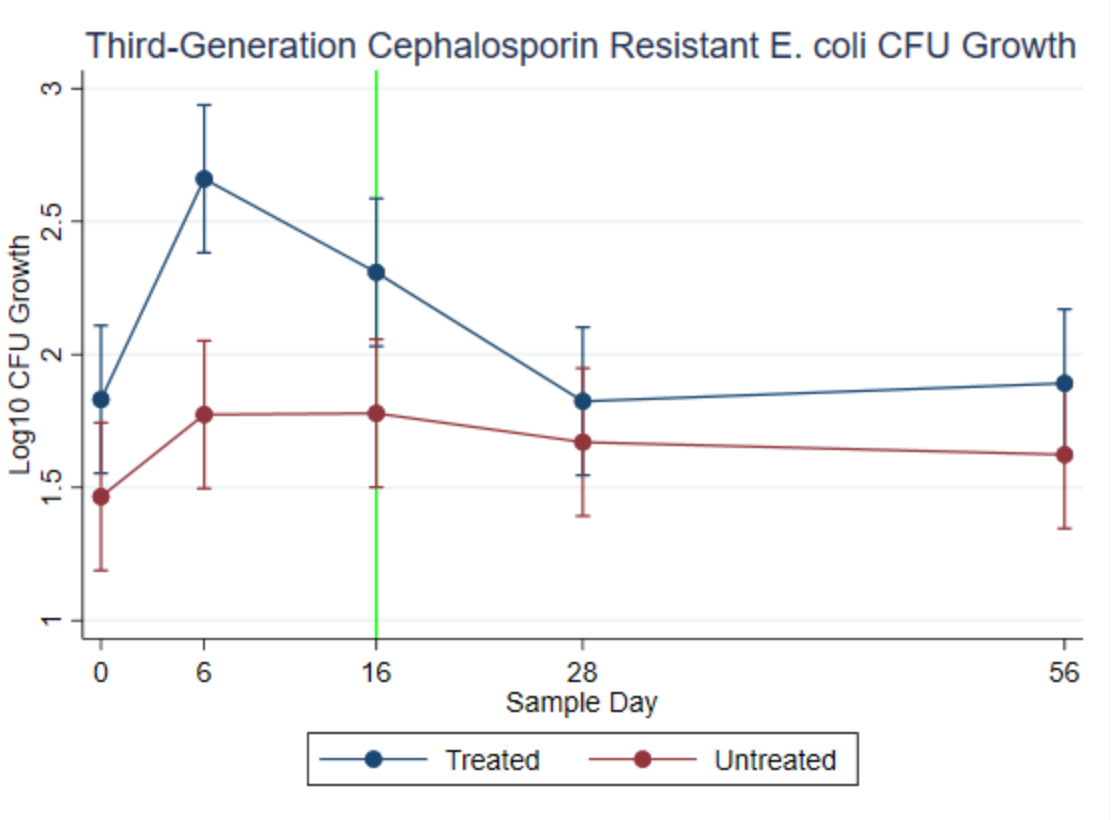

Figure 8: Ceftriaxone-resistant (4µg/ml) *E. coli* growth on a log_{10} scale across study day with 95% marginal means confidence intervals after 0 count data were imputed across levels below detection. The blue line represents animals in the treated group and the red represents animals in the untreated group. The vertical green line represents the first-eligible slaughter date. CFU, colony-forming unit.

To evaluate the differences in 3GC growth among the 3 dairy farms, an additional mixed effect model was run with an interaction between dairy farm, treatment group and sample day. Treated and untreated groups had similar marginal means on study days 0, 28 and 56 across all dairy farms. On day 6, the treatment groups increased to a greater log_{10} CFU growth than their untreated counterparts: Dairy Farm 1 (Treated marginal means = 2.275, 95% CI = 1.981 – 2.568;

Untreated marginal means = 1.616, 95% CI = 1.323 -1.909), Dairy Farm 2 (Treated marginal means = 2.368, 95% CI = 2.075 – 2.662; Untreated marginal means = 1.535, 95% CI = 1.242 – 1.829), and Dairy Farm 3 (Treated marginal means = 3.344, 95% CI = 3.043 – 3.644; Untreated marginal means = 2.131, 95% CI = 1.830 – 2.432). The treated group of Dairy Farm 3 had the highest marginal means on day 6 than all other group. The untreated population at the same location had marginal means at levels similar to that of the treated groups on Dairy Farms 1 and 2 on the same day. On day 16, only Dairy Farm 3 had marginal means in the treatment group elevated above those of the untreated group (Treated marginal means = 2.846, 95% CI = 2.565 – 3.147; Untreated marginal mean = 2.036, 95% CI = 1.735 – 2.336).

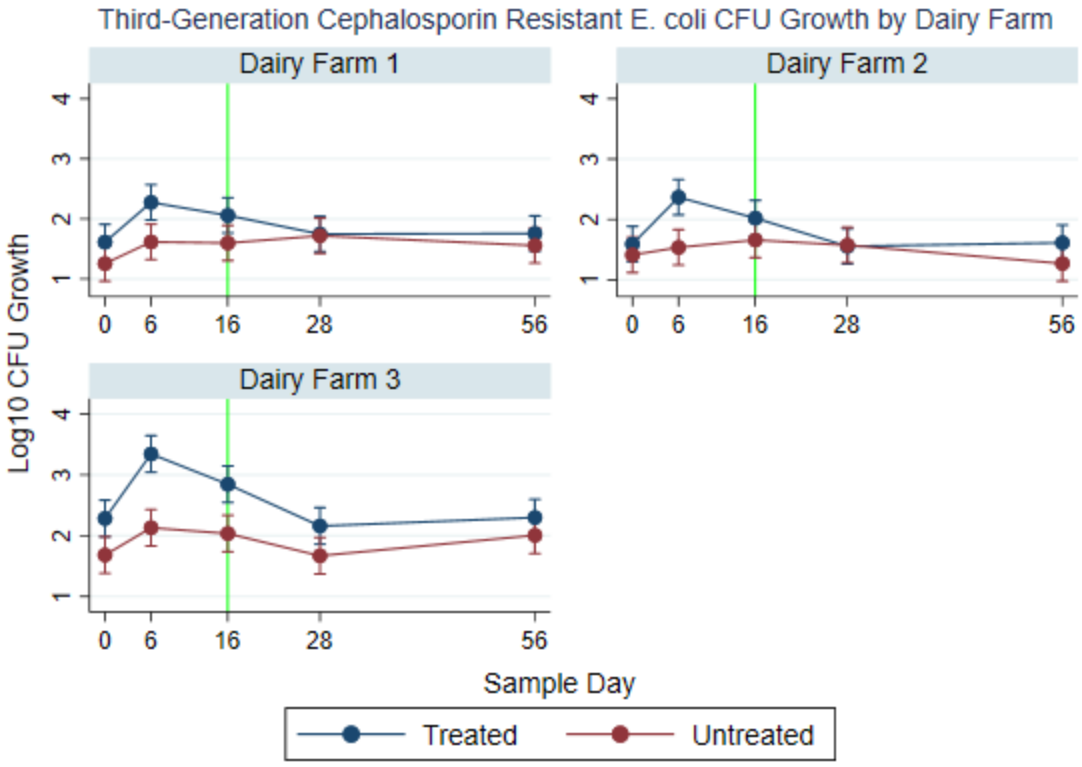

Figure 9: Ceftriaxone-resistant *E. coli* population growth on MacConkey agar containing ceftriaxone (4µg/ml) after 0 count data were imputed across levels below detection is shown on a $\log_{10}$ scale. Individual dairy farm graphs are displayed for better comparison of treatment groups within dairy farm. 95% marginal means are displayed with treated animal values displayed in blue and untreated in red. The vertical green line represents the first-eligible slaughter date. CFU, colony-forming unit.

4.1.4 Mixed Model of Difference between Total and 3GC Resistant E. coli CFU Counts

The outcome modeled as the arithmetic difference between the total enteric *E. coli* (MAC) CFU count and the 3GC resistant enteric *E. coli* (MACCEF) CFU count by treatment

across study days using mixed effects linear regression is displayed in Figure 10. This analysis utilized the original MACCEF data, not imputed values. The rationale for this analysis was that as the baseline levels of total coliforms changes, the observed changes in quantity of resistance resulted from shifts in either the numerator, denominator, or else both. The difference in the relative quantities across sampling days for the untreated group remained constant over time (day 0 95% CI: 3.847-4.520; day 6 95% CI: 3.990-4.673; day 16 95% CI: 4.002-4.679; day 28 95% CI: 4.050-4.735; day 56 95% CI: 4.044-4.729). At baseline day 0, the arithmetic difference in the relative quantities was at similar levels for both treated and untreated groups ($P = 0.775$; Treated: $\log_{10}$ CFU: 4.080; 95% CI: 3.740-4.420; Untreated: $\log_{10}$ CFU: 4.185; 95% CI: 3.846-4.525). However, following treatment with CCFA, the $\log_{10}$ arithmetic difference between total *E. coli* and 3GC resistant *E. coli* CFU was reduced to 1.5 $\log_{10}$; meaning, of bacteria remaining after treatment, approximately 1 in 32 total colony-forming units were resistant to ceftriaxone. Of note, this differed significantly from the untreated group ($P < 0.0001$; Treated 95% CI: 1.111-1.788; Untreated 95% CI: 3.990-4.673). This difference was also observed on the first-eligible slaughter date (day 16) ($P = 0.001$; Treated arithmetic differences in $\log_{10}$ CFU 95% CI: 2.970-3.653; Untreated 95% CI: 4.002-4.680). On study day 16, approximately 1 in 1,250 *E. coli* CFU were 3GC resistant. By study days 28 and 56, the differences in total and resistant *E. coli* populations in the treatment group were not significantly different ($P > 0.05$) from levels from the untreated group (Day 28: $P = 0.808$; Treated 95% CI: 3.877-4.556; Untreated 95% CI: 4.050-4.735; Day 56: $P = 0.775$; Treated 95% CI: 4.029-4.722; Untreated 95% CI: 4.044-4.729). Similar to 3GC resistant *E. coli* models, historical ceftiofur usage with Dairy Farm 2 as referent did not yield significantly different values from Dairy Farm 1 ($P = 0.135$, 95% CI: -0.638 –

0.086), but did differ statistically from Dairy Farm 3 (P = 0.009, 95% CI: -0.848 – -0.122). In this analysis, dairy farm accounted for 2.3% of the total variance and animal accounted for 6.2%.

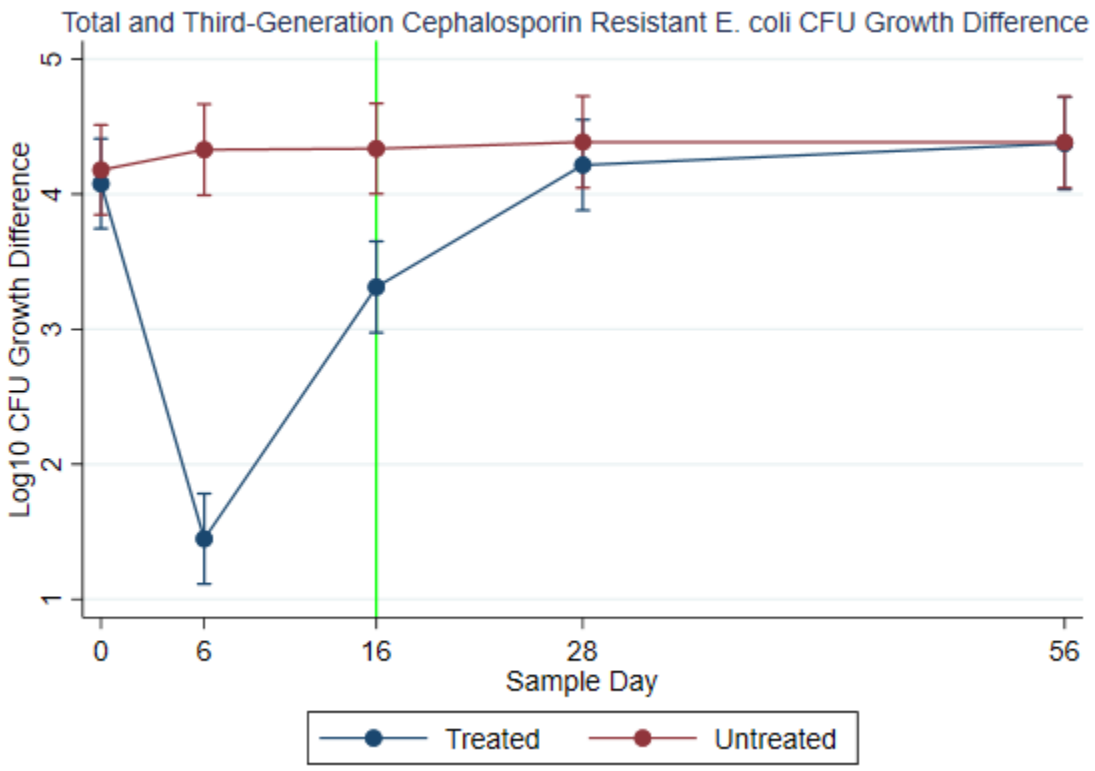

Figure 10: The graph displays the difference in growth between total and ceftriaxone-resistant (4µg/ml) *E. coli* populations across study day. Decreases in growth difference are indicative of higher proportions of ceftriaxone-resistant *E. coli*. 95% confidence intervals are displayed with marginal means. Treated animals are represented by the blue line and the untreated group is represented by the red. The vertical green line indicated the first-eligible slaughter date. CFU, colony-forming unit.

Due to the variability resulting from dairy farm, mixed effect linear regression with three-way interaction of farm, treatment, and sample day was performed on the arithmetic difference in *E. coli* growth on MAC and MACCEF agars. Figure 11 shows the breakdown of the difference of total and resistant *E. coli* populations by dairy farm. The difference in counts for untreated groups remained constant throughout. However, on both study days 6 and 16, the Dairy Farm 3 treatment group exhibited the smallest difference between total and resistant *E. coli* populations, followed by Dairy Farm 1 and Dairy Farm 2. Figure 11 shows the starting $\log_{10}$ CFU arithmetic differences between total and 3GC resistant populations are a single $\log_{10}$ CFU lower on Dairy Farm 3 than those same paired differences on Dairy Farms 1 and 2; meaning, dairy 3 has a higher proportional level of resistant *E. coli* than the other 2 locations. The ratio of 3GC resistant to total *E. coli* increases significantly in the treated group compared to the untreated group on day 6 on all 3 farms: Dairy Farm 1 ($P < 0.0001$; Treated 95% CI: 0.912-1.846; Untreated 95% CI: 3.851-4.785), Dairy Farm 2 ($P < 0.0001$; Treated 95% CI: 1.976-2.945; Untreated 95% CI: 3.859-4.853), and Dairy Farm 3 ($P < 0.0001$; Treated 95% CI: 0.034-0.991; Untreated 95% CI: 3.785-4.753). These differences begin to decrease as time progresses. However, the level of significance concerning these differences on day 16 (slaughter eligibility) varies greatly by farm. The differences observed on day 16 for the treated groups are significantly different from the untreated group on Dairy Farm 1 ($P = 0.027$; Treated 95% CI: 2.728-3.673 Untreated 95% CI: 3.807-4.740) and 3 ($P = 0.008$; Treated 95% CI: 2.352-3.321; Untreated 95% CI: 3.892-4.848), but are non-significantly different on Dairy Farm 2 ($P= 0.081$; Treated 95% CI: 3.385-4.366; Untreated 95% CI: 3.844-4.812). Because Dairy Farm was included in this model as a fixed effect, unlike overall models where it was included as a random effect, historical ceftiofur usage was not considered due to its collinearity with farm.

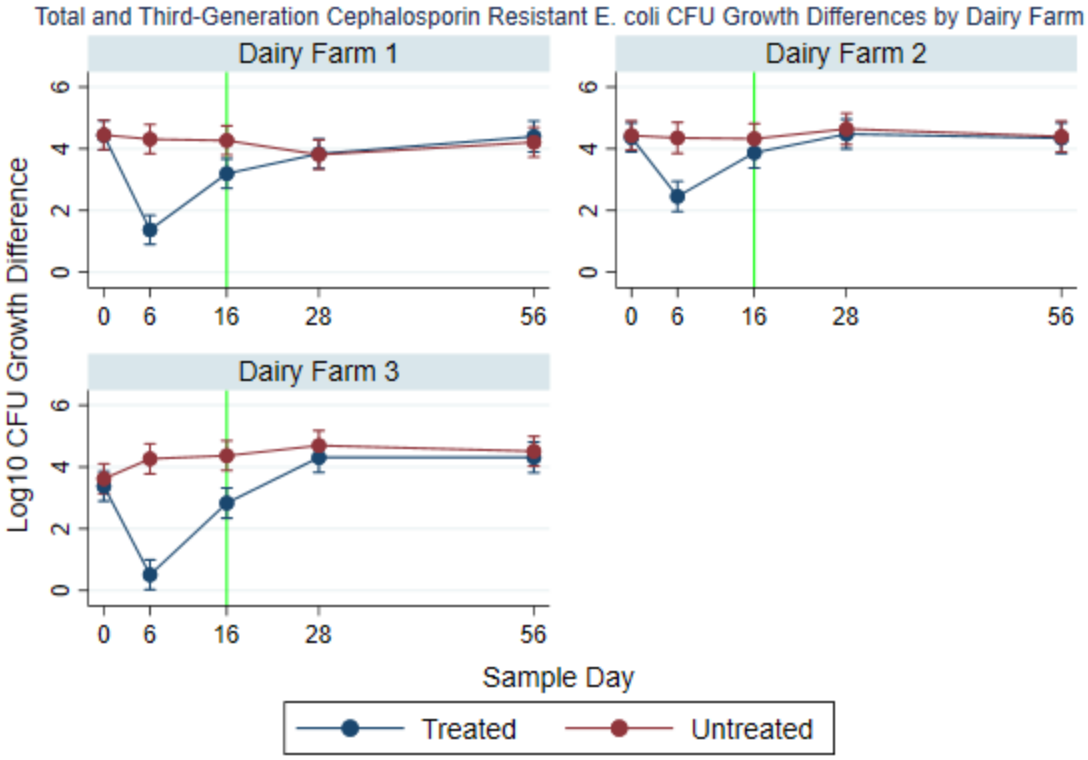

Figure 11: The difference in growth between total and ceftriaxone-resistant (4µg/ml) *E. coli* across study day with 95% marginal means is displayed with individual graphs by dairy farm. Decreases in difference show increases in the proportion of colony-forming units resistant to ceftriaxone. Animals in the treated group are represented by the blue line and the untreated group is represented by red. The vertical green line indicates the first-eligible slaughter date. CFU, colony-forming unit.

4.2 AmpC AND ESBL *E. coli* DIFFERENTIATION

Histograms, resulting from attempts to determine what proportion of 3GC resistant *E. coli* growth possessed AmpC or ESBL resistance, shown in Figure 12 provide a visual depiction

of the frequency of different plate types with varying levels of growth. Due to the large number of samples that either did not display growth or else displayed colonies in a manner that was too numerous to count, the x-axes are displayed as no growth, countable, or overgrown rather than as a $\log_{10}$ scale, as with the quantifiable *E. coli* CFU count data of the previous chapter. The first set of histograms displays growth on the 5 agar types without sample enrichment (Figure 12). The samples that did display detectable levels of growth all displayed countable amounts. Unfortunately, the MACFEP agar did not display any detectable levels of growth for any of the 30 samples; that is, despite the known presence of ESBL bacteria in the samples (see later sections).

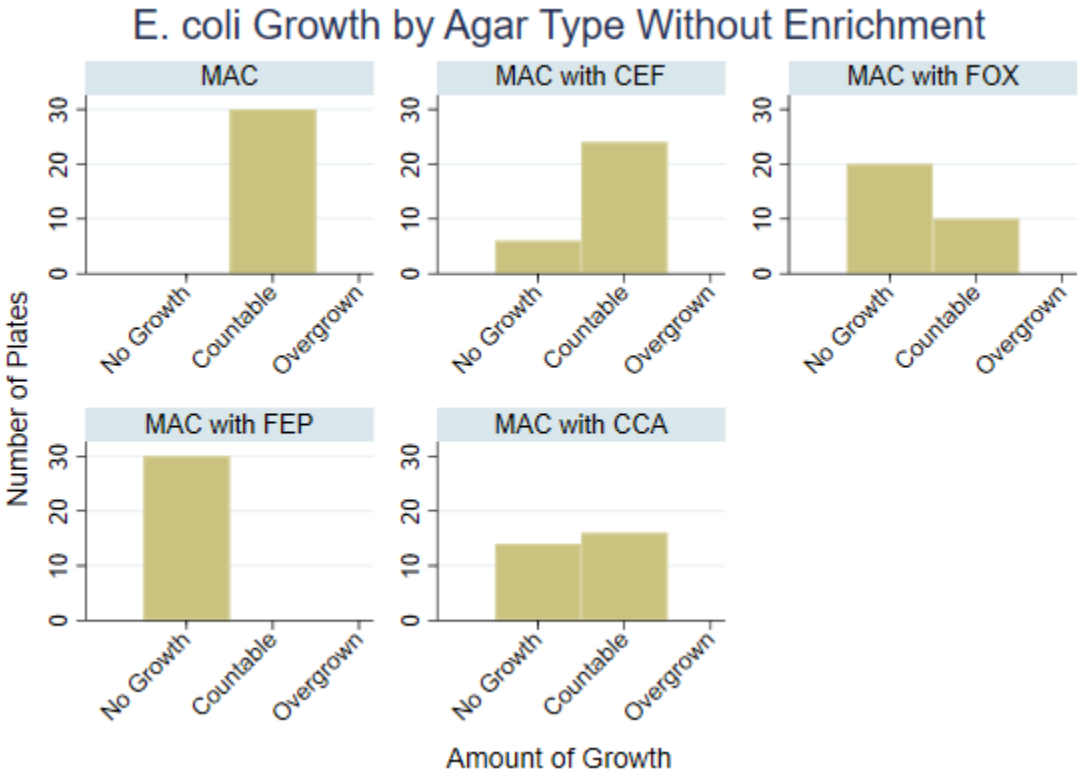

Figure 12: The figures above display the quantity of samples (n = 30) with varying amounts of growth across the 5 agar plate types when the samples are spiral plated without an enrichment step in processing. MAC, plain MacConkey agar; CEF, ceftriaxone (4 µg/mL); FOX, cefoxitin (32 µg/mL); FEP, cefepime (16 µg/mL); CCA, ceftriaxone/clavulanic acid (4/4 µg/mL).

The extended enrichment period of an 18 hr incubation at 37°C in MacConkey broth with 1 µg/mL of ceftriaxone created a large number of agar plates with colony growth too numerous to count. This was the case with a majority of samples on every agar type, but MACFEP (Figure 13). However, the lengthened enrichment produced 6 samples displaying countable levels of growth on MACFEP. A shorter enrichment period (3 hrs) with a higher concentration of

ceftriaxone in the MacConkey broth (2 µg/mL) yielded similar results to the agar plates without sample enrichment (Figure 14). All plates with detectable levels of growth provided countable quantities. Unfortunately, even with the increased levels of ceftriaxone in the MacConkey broth to reduce selection pressure, the shortened enrichment period did not yield detectable levels of growth on the MACFEP agar.

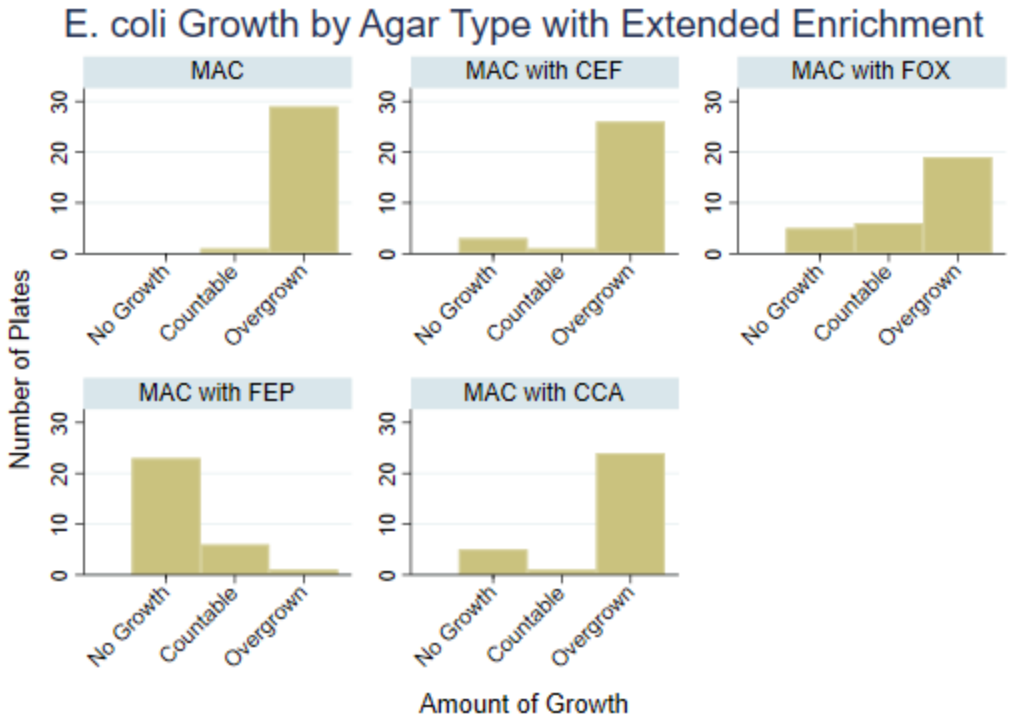

Figure 13: The above figures display the frequency of samples (n = 30) with differing amounts of growth when spiral plated to 5 different agars after undergoing an 18 hr enrichment in MAC broth with 1µg/mL of ceftriaxone. MAC, plain MacConkey agar; CEF, ceftriaxone (4 µg/mL); FOX, cefoxitin (32 µg/mL); FEP, cefepime (16 µg/mL); CCA, ceftriaxone/clavulanic acid (4/4 µg/mL).

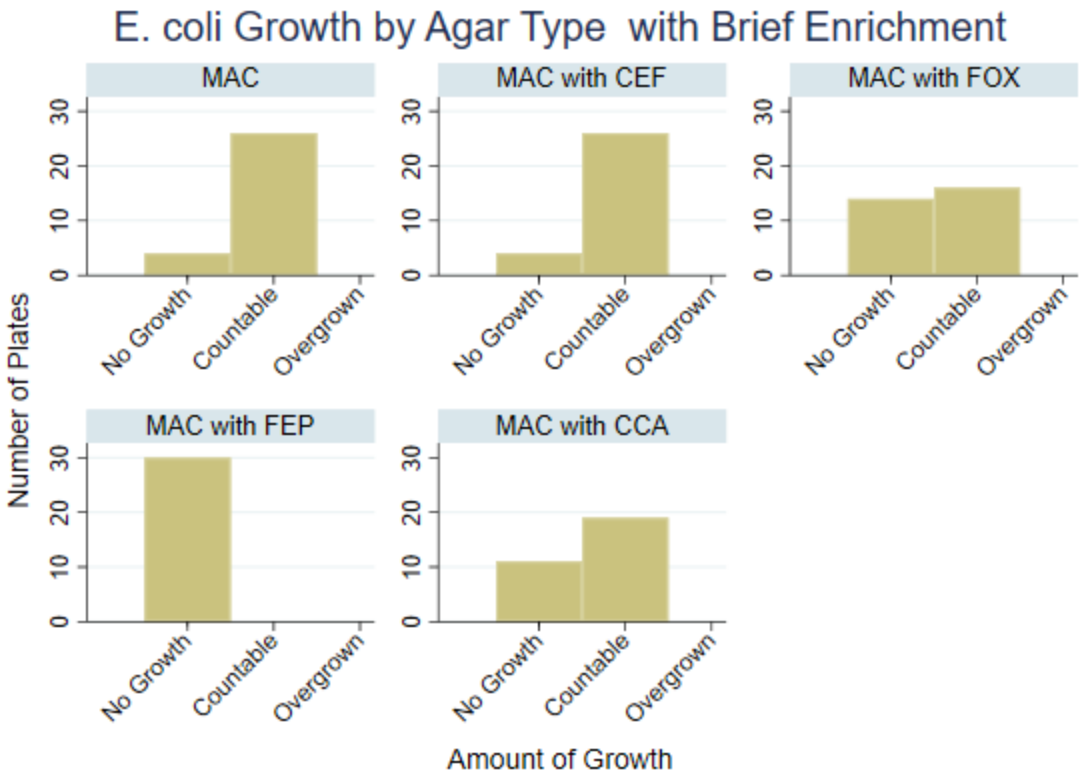

Figure 14: The figure, broken down by agar type, display the number of samples (n = 30) displaying differing levels of growth when spiral plated after a 3 hr incubation in MAC broth with 2 µg/mL. MAC, plain MacConkey agar; CEF, ceftriaxone (4 µg/mL); FOX, cefoxitin (32 µg/mL); FEP, cefepime (16 µg/mL); CCA, ceftriaxone/clavulanic acid (4/4 µg/mL).

4.3 AmpC/ESBL FECAL *E. coli* SHEDDING

4.3.1 Descriptive Statistics

All collected samples (n = 1197) were spiral plated to CHROM-ESBL agar with 25.98% testing positive (n = 311) and 74.02% tested negative. Of the isolates that grew on ESBL agar,

310 out of 311 isolates shown to be *E. coli* via indole testing and MALDI-TOF were tested for their phenotypic MIC for 14 antibiotics via microbroth dilution (1 isolate was misplaced and not tested). Microbroth dilution showed that 74.52% of isolates had a phenotypic ESBL resistance profile, while 25.48% had an AmpC resistance profile. Of the phenotypic ESBL *E. coli* isolates, 58.9% were found in the treated group; however, this was not dependent on treatment when tested by the Pearson Chi-Square Test ($P = 0.923$) (Table 6). The number of samples testing positive for a phenotypic ESBL resistance profile was maintained consistently across study days while decreasing slightly on day 56, but was still dependent upon sampling day with a significant Pearson's Chi-Squared test ($P = 0.019$) (Table 7). The number of AmpC resistance profiles peaked on day 6 and decreased to its lowest level on day 56, similar to the quantitative 3GC *E. coli* counts (Figure 8). The number of isolates exhibiting an ESBL phenotype varied significantly (Pearson's Chi-Squared P = 0.001) by farm with Dairy Farm 3 having the largest number of ESBL isolates (Table 8).

Table 6: Frequency of *E. coli* Isolates Displaying ESBL or AmpC Resistance Profile by Treatment Group

Resistance Phenotype	Animal Group		Total
	Treated	Control	
ESBL	136	95	231
AmpC	47	32	79
Total	183	127	310

The distribution of ESBL and AmpC resistance profiles of *E. coli* isolates are displayed by treatment grouping.

Table 7: Frequency of *E. coli* Isolates Displaying ESBL or AmpC Resistance Profile by Sample Day

Phenotype	Sample Day					Total
	0	6	16	28	56	
ESBL	46	49	48	52	36	231
AmpC	16	28	20	9	6	79
Total	62	77	68	61	42	310

The distribution of antimicrobial resistance profile of *E. coli* isolates grown on CHROM-ESBL agar is displayed by sampling day.

Table 8: Frequency of *E. coli* Isolates Displaying ESBL or AmpC Resistance Profile by Dairy Farm

Phenotype	Dairy Farm Number			Total
	1	2	3	
ESBL	74	38	119	231
AmpC	11	9	59	79
Total	85	47	178	310

The resistance profile of *E. coli* isolates grown on CHROM-ESBL agar is shown by dairy farm.

4.3.2 AmpC/ESBL E. coli Prevalence

The prevalence of AmpC/ESBL fecal *E. coli* shedding over time was modeled (Figure 16). Because CHROM-ESBL agar was used with enrichment, the selection criteria was biased toward the ESBL phenotype. Animals from Dairy Farm 2 had a decreased odds of shedding AmpC/ESBL resistant *E. coli* than Dairy Farm 1 (Odds ratio: 0.508, $P = 0.001$, 95% CI: 0.343 – 0.752), but Dairy Farm 3 had an elevated odds of nearly 3.4 times as likely that an animal tested positive for shedding *E. coli* with an AmpC/ESBL phenotype than the referent Dairy Farm 1 (Odds ratio: 3.35, $P < 0.0001$, 95% CI: 2.438 – 4.608). Although not significant at all levels, the odds of testing positive for an AmpC/ESBL *E. coli* decreased by sampling day after the first treatment sample was taken. The odds on day 6 increased to 1.79 ($P = 0.046$, 95% CI: 1.010 - 3.191) among the treatment group compared to the control group, decreasing slightly to 1.316 ($P = 0.358$, 95% CI: 0.0.733 – 2.362) on day 16, decreasing back to even with the referent day 0 on day 28 at 0.995 ($P = 0.988$, 95% CI: 0.549 – 1.807), and improving to better than the referent

day 0 on day 56 with an odds of 0.760 (P = 0.387, 95% CI: 0.408 – 1.416). Similar trends were observed regarding the prevalence of animals testing positive for shedding an *E. coli* with an AmpC or ESBL form of resistance (Figure 16), while also noting treated animals tended to have a higher prevalence (day 0: 28.7% (95% CI: 21.2% – 36.2%), day 6: 40.1% (95% CI: 32.0% – 48.2%), day 16: 33.9% (95% CI: 25.9% – 41.8%), day 28: 28.6% (95% CI: 21.1% – 36.2%), day 56: 24.0% (95% CI: 16.6% – 31.5%)) than their control counterparts (day 0: 22.2% (95% CI: 15.2% – 29.1%), day 6: 23.1% (95% CI: 16.1% – 30.2%), day 16: 23.9% (95% CI: 16.8% – 31.1%), day 28: 22.6% (95% CI: 15.5% – 29.6%), day 56: 14.4% (95% CI: 8.3% – 20.4%).

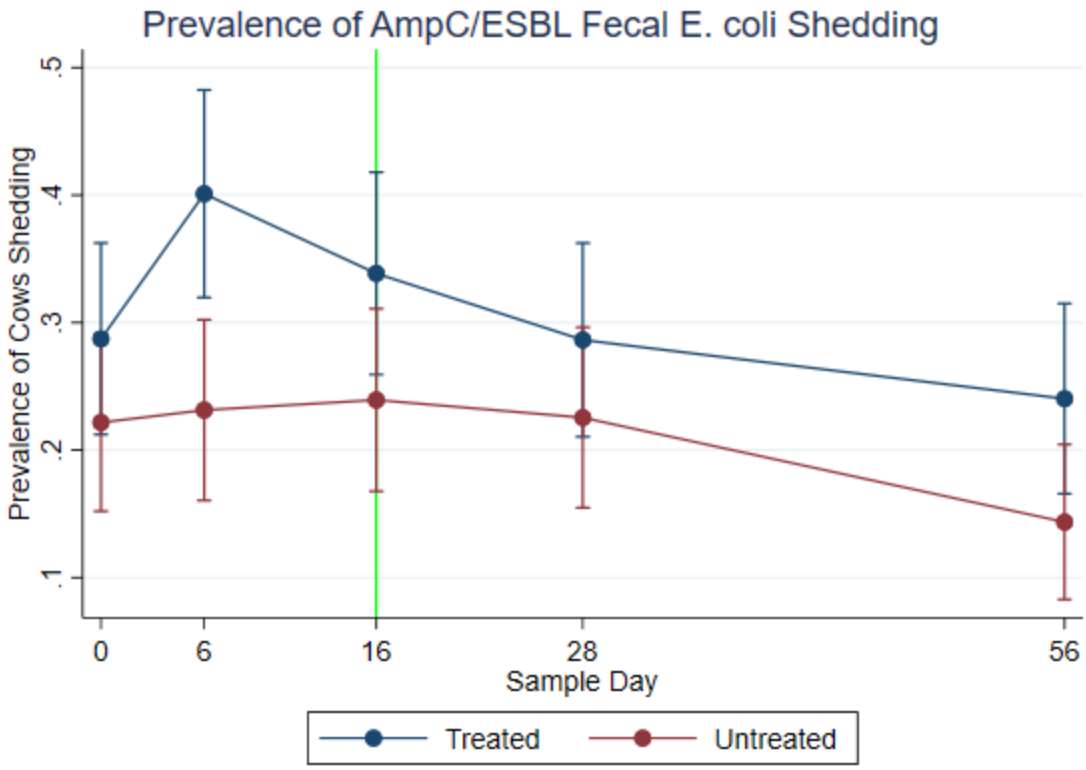

Figure 15: The change of prevalence of cows shedding fecal *E. coli* with an AmpC or ESBL resistance phenotype by sample day is shown with the blue line representing the treatment group and red showing the control group. The vertical green line represents the first-eligible slaughter date.

By adding an interaction of treatment, sample day, and dairy farm, the odds of an animal shedding an AmpC/ESBL-producing *E. coli* increased insignificantly to 1.819 (P = 0.297, 95% CI: 0.591 – 5.598) on Dairy Farm 2 and the odds of having an animal shedding an *E. coli* isolate with an AmpC or ESBL resistance profile based on the CHROM-ESBL agar selection bias increased significantly to 5.346 (P = 0.002; 95% CI: 1.833 – 15.590) on Dairy Farm 3 in

comparison to Dairy Farm 1. The odds varied by sampling day after peaking on day 6. The greatest odds of an animal testing positive for shedding an *E. coli* isolate with an AmpC or ESBL phenotypic resistance based upon the CHROM-ESBL agar selection criteria was an odds ratio of 3.291 (*P* = 0.030, 95% CI: 1.122 – 9.654) on day 6, before steadily decreasing to an odds ratio of 2.733 (*P* = 0.071, 95% CI: 0.917 – 8.146) on day 16 and 2.153 (*P* = 0.177, 95% CI: 0.708 – 6.548) on day 28 with a slight increase to 2.594 on day 56 (*P* = 0.096, 95% CI: 0.843 – 7.984). The starting prevalence of treated and control cow groups were the same on Dairy Farms 1 (*Treated*: Prevalence = 14.7%, 95% CI: 3.9% - 25.4%; *Control*: Prevalence = 9.8%, 95% CI: 0.7% – 18.8%) and 2 (*Treated*: Prevalence = 23.7%, 95% CI: 10.9% – 36.5%; *Control*: Prevalence = 16.6%, 95% CI: 5.4% – 27.8%), while the prevalence of AmpC/ESBL *E. coli* was slightly higher among the treated group than the control on day 0 on Dairy Farm 3 (*Treated*: Prevalence = 47.4%, 95% CI: 32.1% – 62.8%; *Control*: Prevalence = 39.9%, 95% CI: 24.9% – 55.0%) (Figure 17). The prevalence increased in both groups on Dairy Farms 1 (*Treated*: Prevalence = 35.8%, 95% CI: 21.4% – 50.3%; *Control*: Prevalence = 21.5%, 95% CI: 9.1% – 33.9%) and 3 (*Treated*: Prevalence = 63.9%, 95% CI: 48.9% – 78.9%; *Control*: Prevalence = 39.9%, 95% CI: 24.9% – 55.0%) on day 6, but were maintained the same or decreased on Dairy Farm 2 (*Treated*: Prevalence = 20.4%, 95% CI: 7.8% – 33.0%; *Control*: Prevalence = 7.7%, 95% CI: -0.6% – 16.1%). Advancing to day 16, the prevalence of animals testing positive for AmpC/ESBL *E. coli* began to decrease among both groups from Dairy Farms 1 (*Treated*: Prevalence = 31.7%, 95% CI: 17.6% – 45.9%; *Control*: Prevalence = 7.2%, 95% CI: -0.6% – 15.0%) and 2 (*Treated*: Prevalence = 7.8%, 95% CI: -0.7% – 16.3%; *Control*: Prevalence = 10.2%, 95% CI: 0.7% – 19.6%), while remaining stable in the treatment (Prevalence = 61.6%,

95% CI: 46.5% – 76.8%) group and increasing in the control (Prevalence = 54.9%, 95% CI: 39.6% – 70.3%) group on Dairy Farm 3 before decreasing toward days 28 and 56.

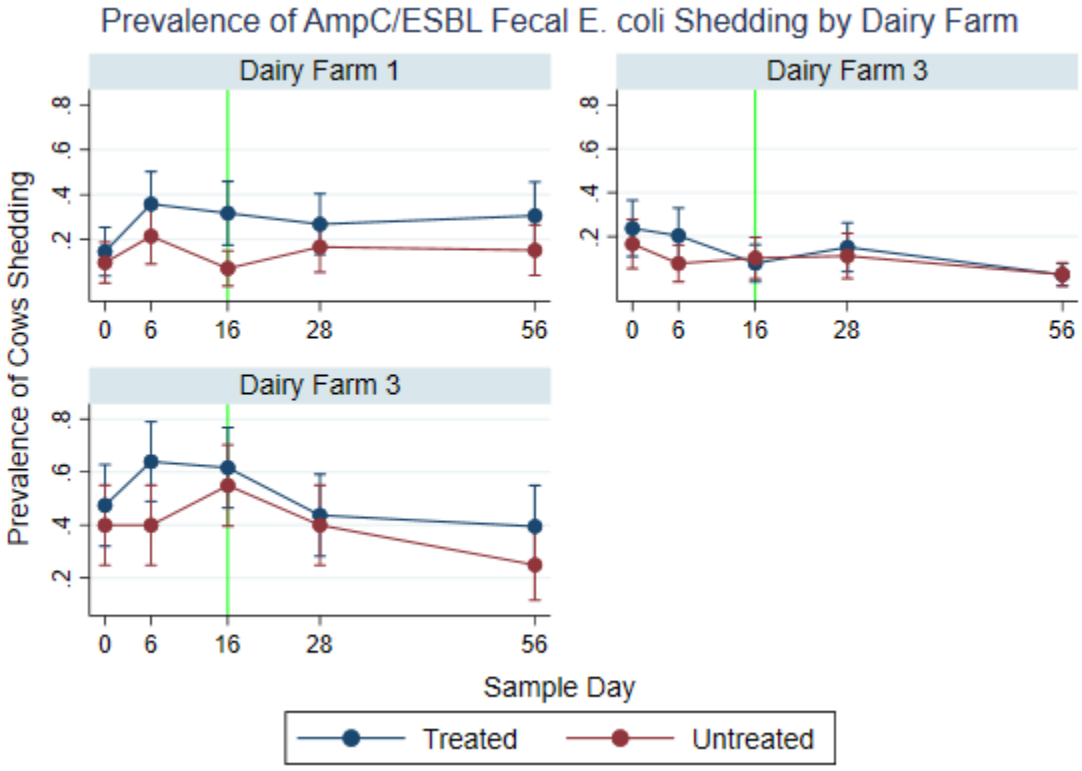

Figure 16: The change of prevalence regarding phenotypic AmpC/ESBL-producing resistant *E. coli* by sample day is shown with the blue line representing the treatment group and red showing the control. The vertical green line represents the first-eligible slaughter date. Individual graphs are shown for each dairy farm.

4.3.3 Antibiotic Class Resistance

The percentage of isolates with resistance to the antibiotics and antibiotic classes on the NARMS gram-negative Sensititre™ plate is shown with a breakdown by treatment group (Table 13). Due to the selection criteria and process, there was no difference in the percentage of isolates with resistance to a certain antibiotic or class among treated or untreated animal groups. All isolates showed resistance to ampicillin and ceftriaxone with nearly 100% having phenotypic resistance to ceftiofur (due to AmpC and ESBL isolates specifically being selected on CHROM-ESBL media). Furthermore, nearly 27% of all isolates had phenotypic resistance to amoxicillin/clavulanic acid and cefoxitin, consistent with an AmpC form of resistance. Of further concern were the levels of resistance or decreased susceptibility observed to ciprofloxacin and azithromycin, as both quinolones and macrolides are listed among the antibiotics that are critically important and of highest priority to human medicine as determined by the WHO (2019). While roughly 11% of the isolates selected tested as having phenotypic resistance to ciprofloxacin, approximately 25% also had reduced susceptibility with MICs in the range of 0.25 – 0.50 µg/mL. Furthermore, nearly 11% had reduced susceptibility to azithromycin with macrolides not readily used in adult cow dairy production settings. It is important to remember these isolates were selected for an ESBL resistance profile and were not selected from the general population, so the isolation approach was biased.

Table 9: Resistance Distribution by Breakpoint and MIC values

Antibiotic class	Antibiotics	Animal Group	Exact (Clopper-Pearson)		MIC (µg/mL) distribution and classification of resistance (%)														
			Resistant	95% CI	0.015	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
B-Lactam Combination Agents	Amoxicillin/ clavulanic acid[a]	Treated	27.3	21.0-34.4						0.0	0.0	19.7	47.0	7.7	8.2	19.1			
		Untreated	26.0	18.6-34.5						0.0	3.9	20.5	39.4	10.2	7.9	18.1			
Penicillins	Ampicillin	Treated	100.0	98.0-1.0*						0.0	0.0	0.0	0.0	0.0	0.0	100.0			
		Untreated	100.0	97.1-1.0*						0.0	0.0	0.0	0.0	0.0	0.0	100.0			
Macrolides	Azithromycin[b]	Treated	10.4	6.4-15.7			0.0	0.0	0.0	0.0	7.7	45.9	19.7	16.4	10.4				
		Untreated	11.8	6.8-18.7			0.0	0.0	0.0	1.6	4.7	47.2	19.7	15.0	11.8				
Cephems	Cefoxitin	Treated	27.8	21.5-35.0					0.0	0.0	5.5	35.5	24.6	4.9	2.7	25.1			
		Untreated	26.0	18.6-34.5					0.0	0.0	8.7	38.9	26.8	0.0	1.6	24.4			
	Ceftiofur	Treated	98.4	95.3-99.7			0.0	0.0	0.0	0.0	0.0	1.6	6.6	91.8					
		Untreated	96.9	93.3-99.5			0.0	0.0	0.0	0.0	1.6	1.6	6.3	90.6					
	Ceftriaxone	Treated	100.0	98.0-1.0*				0.0	0.0	0.0	0.0	1.6	1.1	6.6	20.2	20.2	50.3		
		Untreated	100.0	97.1-1*				0.0	0.0	0.0	0.0	3.1	3.1	13.4	10.2	24.4	45.7		
Phenicols	Chloramphenicol	Treated	48.1	40.7-55.6							2.7	24.0	21.9	3.3	1.1	47.0			
		Untreated	47.9	39.1-57.1							3.1	28.3	15.7	4.7	0.7	47.2			
Quinolones	Ciprofloxacin	Treated	12	7.7-17.6	50.8	1.1	0.00	7.7	16.9	4.9	0.00	0.5	6.6						
		Untreated	10.3	6.2-17.8	53.5	0.1	0.00	9.4	17.3	0.1	0.00	0.0	10.2						
	Nalidixic acid	Treated	13.8	9.0-19.5					0.0	4.4	43.2	18.0	6.6	14.2	0.7	13.1			
		Untreated	14.1	8.6-21.5					0.0	1.6	47.2	14.2	8.7	14.2	0.0	14.1			
Aminoglycosides	Gentamicin	Treated	4.9	2.3-9.1				0.5	29.0	54.1	7.7	3.3	0.5	0.0	4.9				
		Untreated	1.6	0.1-5.6				2.4	26.0	62.2	6.3	0.0	0.7	0.0	1.6				
	Streptomycin[b]	Treated	50.3	42.8-57.7							0.0	7.1	33.3	9.3	5.5	44.8			
		Untreated	49.6	40.6-58.6							0.7	6.3	31.5	11.8	2.4	47.2			
Folate Pathway Antagonists	Sulfisoxazole	Treated	54.6	47.1-62.0										41.0	3.8	0.0	0.5	0.0	54.6
		Untreated	52.0	42.9-60.9										44.9	2.4	0.0	0.8	0.0	52.0
	Trimethoprim/ Sulfamethoxazole[c]	Treated	16.4	11.3-22.6			55.2	20.2	6.6	1.1	0.5	0.00	16.4						
		Untreated	16.5	10.5-24.2			56.7	18.6	5.5	2.4	0.0	0.00	16.5						
Tetracyclines	Tetracycline	Treated	71.6	64.4-78.0								27.3	1.1	1.1	5.5	65.0			
		Untreated	75.5	67.1-82.8								23.6	0.8	0.8	8.6	66.1			

The MIC distribution of isolates and resistance to each antibiotic and antibiotic class are broken down by treatment group. The vertical black line represents the Clinical Laboratory Standards Institute or NARMS consensus breakpoint for resistance. Numbers in red represent the percentage of resistant isolates at each MIC. Red numbers displayed on a grey background indicate the percentage of isolates with a minimum inhibitory concentration greater than that of the plate (right-censored)

4.3.4 Survival Analysis

In Figure 17, the minimum inhibitory concentration for 50% of the isolates (MIC 50) had an MIC less than or equal to 8/4 µg/mL of amoxicillin/clavulanic acid, while 90% of isolates fell less than or equal to the upper limit of 32/16 µg/mL These trends remained the same regardless of treatment, while isolates from the treated group maintained a slightly higher proportion of *E. coli* with higher MIC levels, as the Mantel-Haenszel rate ratio increased slightly to 1.031 (P = 0.8130, 95% CI: 0.802 – 1.325) in the control group (Figure 18). Both groups had the same MIC 50 (8 µg/4 µg/mL) and MIC 90 (> 32 µg/16 µg/mL). Dairy Farm 1 had isolates exhibiting a greater proportion of lower MICs indicated by a rate ratio of 0.794 (P = 0.0023, 95% CI: 0.685 – 0.921) above Dairy Farm 2. Dairy Farm 3 had the lowest rate of isolates reaching their MIC and a greater proportion of isolates with higher-level MICs than the other 2 dairy farms (Figure 19). However, the isolates from each farm still maintained the same MIC 50 (8 µg/4 µg/mL) and MIC 90 (> 32 µg/16 µg/mL). Unsurprisingly, there was an increase in the rate at which isolates reached their MIC by day at a rate of 1.012 (P = 0.0021, 95% CI: 1.004 – 1.019). It was observed that day 6 had a greater proportion of isolates with an increased MIC than days 0 and 16 or day 28 and 56, which had the lowest proportion of isolates with an MIC of 8 µg/4 µg/mL or greater (Figure 20). Isolates from day 6 had an increased MIC 50 over isolates from the other days (16 µg/8 µg/mL versus 8 µg/4 µg/mL). Isolates from days 28 and 56 had MIC 90s of 32 µg/16 µg/mL, whereas those from the other sampling days had MIC 90s greater than 32 µg/16 µg/mL.

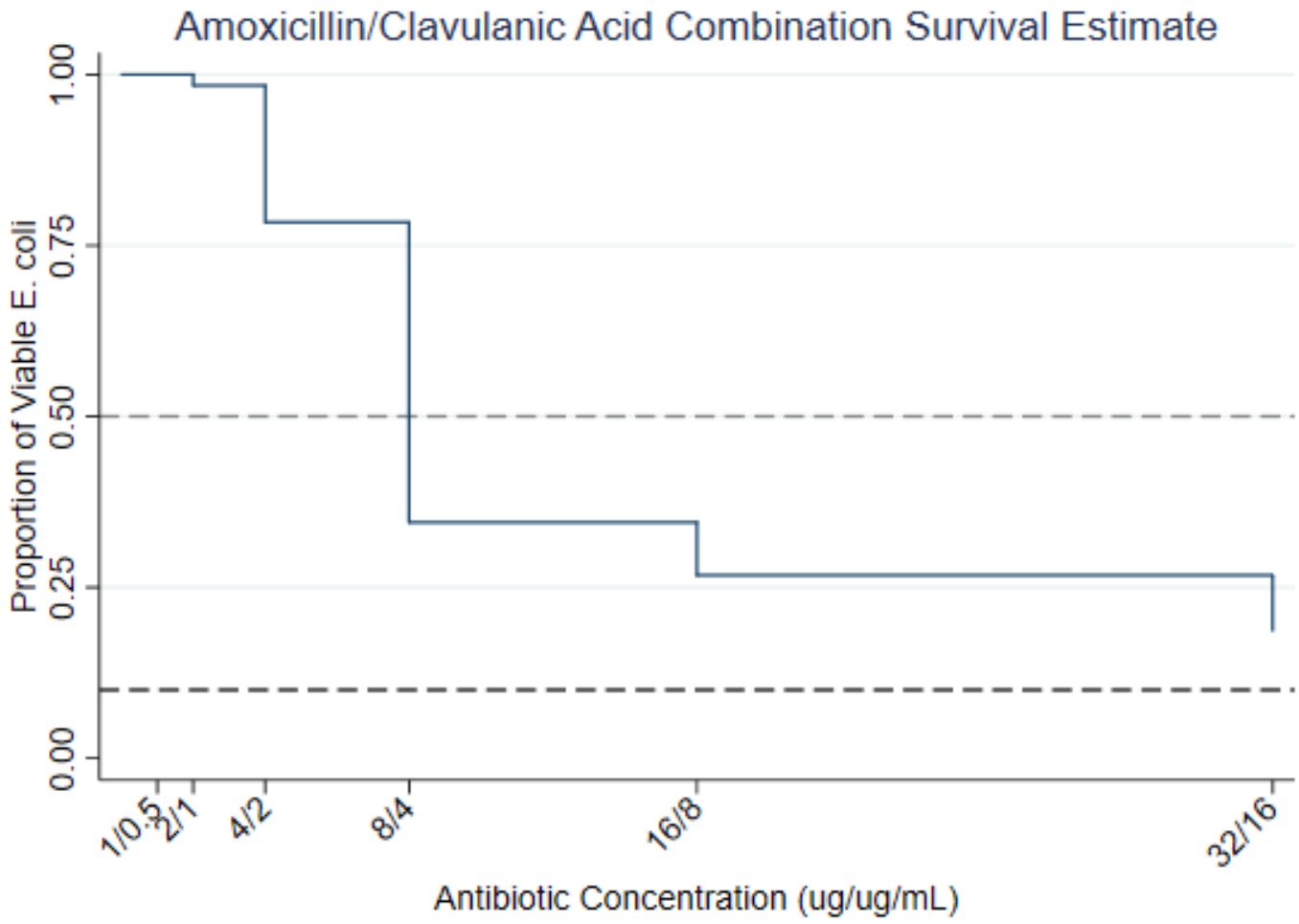

Figure 17: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to an amoxicillin and clavulanic acid combination. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

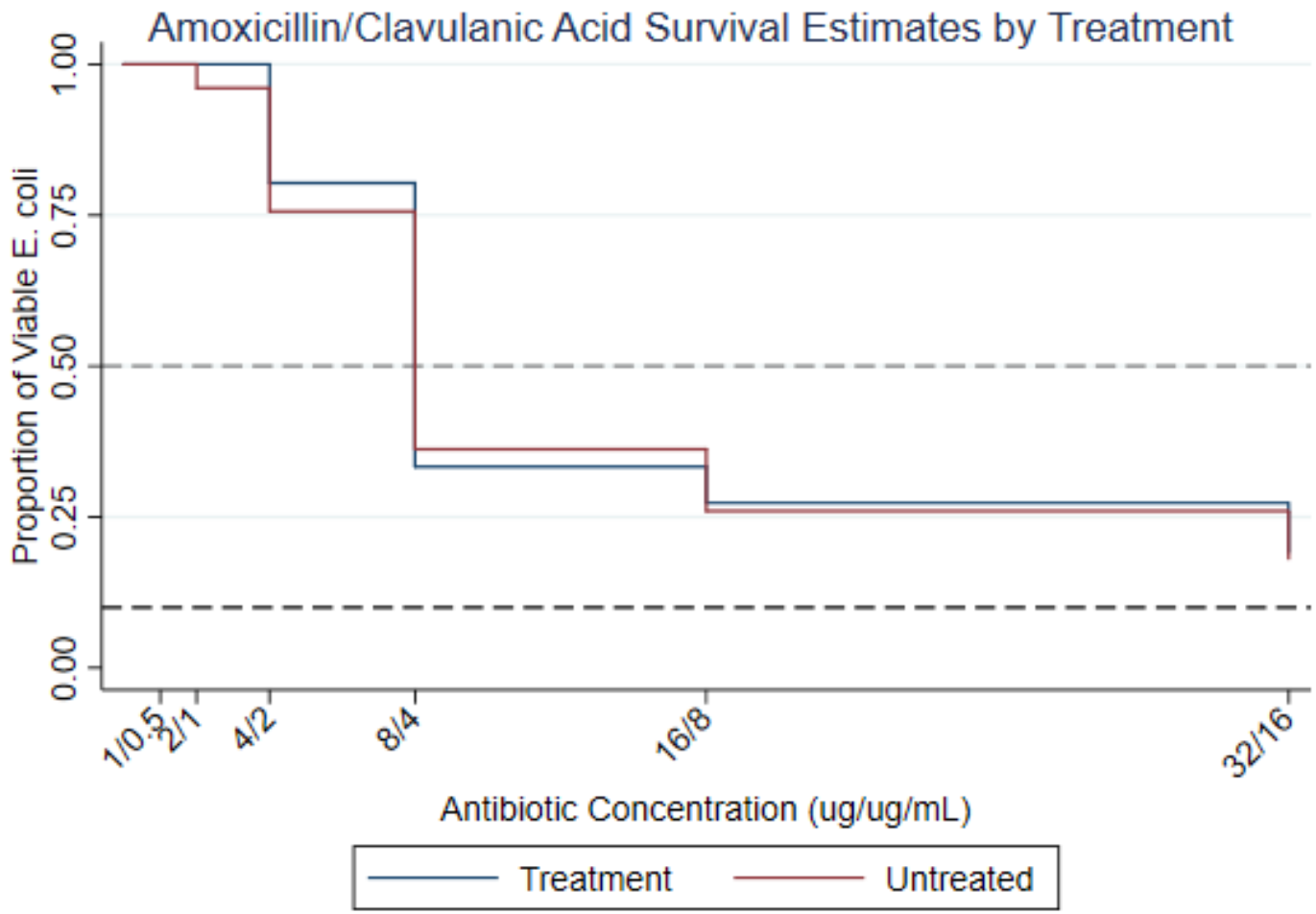

Figure 18: The Kaplan-Meier survival estimate is shown for all isolates based on treatment regarding the phenotypic MIC to an amoxicillin and clavulanic acid combination. The treatment group is represented by the blue line and the untreated red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

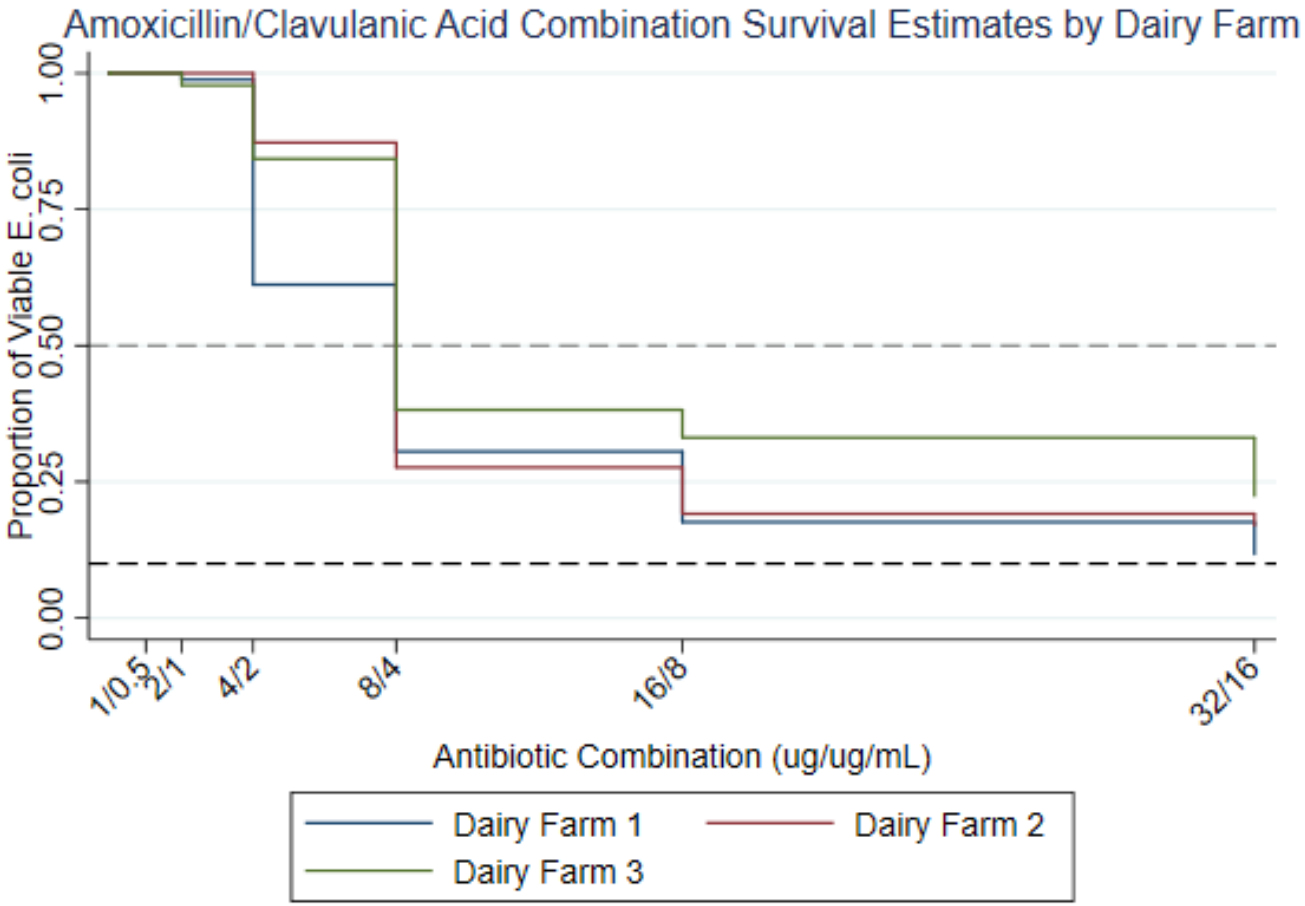

Figure 19: The Kaplan-Meier survival estimate is shown for isolates based on dairy farm regarding the phenotypic MIC to an amoxicillin and clavulanic acid combination. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

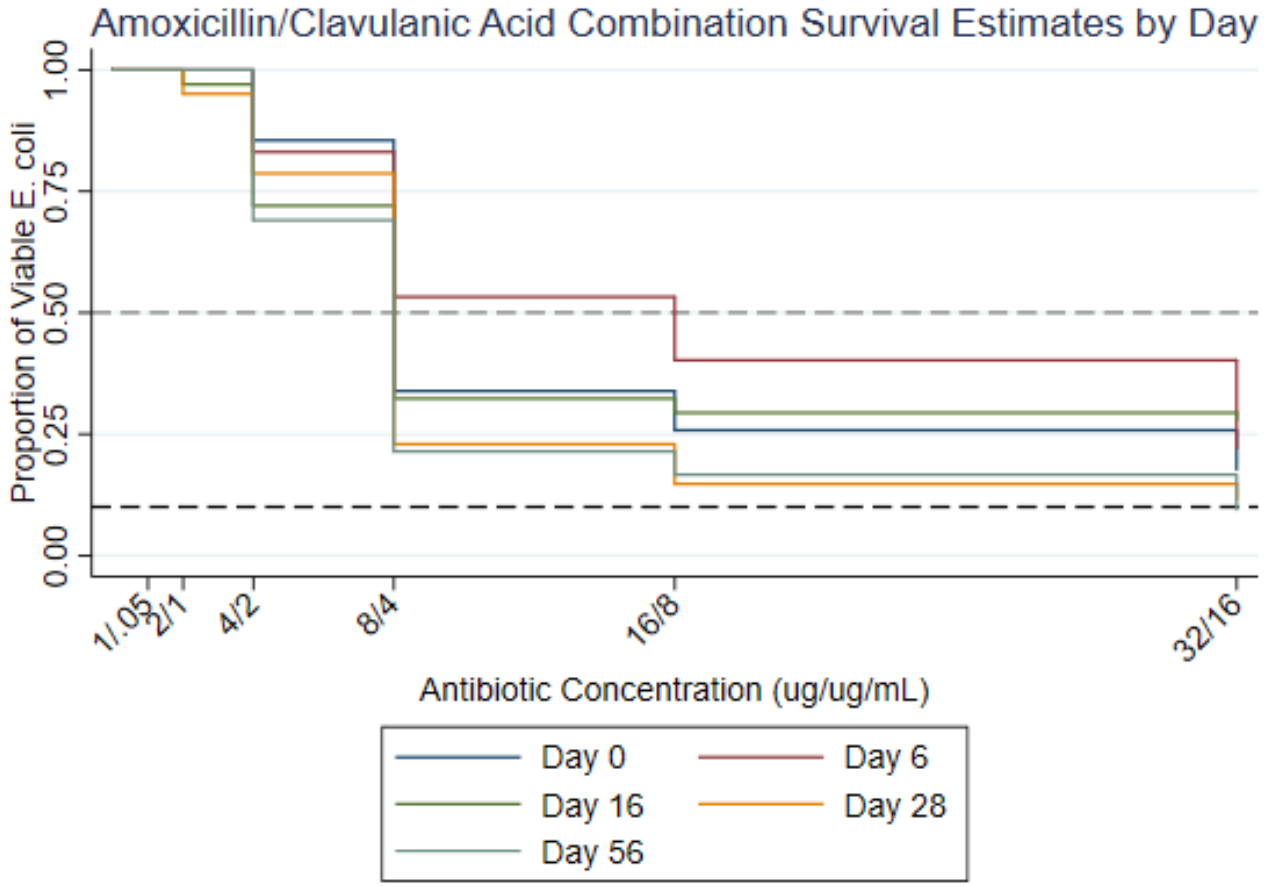

Figure 20: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to an amoxicillin and clavulanic acid combination. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

Regarding azithromycin, the greatest proportion of isolates in general were inhibited at a concentration of 4 µg/mL, which also happens to be the MIC 50 (Figure 21). Meanwhile, the MIC 90 was 16 µg/mL. It can be observed from Figure 22 the rate at which isolates from the treated and untreated groups reached their MIC was consistent, as indicated by a rate ratio of 0.983 (P = 0.8864, 95% CI: 0.773 – 1.250) and their MIC 50 and 90 was the same as the isolates in general. Yet again, the rate at which isolates were inhibited decreased from Dairy Farm 1 to Dairy Farm 2 and Dairy Farm 3, although not at levels of significance (Rate ratio = 0.873, P = 0.0639, 95% CI: 0.756 – 1.008). The MIC 50 of isolates from Dairy Farm 1 (4 µg/mL) was lower than that of isolates from Dairy Farms 2 and 3 (8 µg/mL) (Figure 23). While the MIC 90 of isolates from Dairy Farm 1 and 3 was 16 µg/mL, the MIC 90 of isolates from Dairy Farm 2 was greater than the 16 µg/mL limiting concentration of the plate. Dairy Farm 2 also maintained the highest proportion of isolates with MICs at or above 4 µg/mL. The rate ratio for isolates reaching their MIC with increases in day remained consistent at 1.004 (P = 0.2359, 95% CI: 0.997 – 1.011). However, the MIC 50 for isolates from day 6 was elevated to 8 µg/mL, while those from the other days was 4 µg/mL (Figure 24).

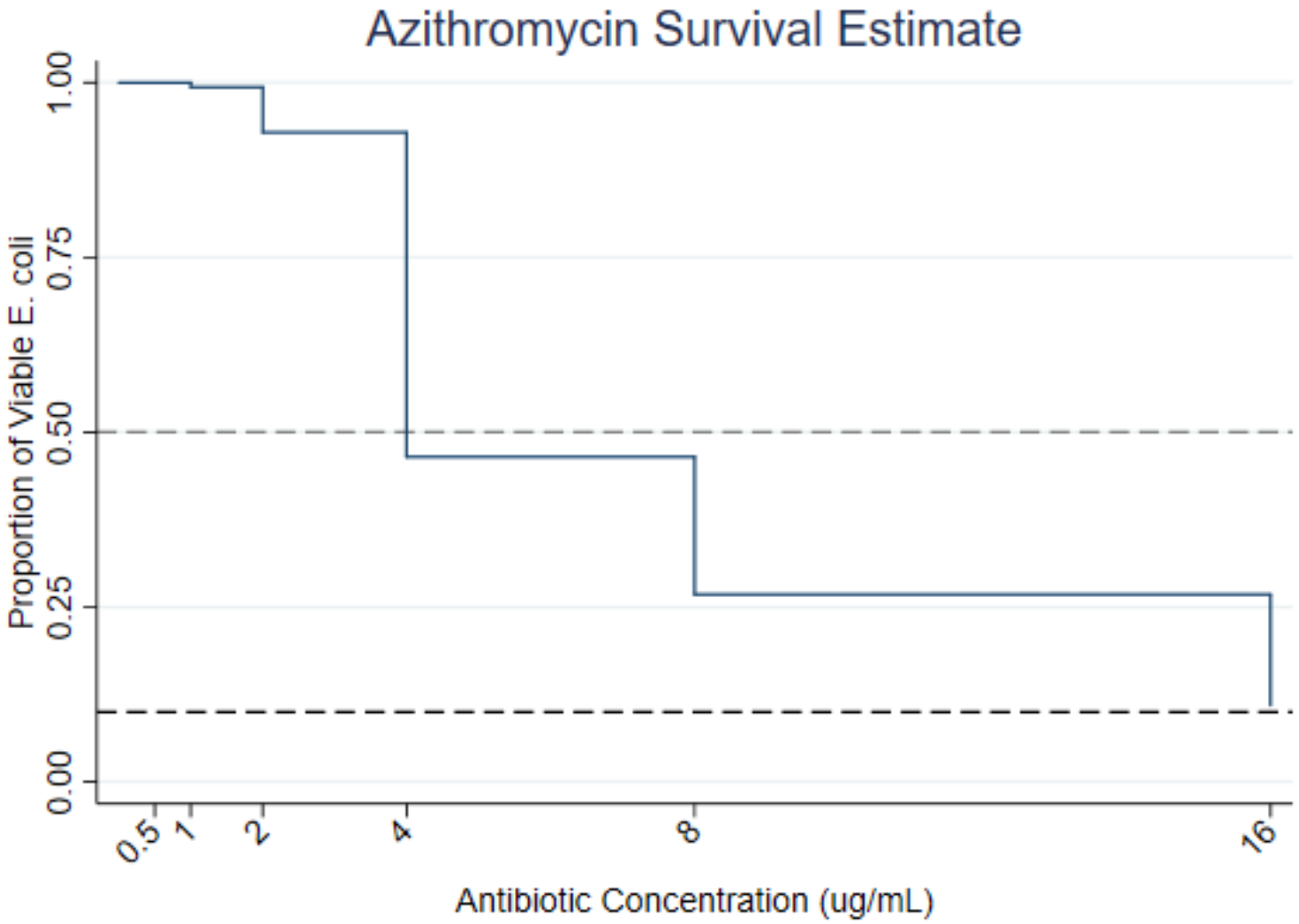

Figure 21: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to azithromycin. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

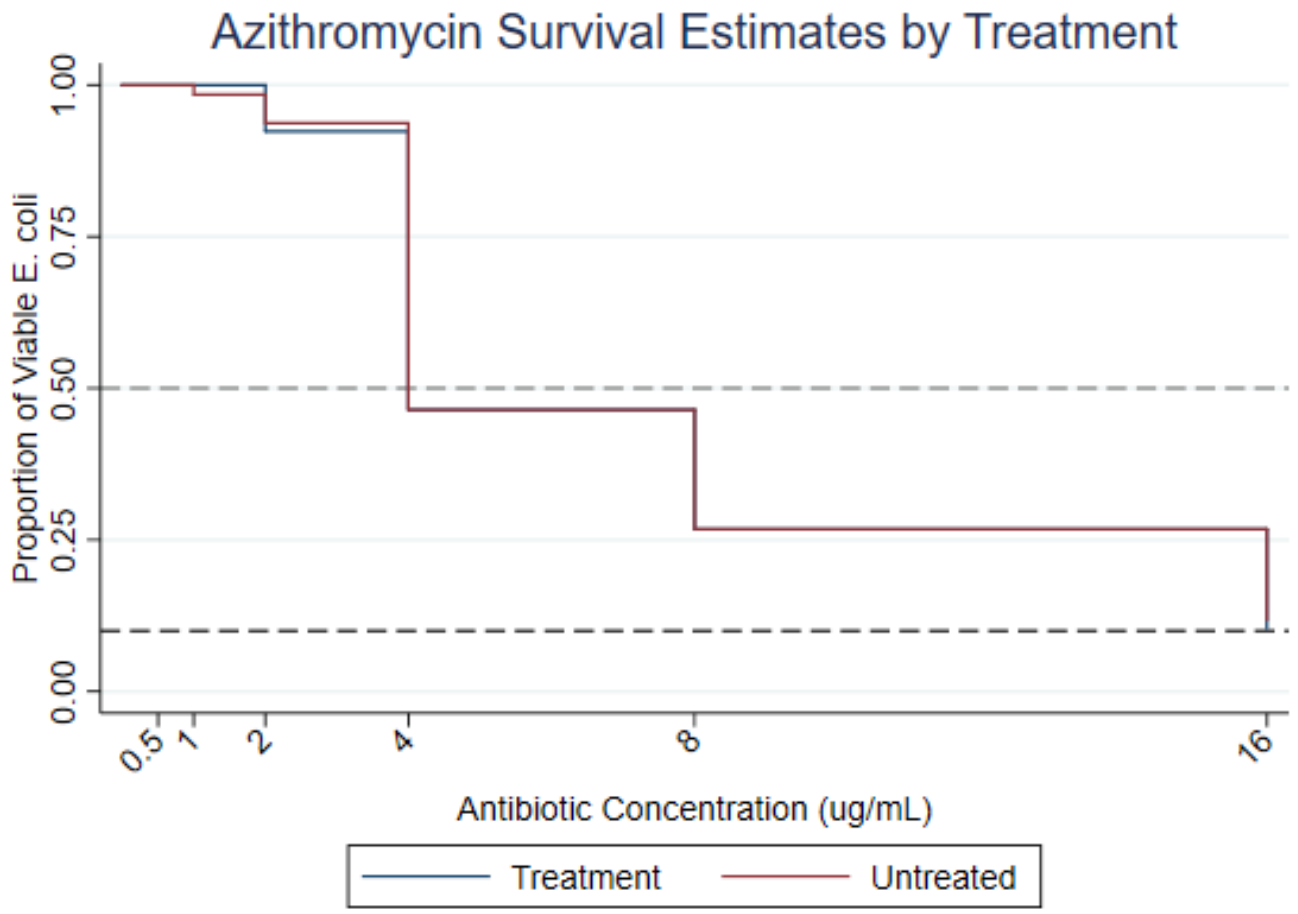

Figure 22: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to azithromycin. The treatment group is represented by the blue line and the untreated red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

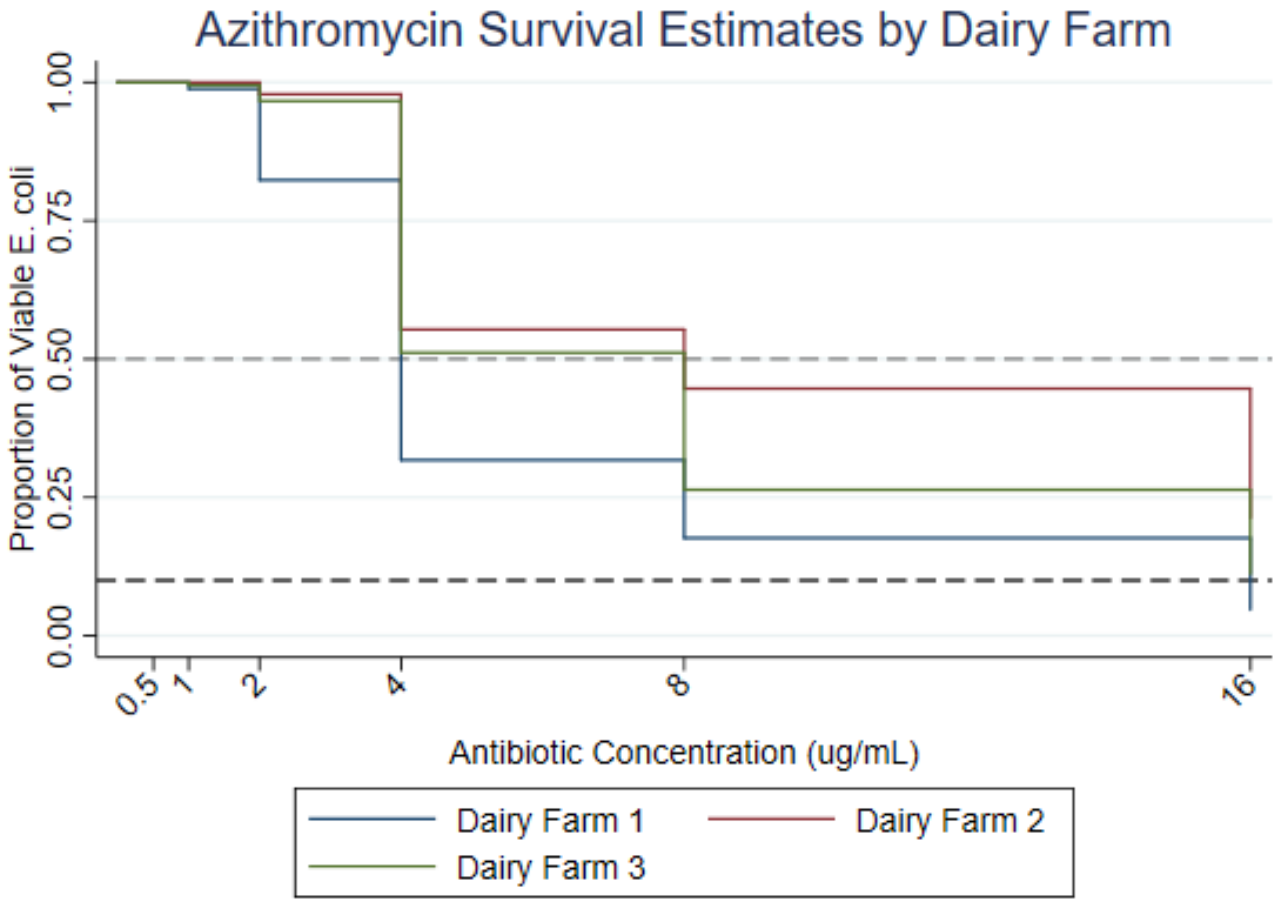

Figure 23: The Kaplan-Meier survival estimate is shown for isolates based on dairy farm regarding the phenotypic MIC to azithromycin. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

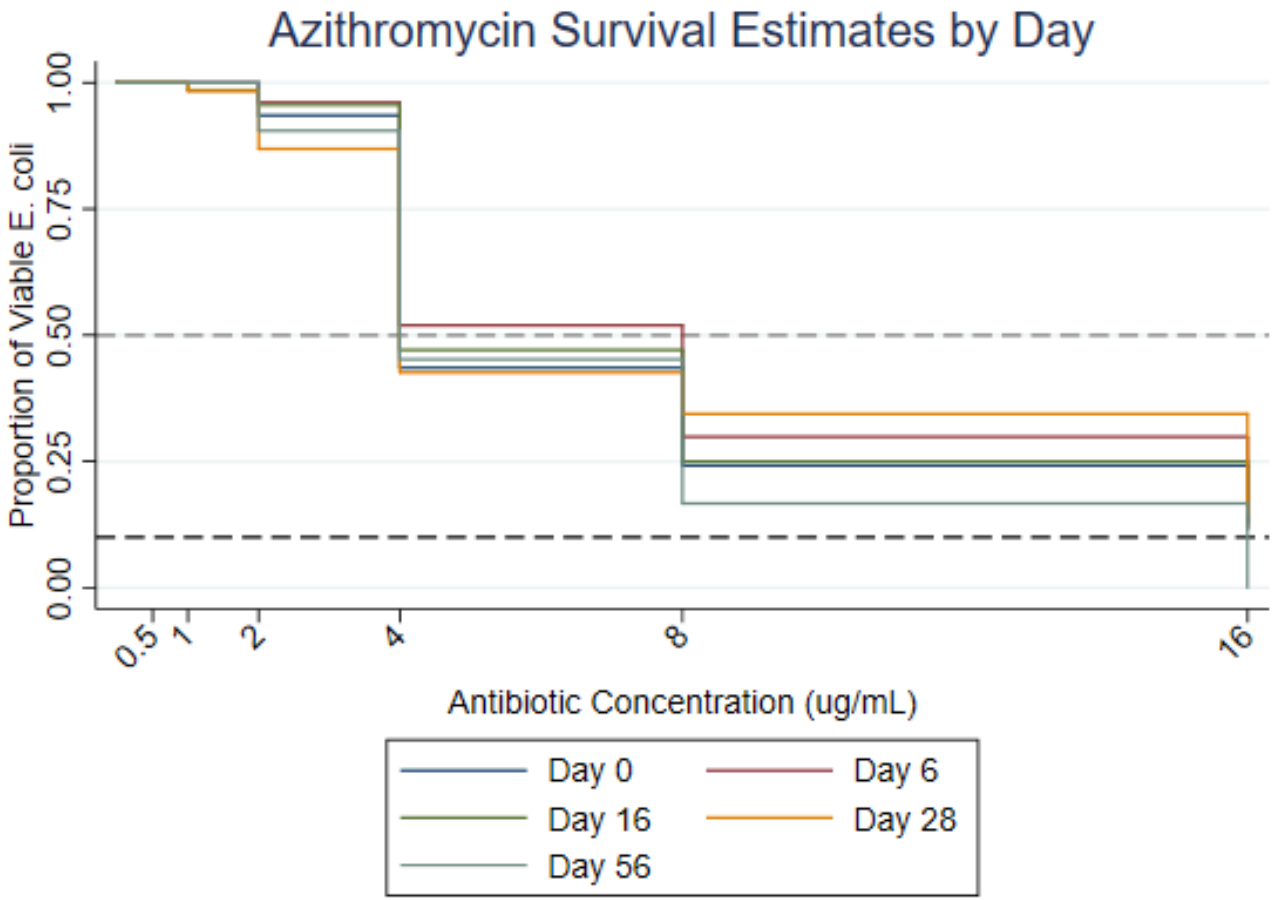

Figure 24: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to azithromycin. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

The MIC 50 of all isolates to cefoxitin is 8 µg/mL with an MIC 90 of greater than 32 µg/mL (Figure 25). While the MIC 50 for both the treatment and control groups were the same at 8 µg/mL and the MIC90 was greater than 32 µg/mL, there was a greater proportion of isolates in the treated group with MICs above 8 µg/mL than the control group (Figure 26). The rate ratio increases slightly to 1.164 in the control group (P = 0.2554, 95% CI: 0.896 – 1.513). Similar to treatment, the MIC 50 and 90 maintained the same at 8 µg/mL and greater than 32 µg/mL; however, the proportion of isolates from Dairy Farm 3 was consistently greater than those of Dairy Farm 1 and 2 (Figure 27). This was evidenced by a decrease in the rate ratio of 0.767 between Dairy Farms 1 and 2 and Dairy Farms 2 and 3 (P = 0.0007, 95% CI: 0.658 – 0.894). Much like what was observed in the amoxicillin/clavulanic acid combination, the rate ratio slightly increased by sample day at 1.015 (P = 0.0003, 95% CI: 1.007 – 1.023). Results from days 28 and 56 mirrored each other as do those of day 0 and 16 with a greater proportion of isolates from day 6 having an MIC greater than 4 µg/mL in comparison to the other 4 sampling days (Figure 28). The MIC 50 for days 28 and 56 was 4 µg/mL, while the MIC 50 for the other sampling days was 8 µg/mL. The MIC 90 for isolates from all days was greater than 32 µg/mL.

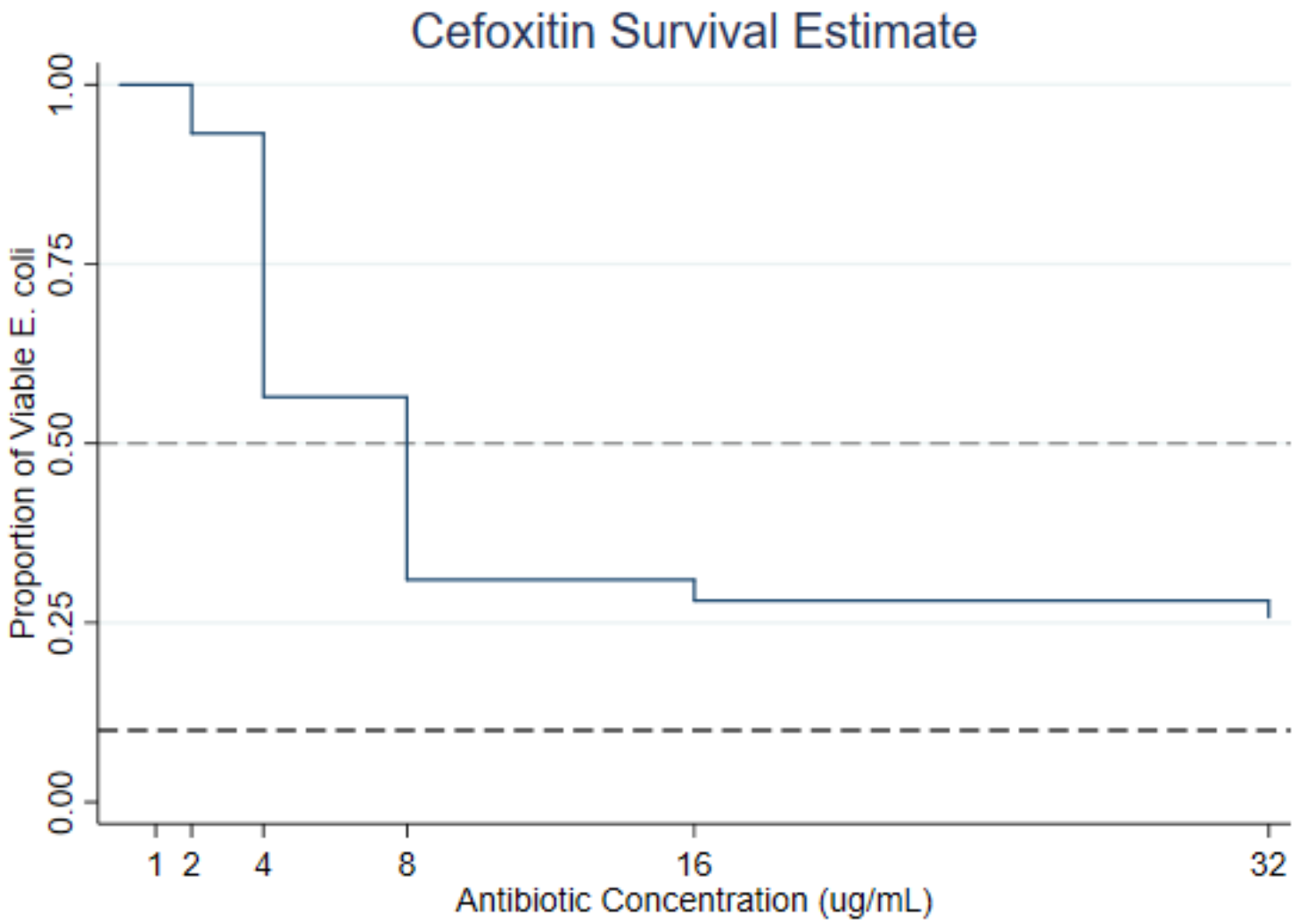

Figure 25: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to cefoxitin. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

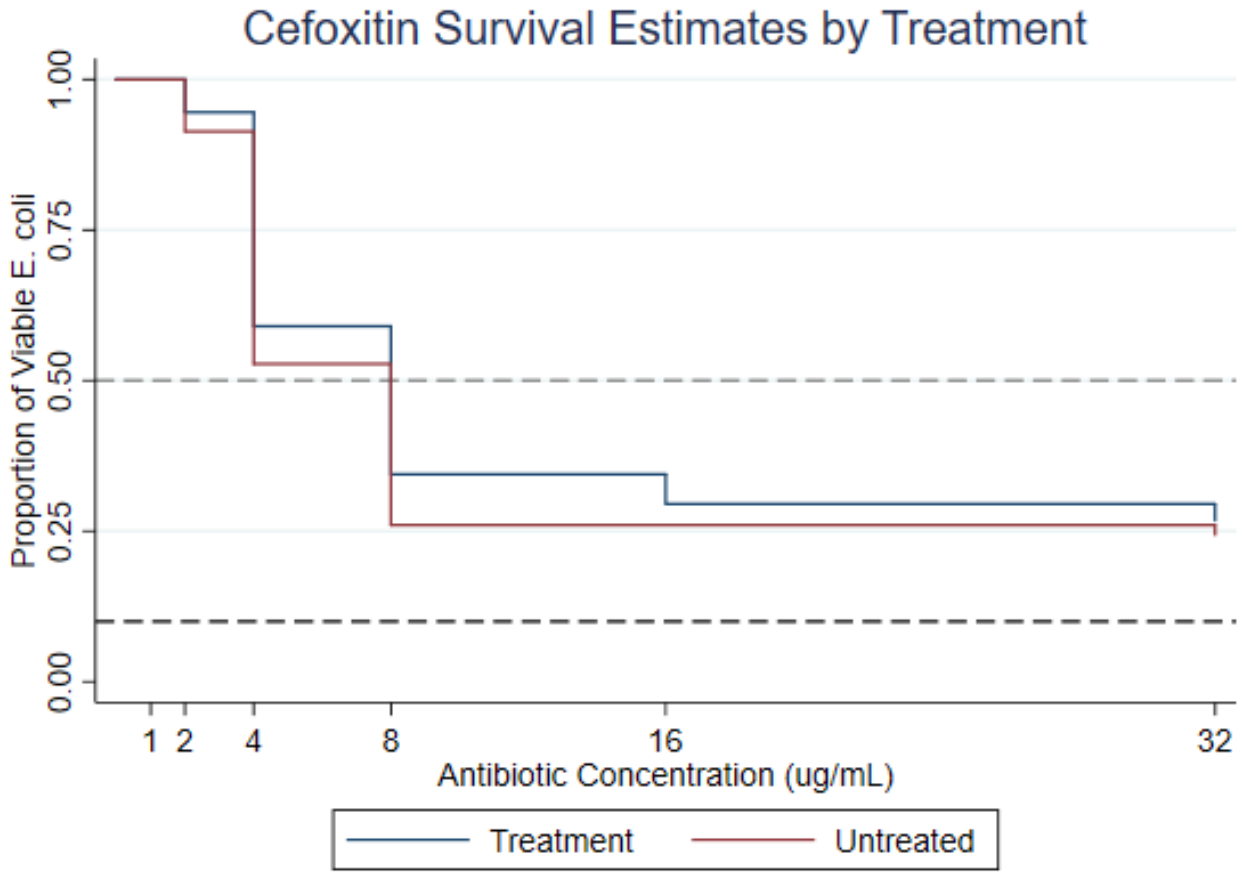

Figure 26: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to cefoxitin. The treatment group is represented by the blue line and the control red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

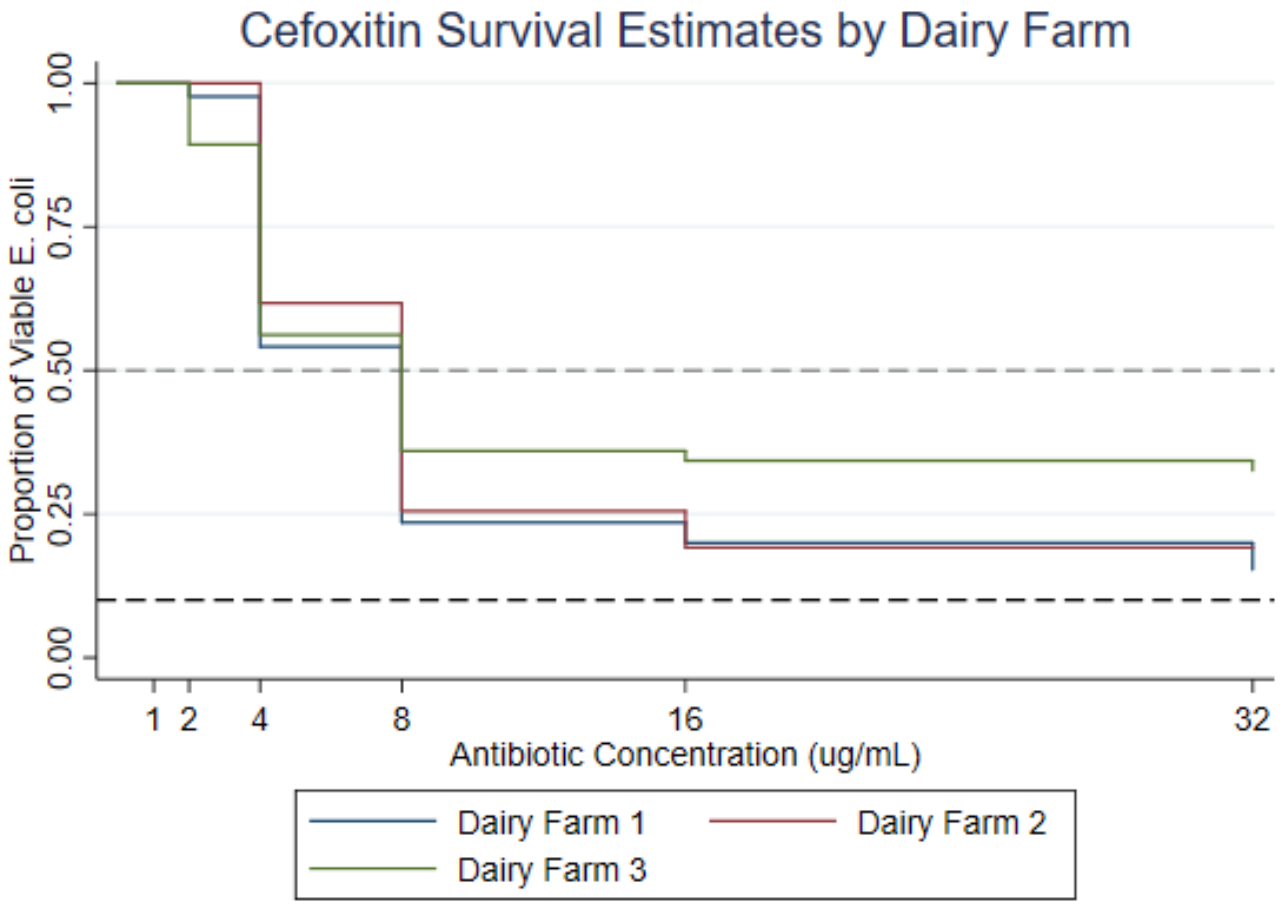

Figure 27: The Kaplan-Meier survival estimate is shown for isolates based on dairy farm regarding the phenotypic MIC to cefoxitin. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

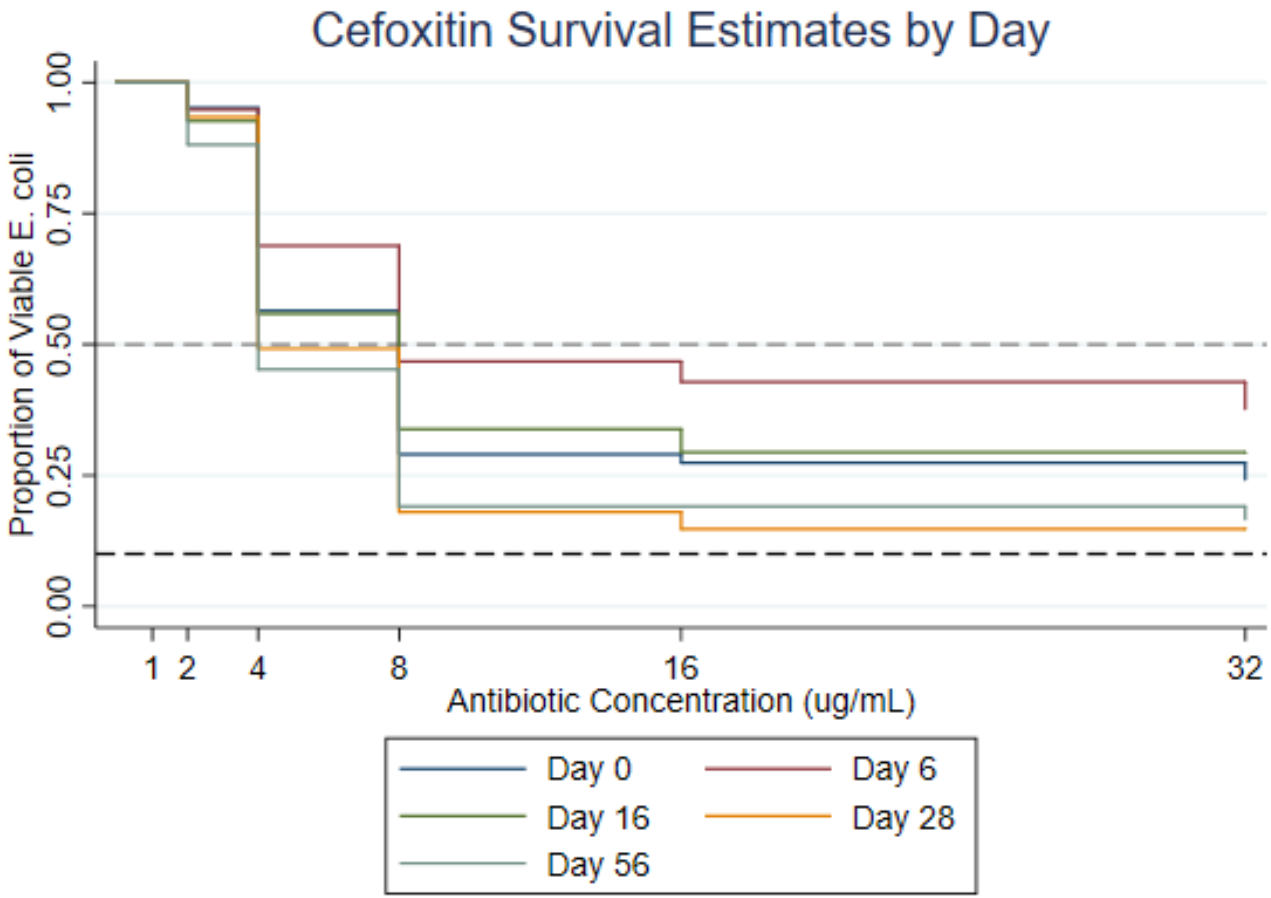

Figure 28: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to cefoxitin. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

In general, 50 and 90% of the isolates had a ceftiofur MIC greater than 8 µg/mL (Figure 29), which was consistent regardless of treatment, farm, or day (Figure 29 - 37). While treatment was associated with an increased Mantel-Haenszel rate ratio by 1.166 in the control group (P = 0.6910, 95% CI: 0.546 – 2.492), a rate ratio decrease of 0.786 (P = 0.2753, 95% CI: 0.510 – 1.212) between Dairy Farms 1 and 2 and Dairy Farms 2 and 3, and a slightly decreased rate ratio of 0.993 (P = 0.5315, 95% CI: 0.973 – 1.014) with an increase in sampling day, none of those changes are significant at a P-value of 0.05.

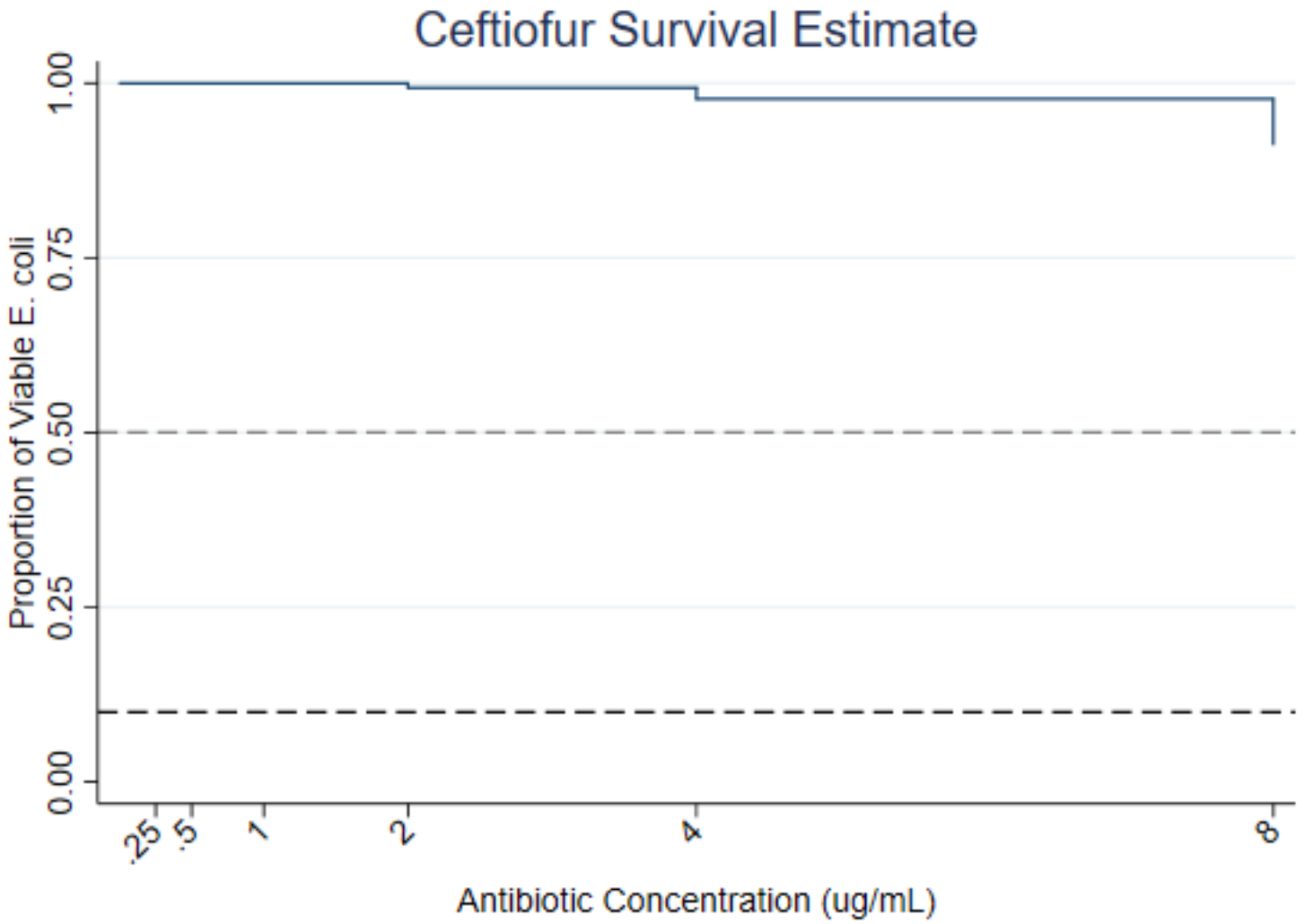

Figure 29: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to ceftiofur. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

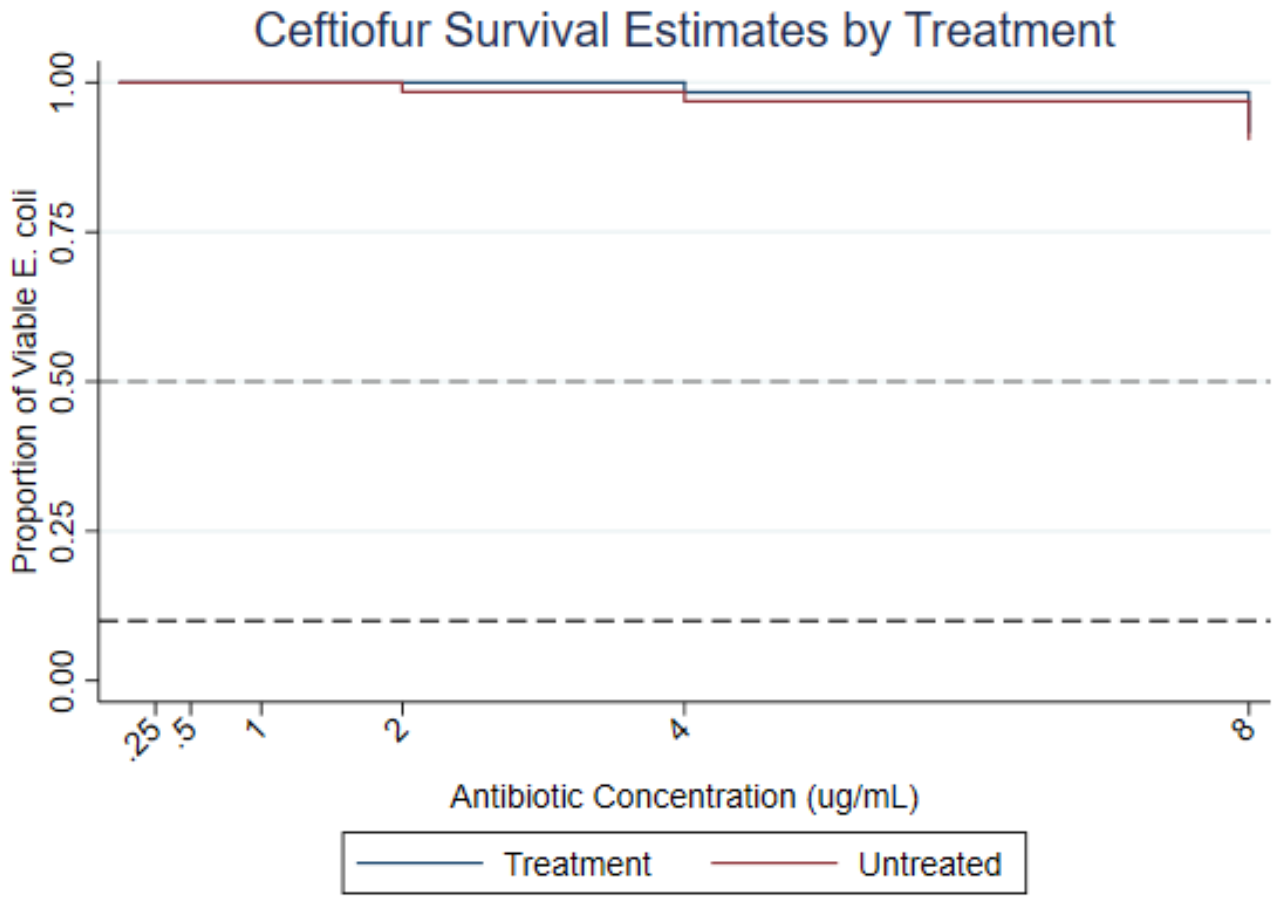

Figure 30: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to ceftiofur. The treatment group is represented by the blue line and the untreated red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

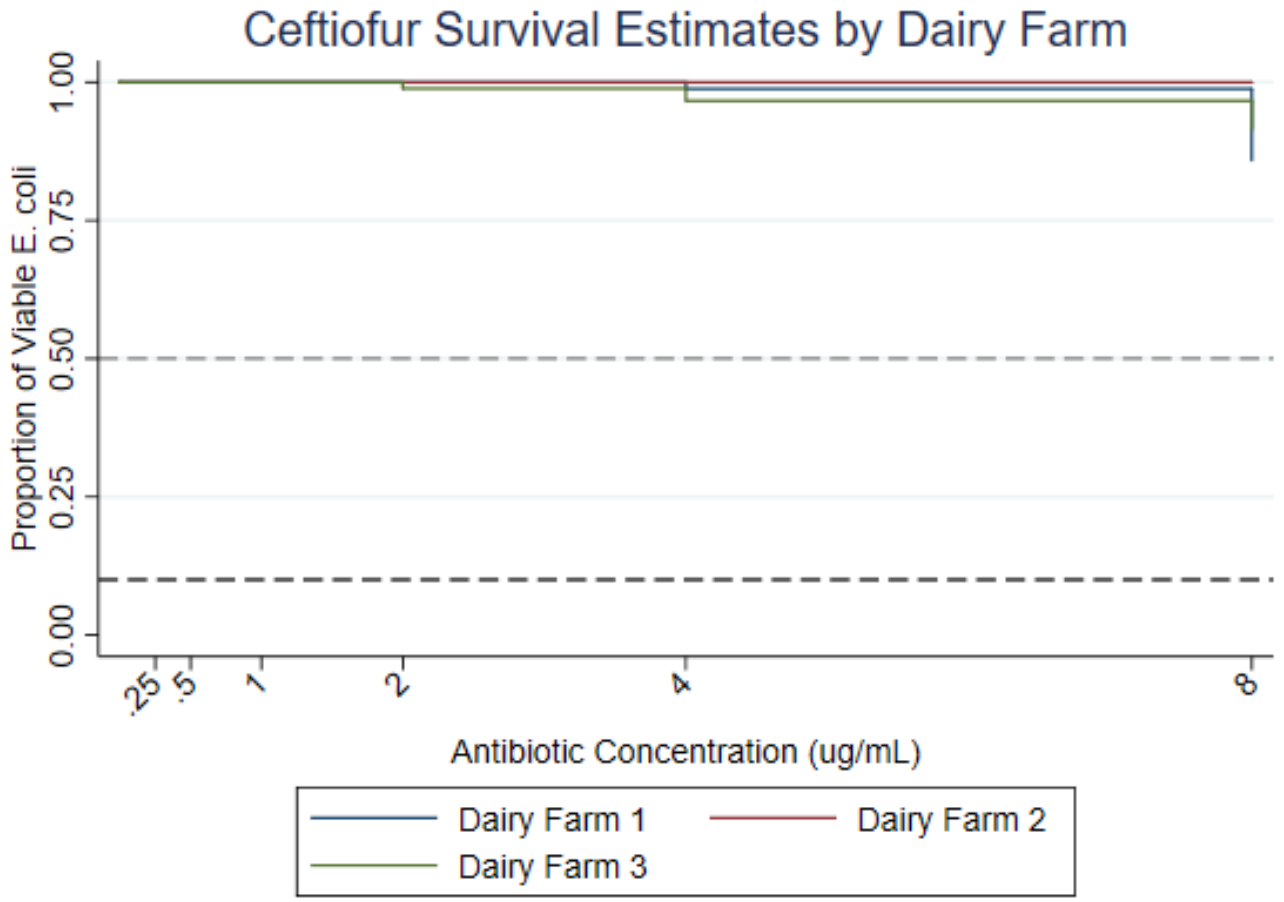

Figure 31: The Kaplan-Meier survival estimate is shown for isolates based on dairy farm regarding the phenotypic MIC to ceftiofur. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

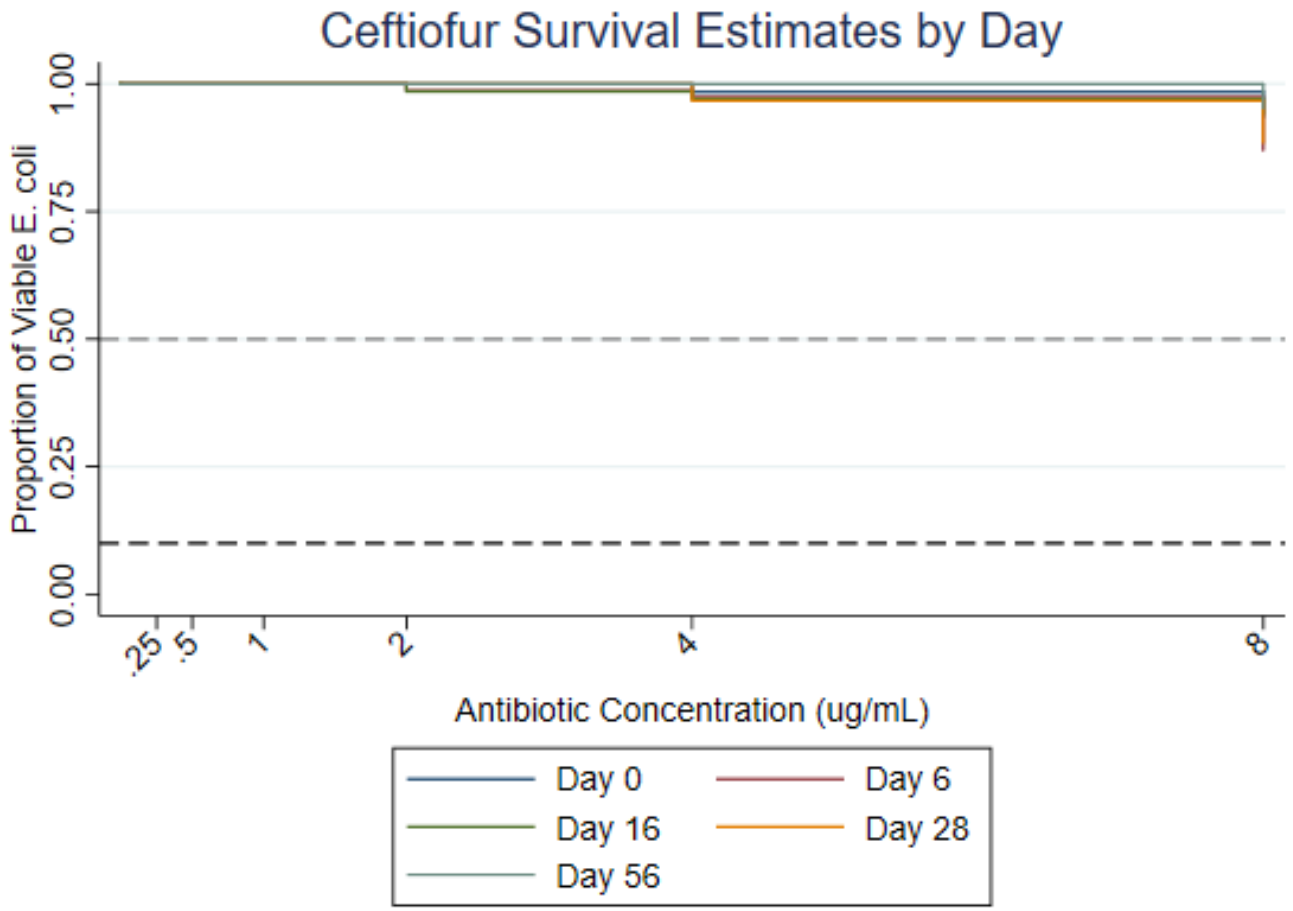

Figure 32: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to ceftiofur. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

The MIC 50 for isolates regardless of treatment, dairy farm, or day was 64 µg/mL for ceftriaxone with an MIC 90 greater than the 64 µg/mL limitation of the plate (Figure 33). The Mantel-Haenszel rate ratio increased by 1.139 (P = 0.833, 95% CI: 0.833 – 1.557) in the control group, but the greatest differences among the groups was observed prior to the MIC of 32 µg/mL (Figure 34). The MIC 50 and 90 remained the same for the treated and control group as is the general isolates regardless of grouping. The rate ratio decreased between Dairy Farms 1, 2, and 3 by 0.736 (P = 0.0011, 95% CI: 0.613 – 0.885). The MIC 50 of isolates from Dairy Farms 1 and 3 was 64 µg/mL with an MIC 90 greater than 64 µg/mL, whereas both the MIC 50 and 90 were greater than 64 µg/mL among Dairy Farm 2 isolates (Figure 35). Dairy Farm 2 maintained a higher proportion of isolates with an MIC greater than 16 µg/mL in comparison to the other dairy farms. The rate ratio relating to day maintained consistent as day increased (Rate ratio = 1.001, P = 0.8620, 95% CI: 0.992 – 1.009) (Figure 36). Interestingly, isolates from days 6 and 28 had MIC 50s of 64 µg/mL, but isolates from other days had an MIC 50 greater than 64 µg/mL.

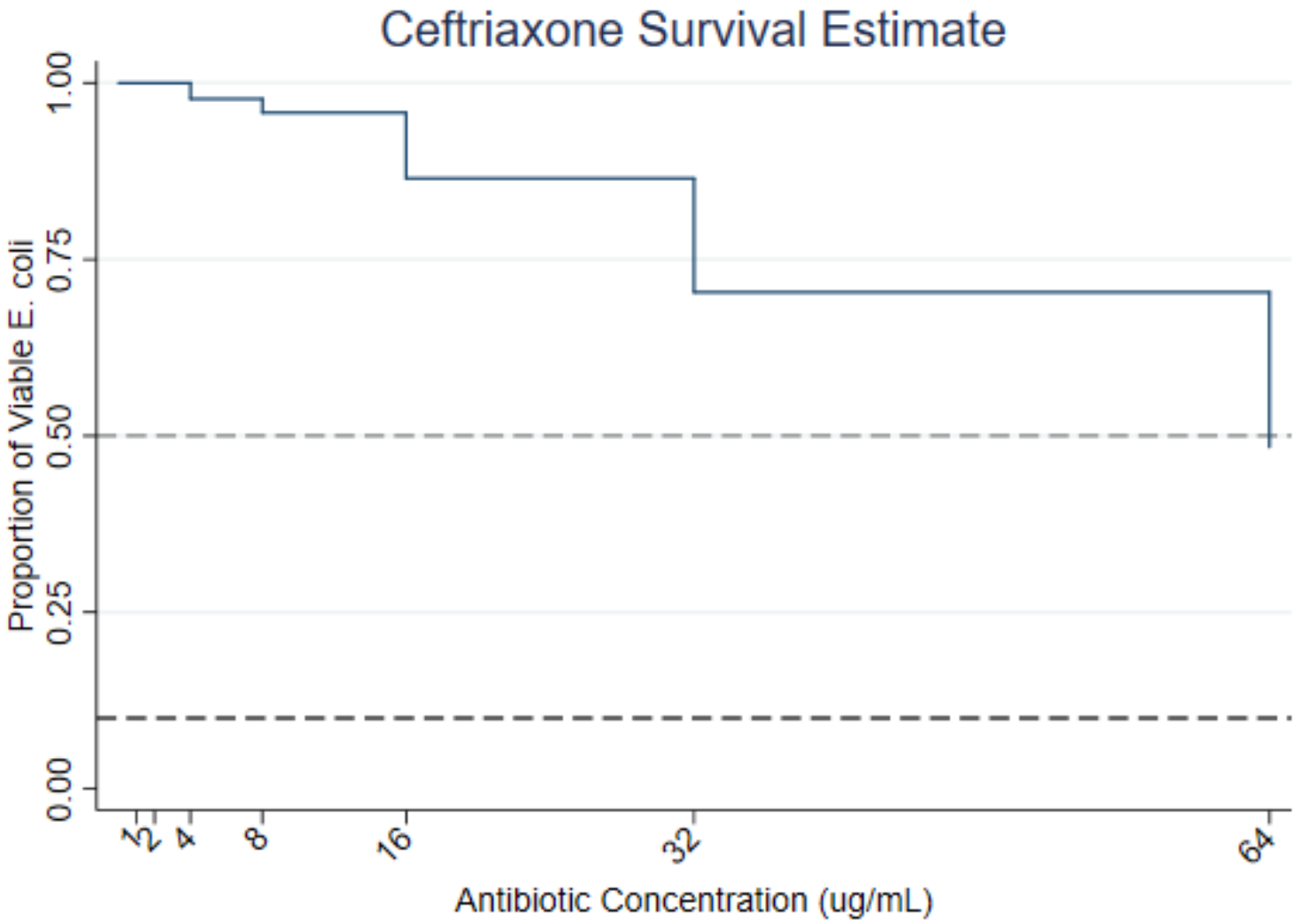

Figure 33: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to ceftriaxone. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

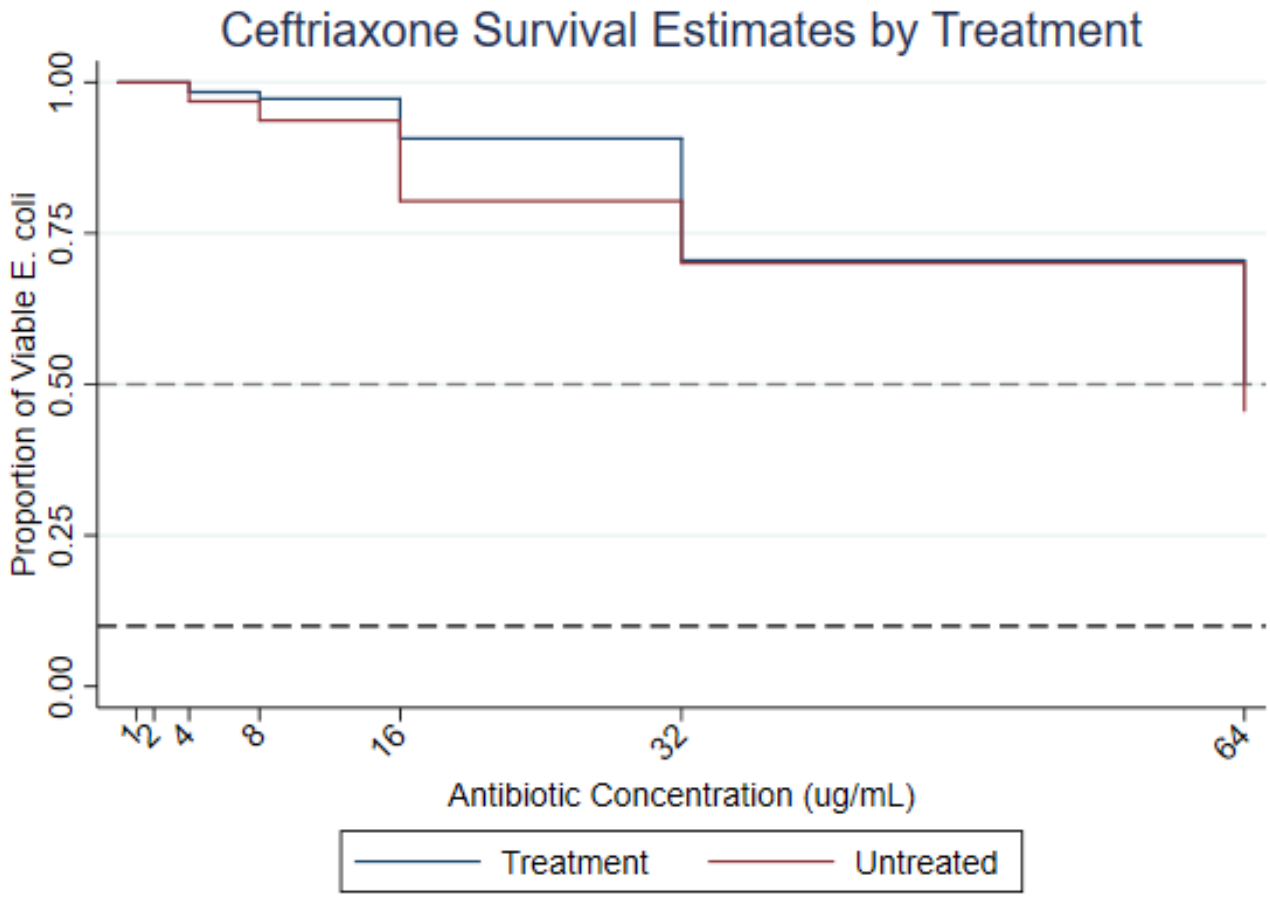

Figure 34: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to ceftriaxone. The treatment group is represented by the blue line and the untreated red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

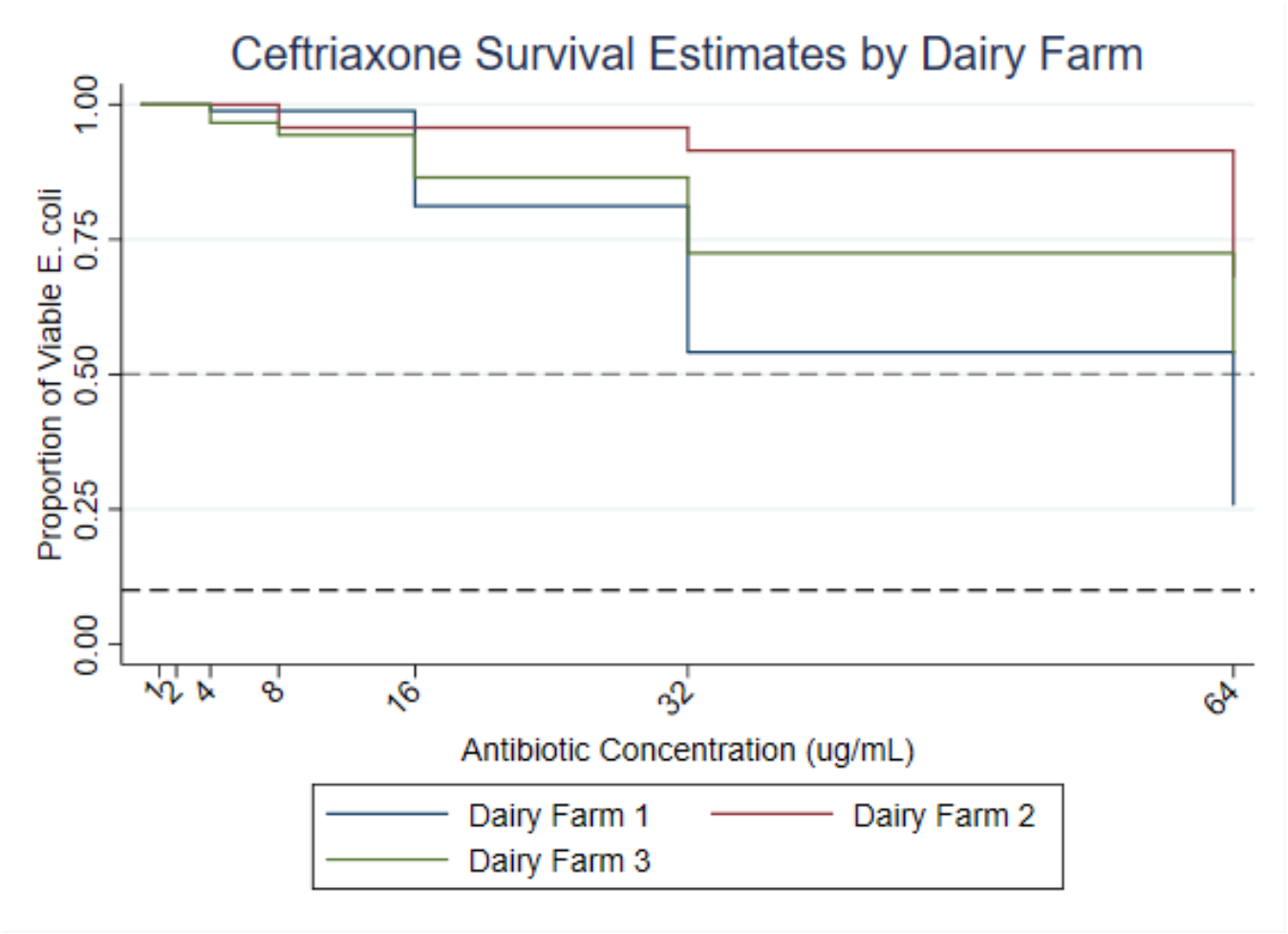

Figure 35: The Kaplan-Meier survival estimate is shown for isolates based on farm regarding the phenotypic MIC to ceftriaxone. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

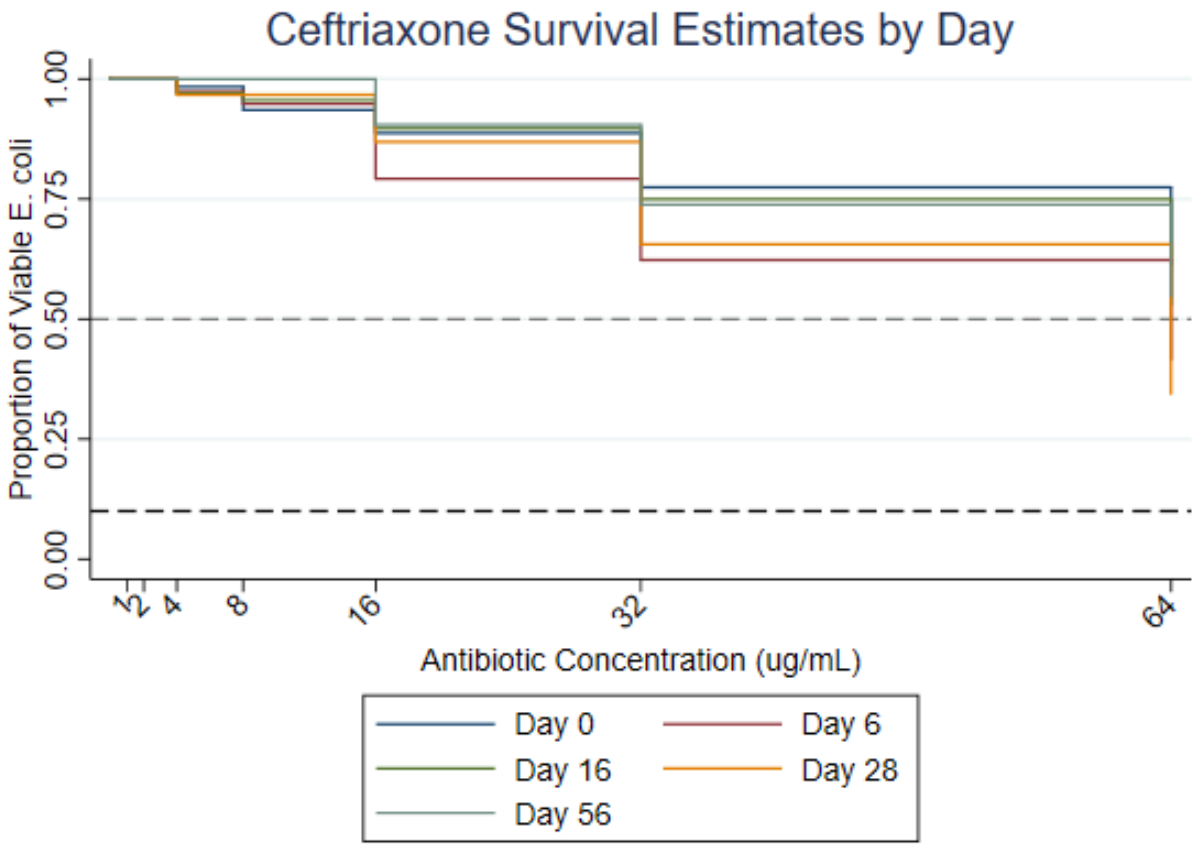

Figure 36: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to ceftriaxone. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

The MIC 50 of isolates to chloramphenicol was 16 µg/mL with an MIC 90 of 32 µg/mL (Figure 37). The rate ratio remained the same (Rate ratio = 1.000, *P* = 0.9981, 95% CI: 0.773 – 1.366) regardless of treatment or control group with MIC 50 and 90 the same as the isolates in general (Figure 38). The Mantel-Haenszel rate ratio decreased by 0.827 (*P* = 0.0435, 95% CI: 0.688 – 0.994) between the 3 dairies. Chloramphenicol is the first antibiotic which had a different MIC 50 for each of their isolate groups. The MIC 50 for isolates to Dairy Farm 1 was 8 µg/mL, 16 µg/mL for Dairy Farm 3, and greater than 32 µg/mL for Dairy Farm 2 (Figure 39). The MIC 90 for isolates was greater than 32 µg/mL for all farm groups. Increases in sample day did not lead to any variance in the rate ratio (Rate ratio = 1.000, *P* = 0.733, 95% CI: 0.733 – 1.366). The MIC 50 tended to vary by day while the MIC 90 was greater than 32 µg/mL (Figure 40). The MIC 50 for isolates from day 0 and 16 was 8 µg/mL, 16 µg/mL for day 6, greater than 32 µg/mL for isolates from days 28 and 56.

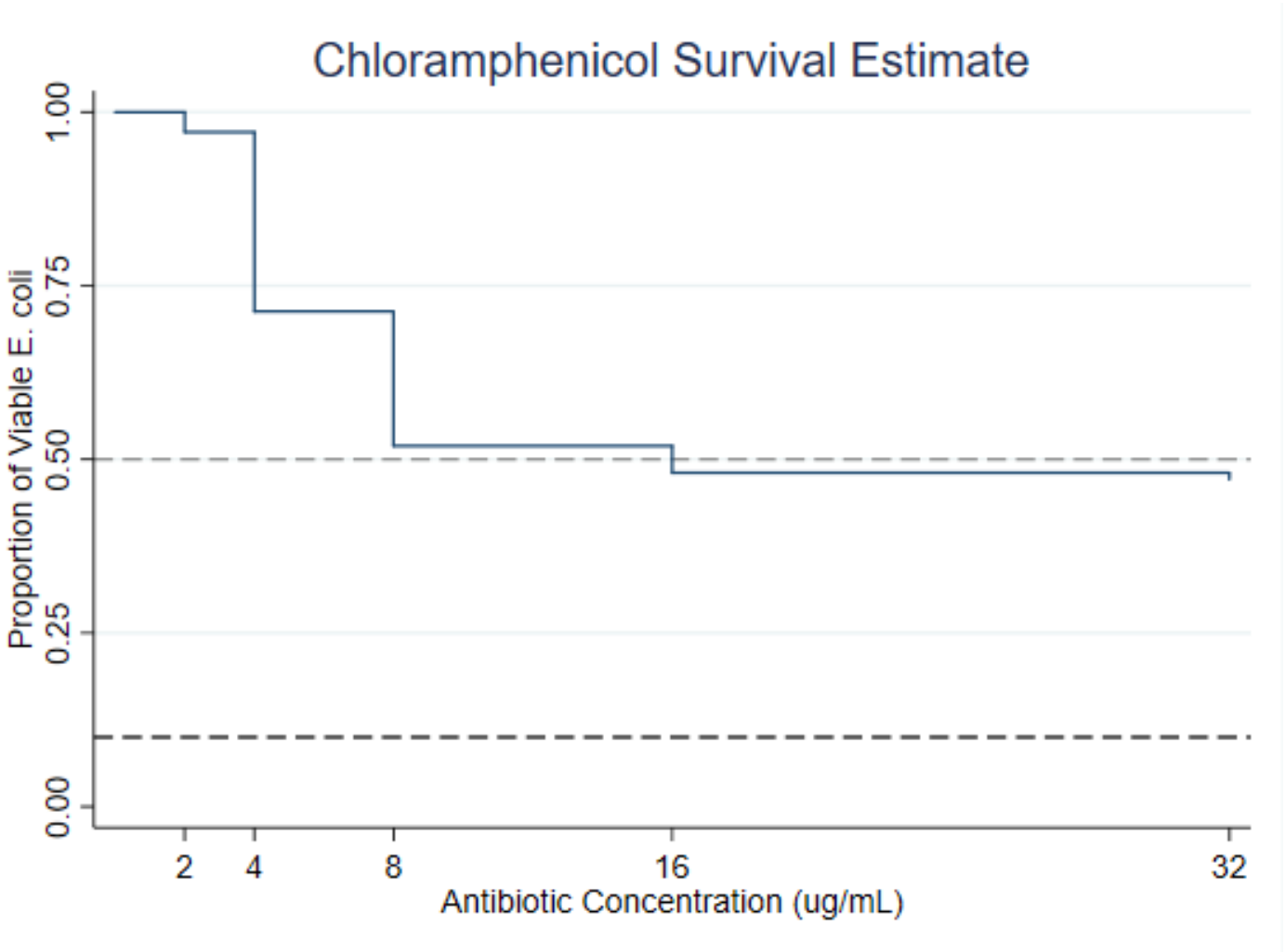

Figure 37: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to chloraphenicol. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

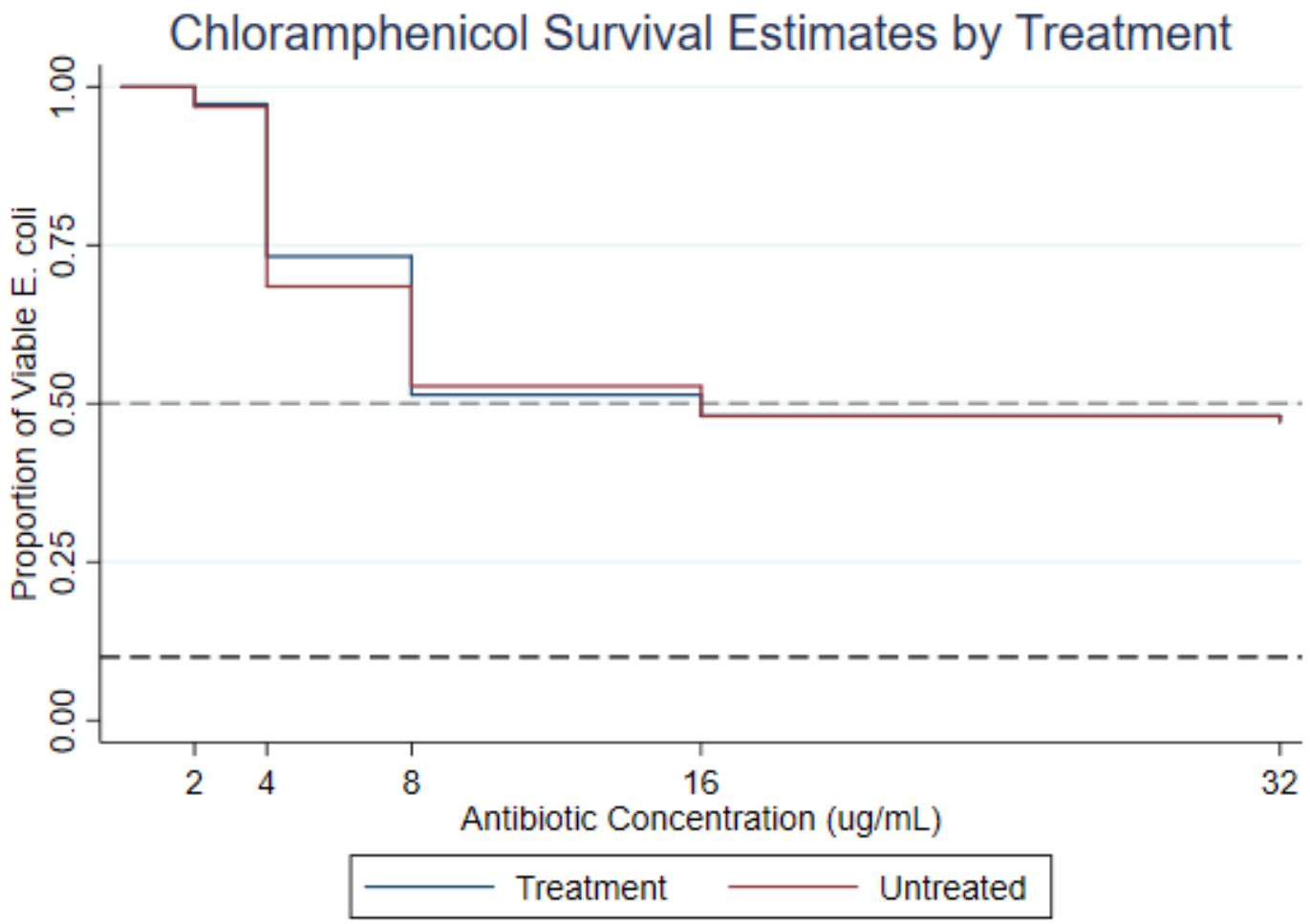

Figure 38: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to chloramphenicol. The treatment group is represented by the blue line and the control red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

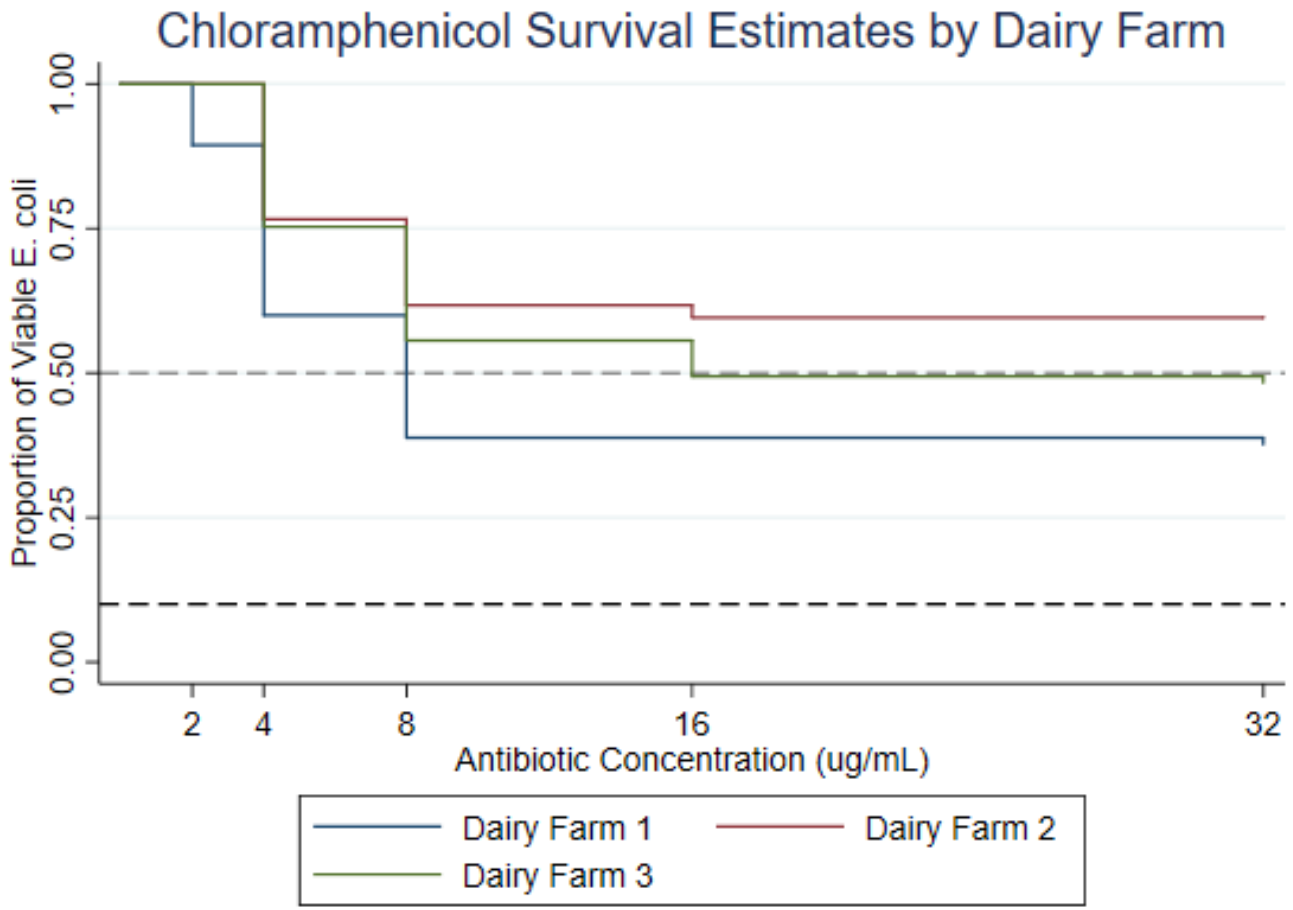

Figure 39: The Kaplan-Meier survival estimate is shown for isolates based on dairy farm regarding the phenotypic MIC to chloramphenicol. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

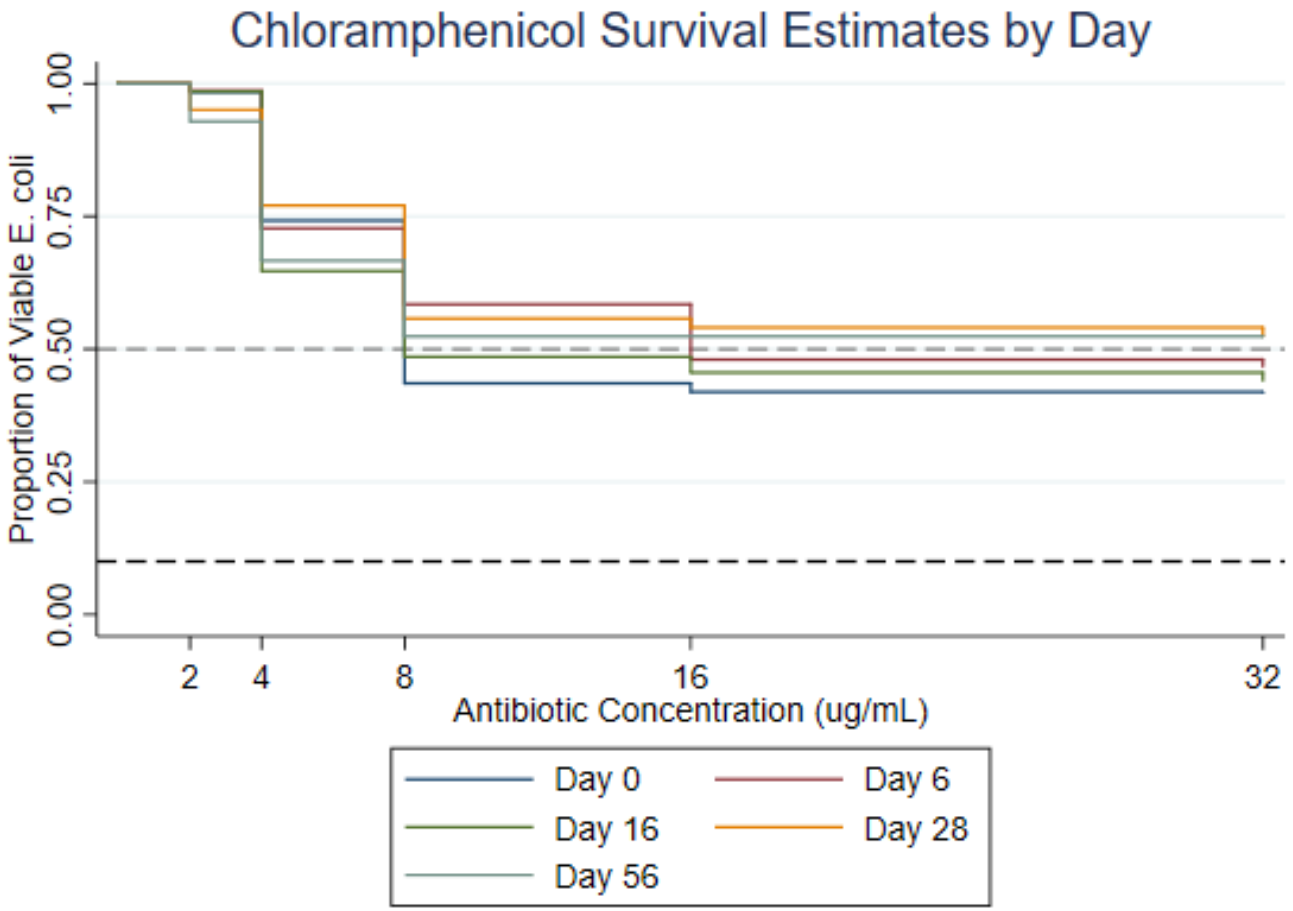

Figure 40: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to chloramphenicol. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

The MIC 50 for ciprofloxacin among all isolates from the CHROM-ESBL agar was 0.015 µg/mL with an MIC 90 of 1 µg/mL (Figure 41). The Mantel-Haenszel rate ratio decreased insignificantly by 0.801 (P = 0.632, 95% CI: 0.632 -1.015) in the control group, but the main difference was observed in the MIC 90. The MIC 50 was 0.015 µg/mL for isolates regardless of group, whereas the MIC 90 for isolates in the treated group was 1 µg/mL and greater than 4 µg/mL in the untreated group (Figure 42). The rate ratio decreased by 0.410 (P = 0.0000, 95% CI: 0.347 – 0.486) from Dairy Farm 1 to 2 to 3, but was impacted largely by Dairy Farm 3. The MIC 50 for isolates from both Dairy Farm 1 and 3 was 0.015 µg/mL and 0.5 µg/mL for Dairy Farm 2; however, this was also the MIC 90 for Dairy 2, whereas the MIC 90 for isolates from Dairy Farm 1 was 1 µg/mL and greater than 4 on Dairy Farm 3 (Figure 43). The rate ratio slightly decreased by 0.980 (P = 0.0000, 95% CI: 0.974 – 0.986) as day increased. This observation was most noticeable among the proportion of isolates with an MIC of 1 µg/mL or greater (Figure 44). At these MICs, day 56 had the highest proportion of isolates followed by day 28, 16, 0, and 6.

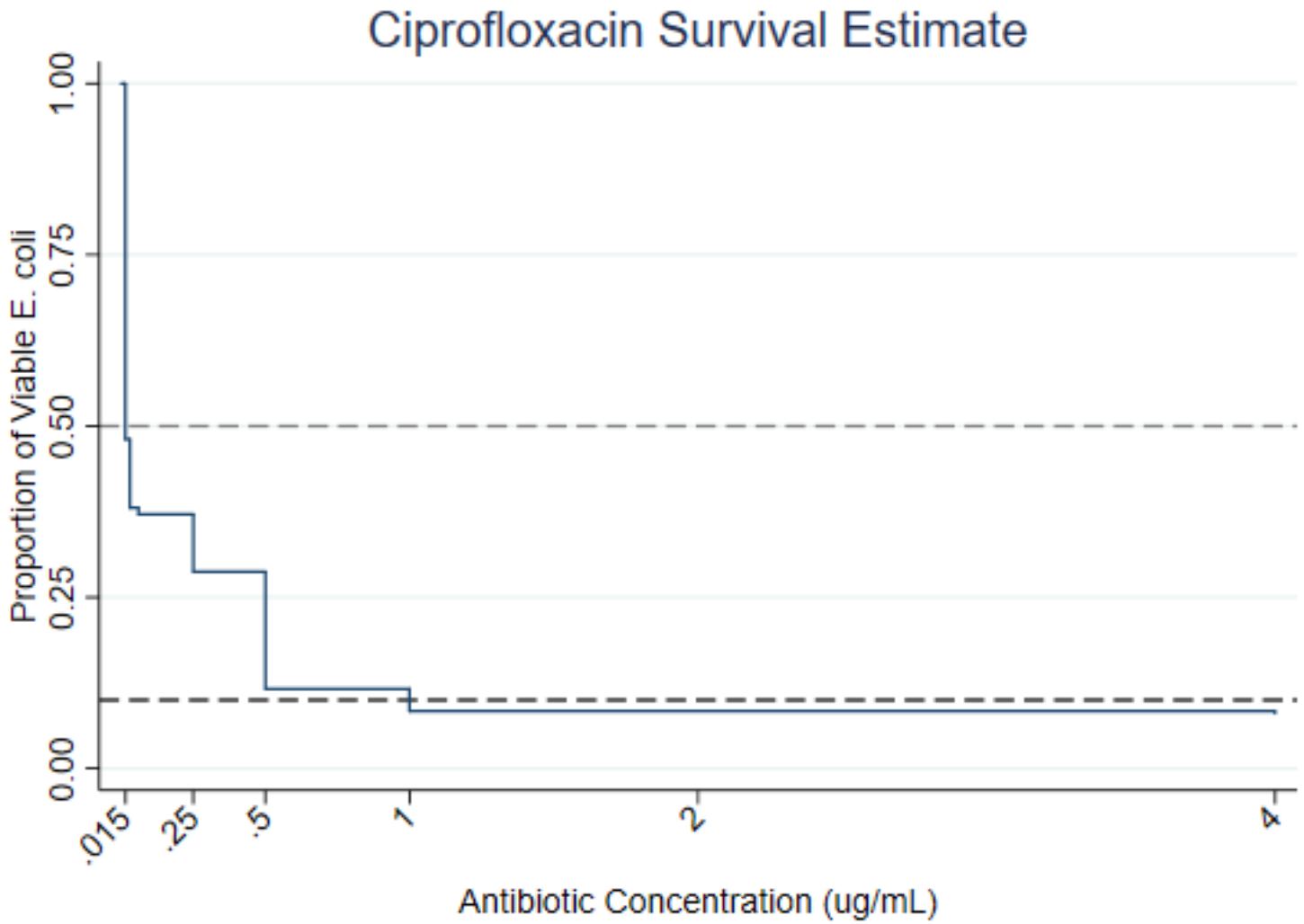

Figure 41: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to ciprofloxacin. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

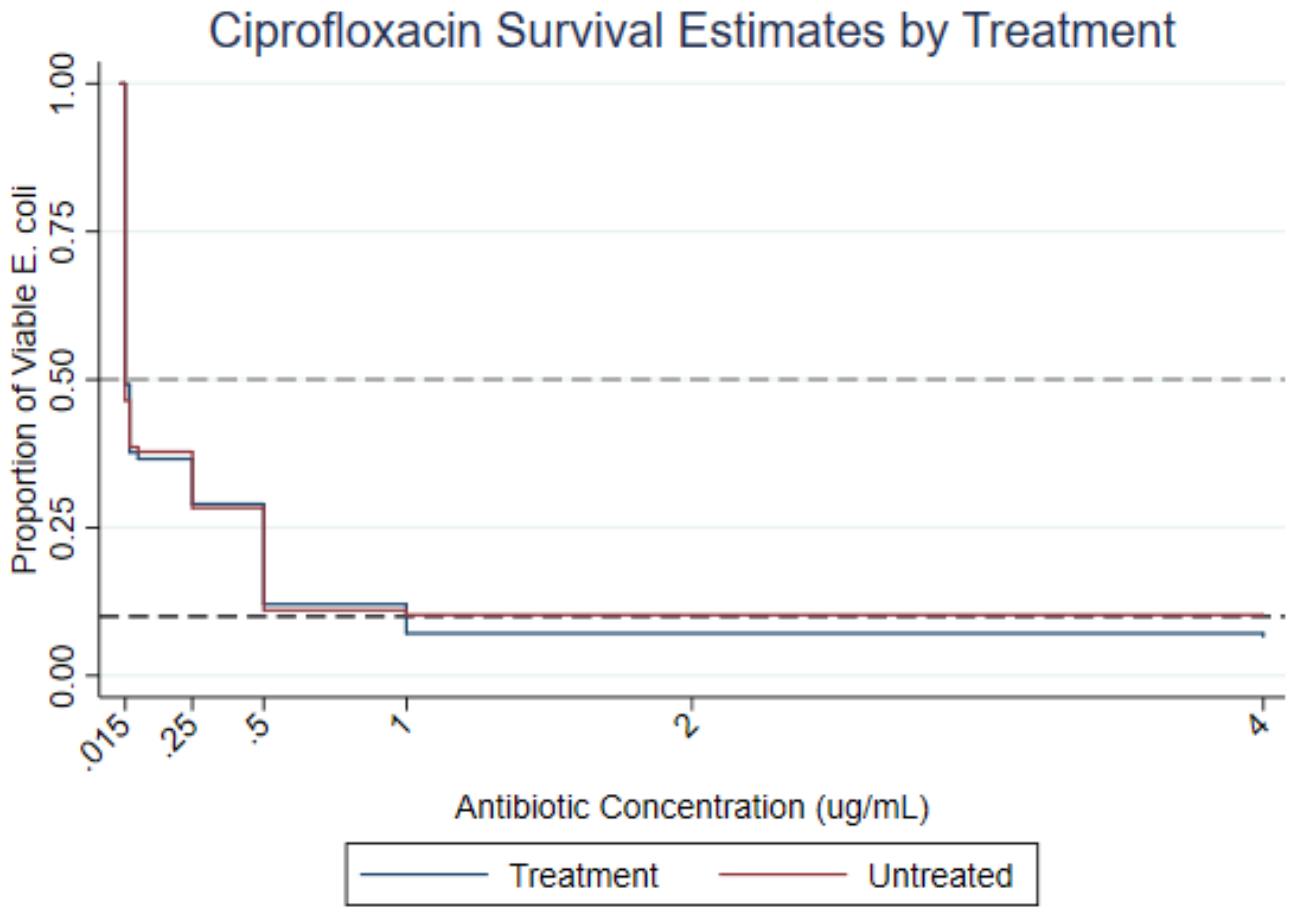

Figure 42: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to ciprofloxacin. The treatment group is represented by the blue line and the untreated red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

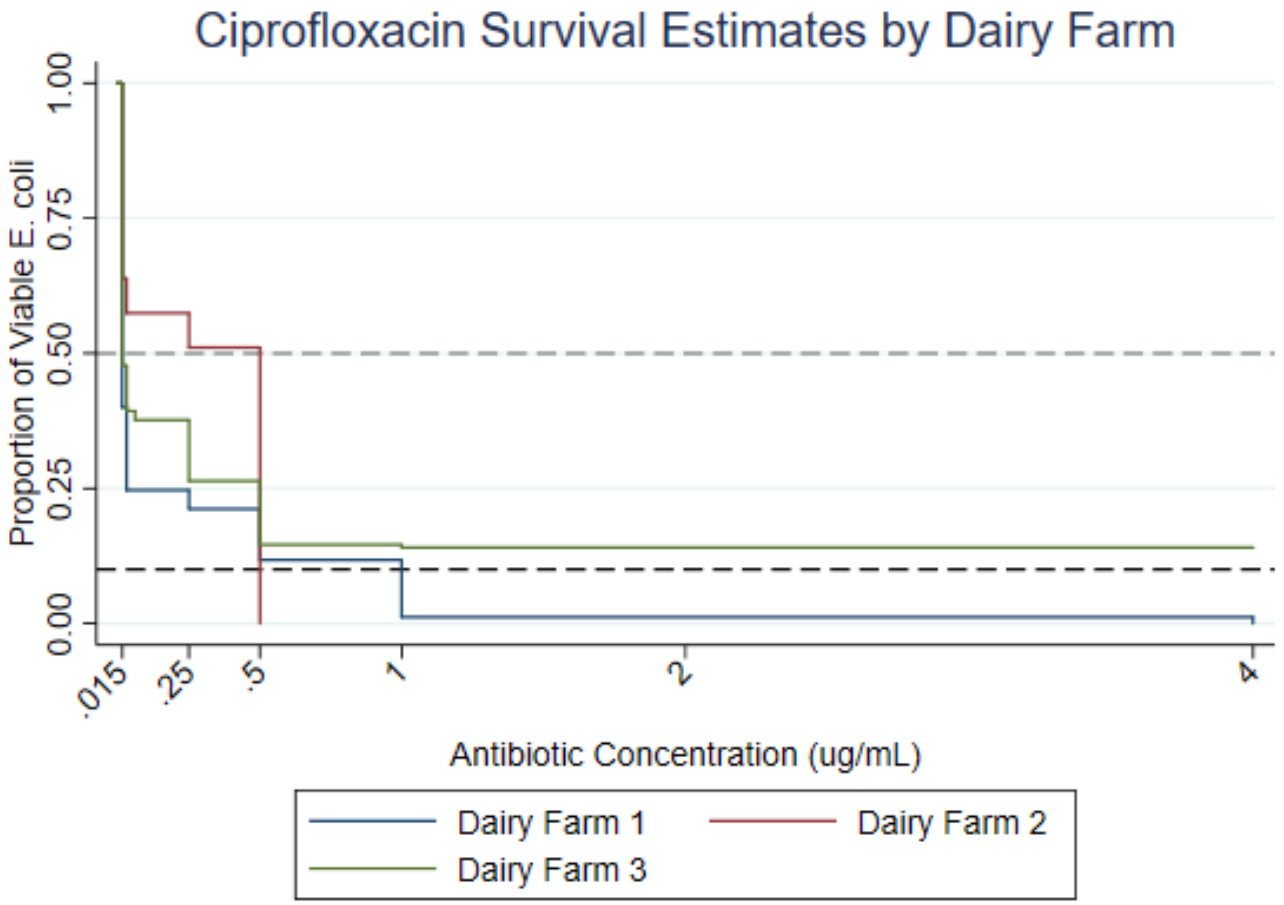

Figure 43: The Kaplan-Meier survival estimate is shown for isolates based on dairy farm regarding the phenotypic MIC to ciprofloxacin. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

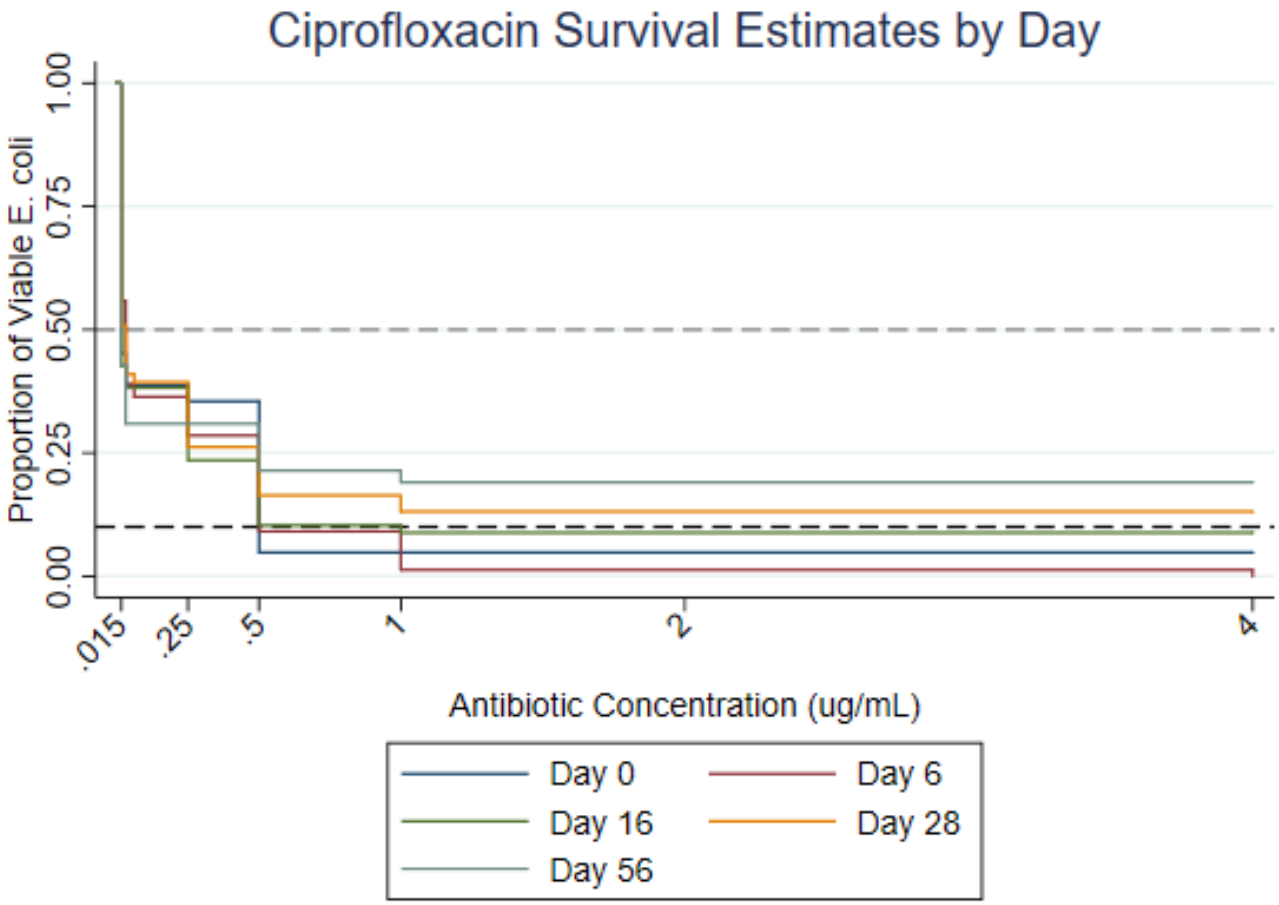

Figure 44: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to ciprofloxacin. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

For isolates in general, the MIC 50 to gentamycin was 1 µg/mL and the MIC 90 is 2 µg/mL (Figure 45). The rate at which isolates from the control group reached their MIC was 1.543 (P = 0.0002, 95% CI: 1.226 – 1.943) times greater than the treatment group. The MIC 50 was 1 µg/mL in both treated and control groups, but the MIC 90 was 1 µg/mL for isolates from the control group and 2 µg/mL in the treated group (Figure 46). The rate ratio decreased by 0.939 (P = 0.3339, 95% CI: 0.827 – 1.067) between the 3 dairies. The MIC 50 was the same among isolates from all dairy farms at 1 µg/mL (Figure 47). The MIC 90 varied by farm, as Dairy Farm 2 had an MIC 90 of 1 µg/mL, 2 µg/mL on Dairy Farm 3, and 4 µg/mL on Dairy Farm 1. The rate ratio remained constant by day with only slight variation among the MIC 90 (Figure 48).

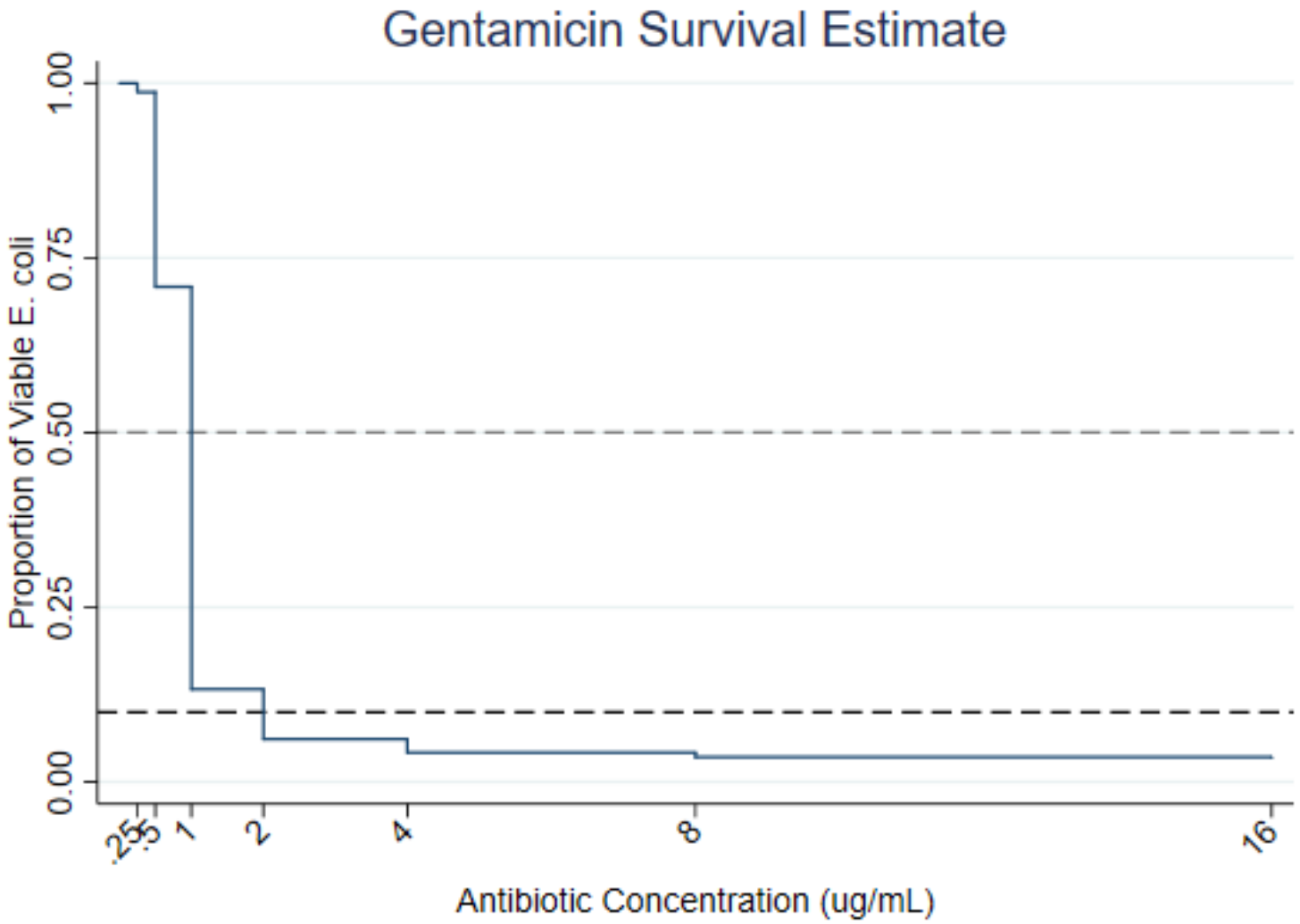

Figure 45: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to gentamicin. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

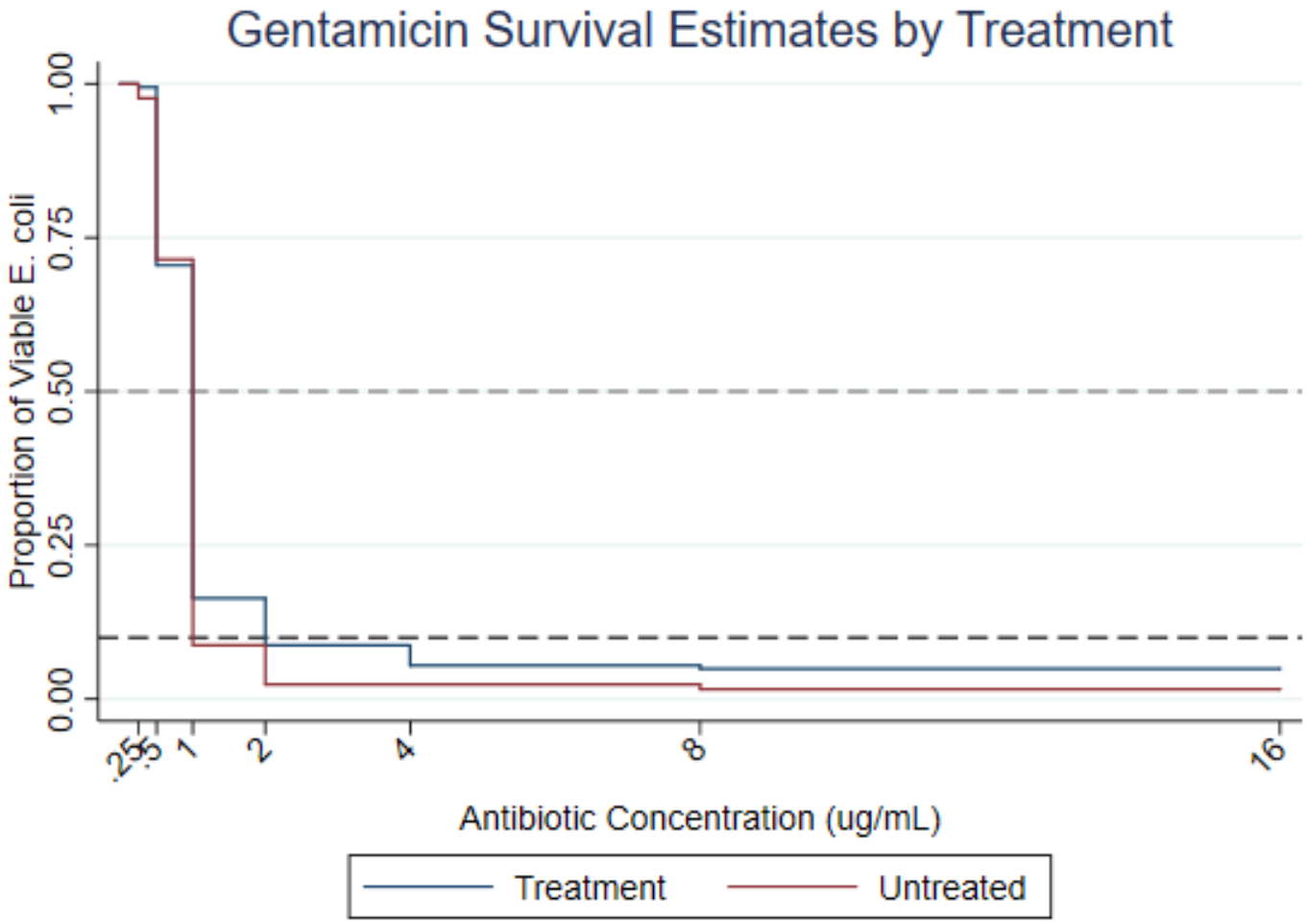

Figure 46: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to gentamicin. The treatment group is represented by the blue line and the untreated red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

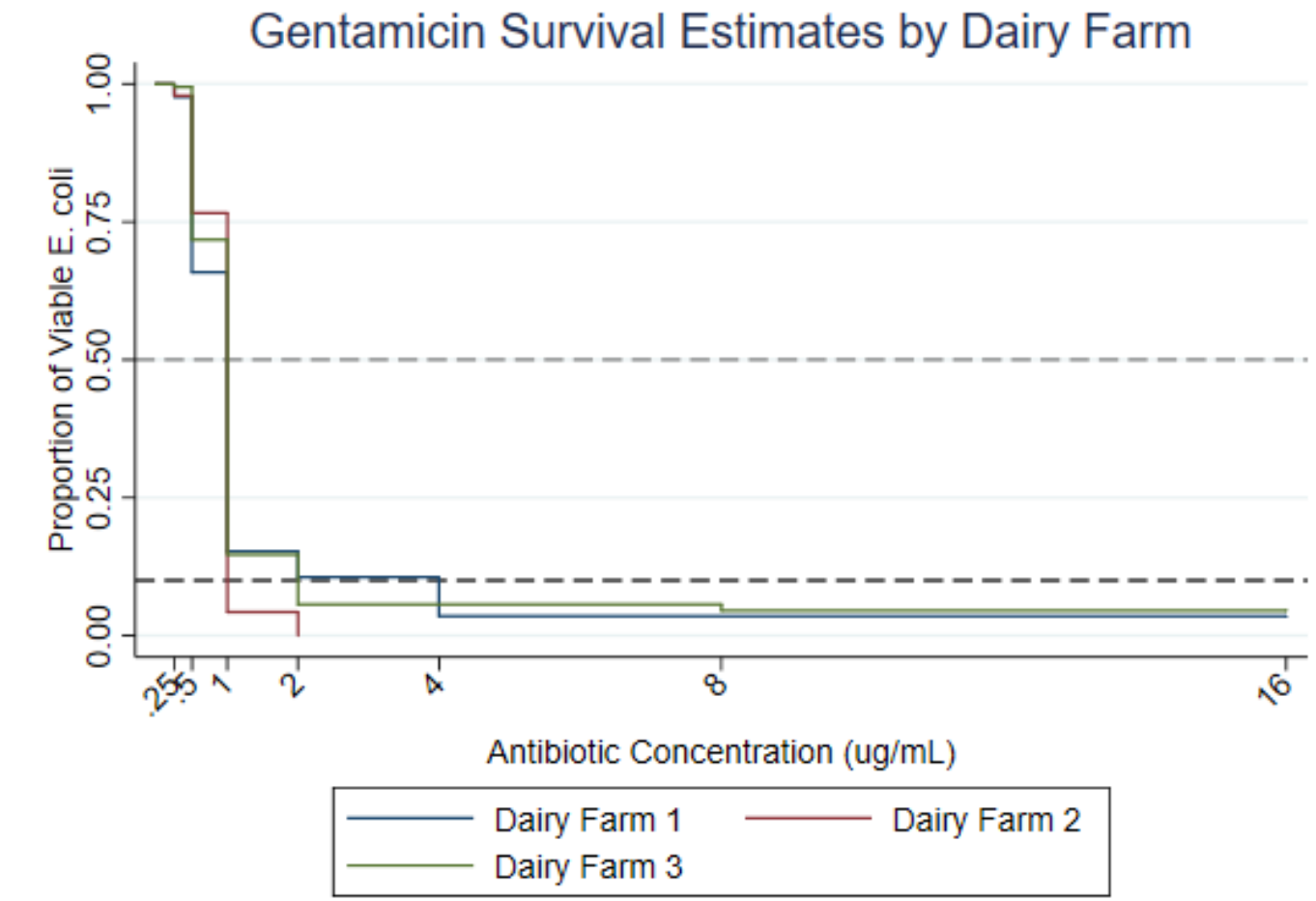

Figure 47: The Kaplan-Meier survival estimate is shown for isolates based on dairy farm regarding the phenotypic MIC to gentamicin. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

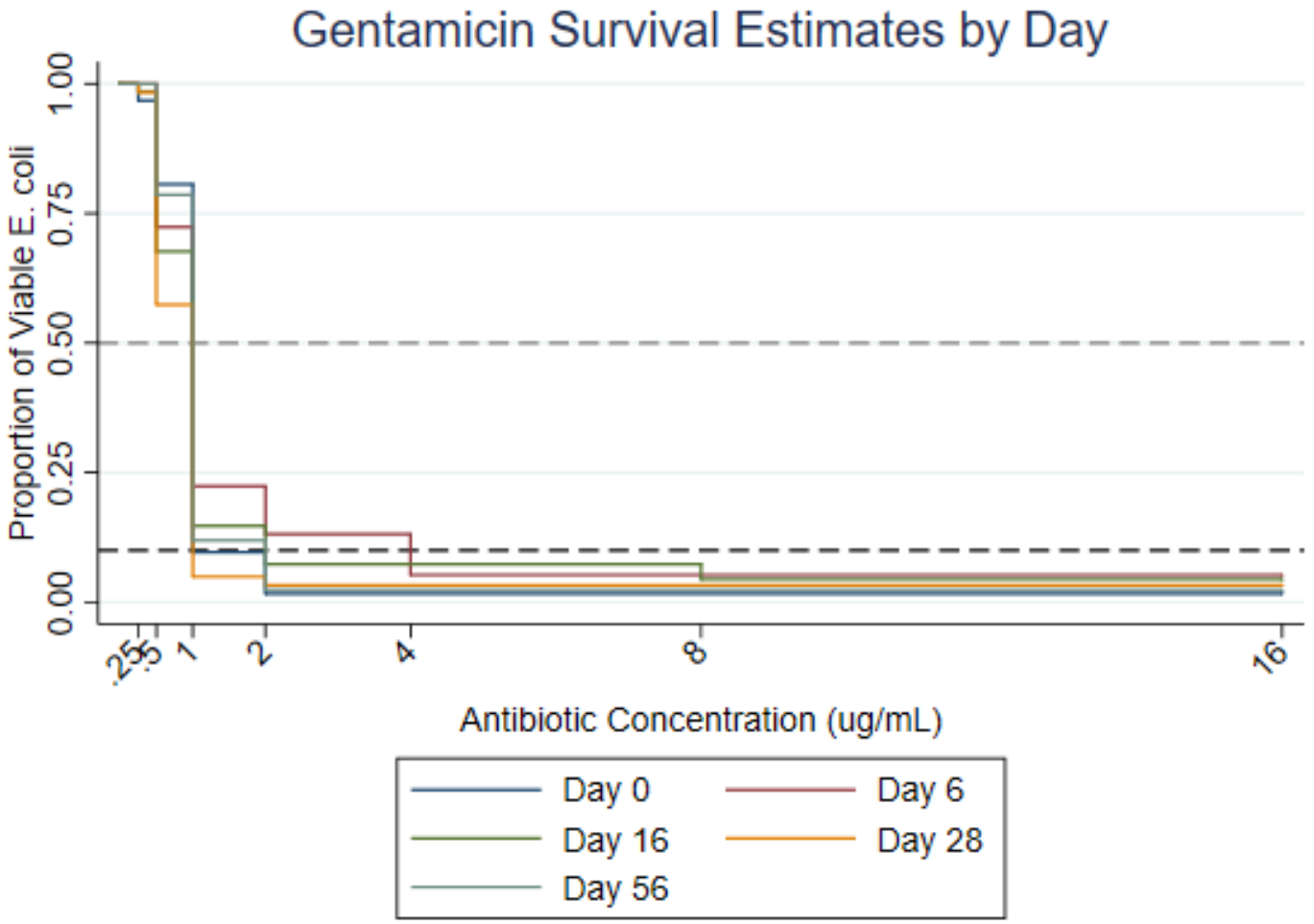

Figure 48: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to gentamicin. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

The MIC 50 for nalidixic acid was 4 µg/mL with an MIC 90 greater than 32 µg/mL among the isolates in general (Figure 49). The rate ratio was 0.963 (*P* = 0.7624, 95% CI: 0.755 – 1.229) for the control group, but isolates from both groups maintain the MIC 50 and 90 of the isolates not broken down into categories (Figure 50). Similar to previous antibiotics, the rate ratio decreased by 0.715 (*P* = 0.0000, 95% CI: 0.616 – 0.829) among isolates from Dairy Farms 1, 2, and 3. This yielded variability in the MIC 50 and 90 of the isolate groupings. The MIC 50 among isolates from Dairy Farm 1 was 2 µg/mL, followed by Dairy Farm 3 at 4 µg/mL, and Dairy Farm 2 at 8 µg/mL (Figure 51). The MIC 90 for isolates from Dairy Farms 1 and 2 was 16 µg/mL, but greater than 32 µg/mL on Dairy Farm 3. The MIC 50 among isolates from day 28 was 2 µg/mL and 4 µg/mL for all other days (Figure 52). Similar to ciprofloxacin, the MIC 90 for isolates from day 0 and 6 (16 µg/mL) was less than that of isolates from days 16, 28, and 56 (> 32 µg/mL). This was consistent with the rate ratio decreasing by 0.991 as day increased (*P* = 0.0058, 95% CI: 0.984 – 0.997).

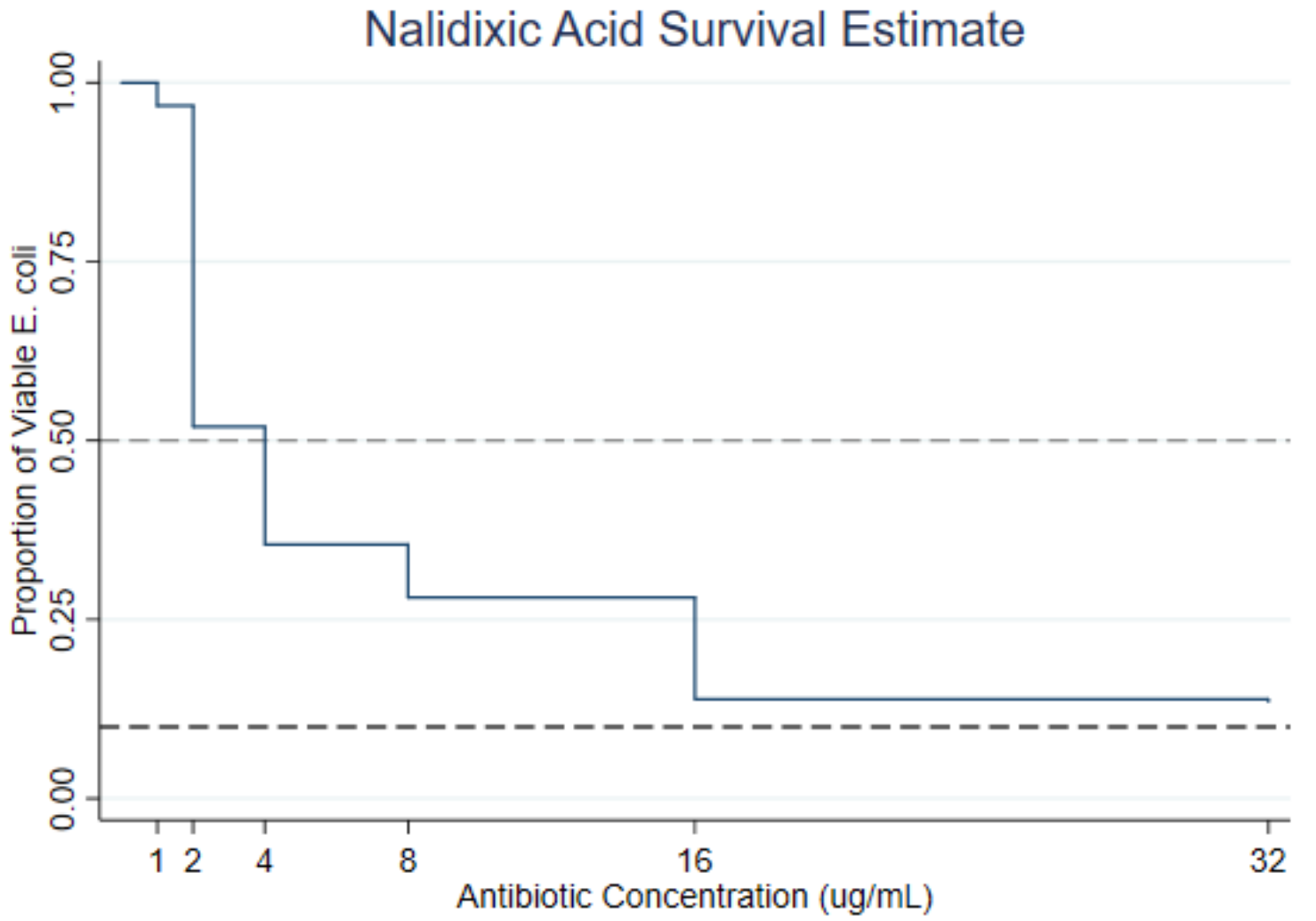

Figure 49: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to nalidixic acid. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

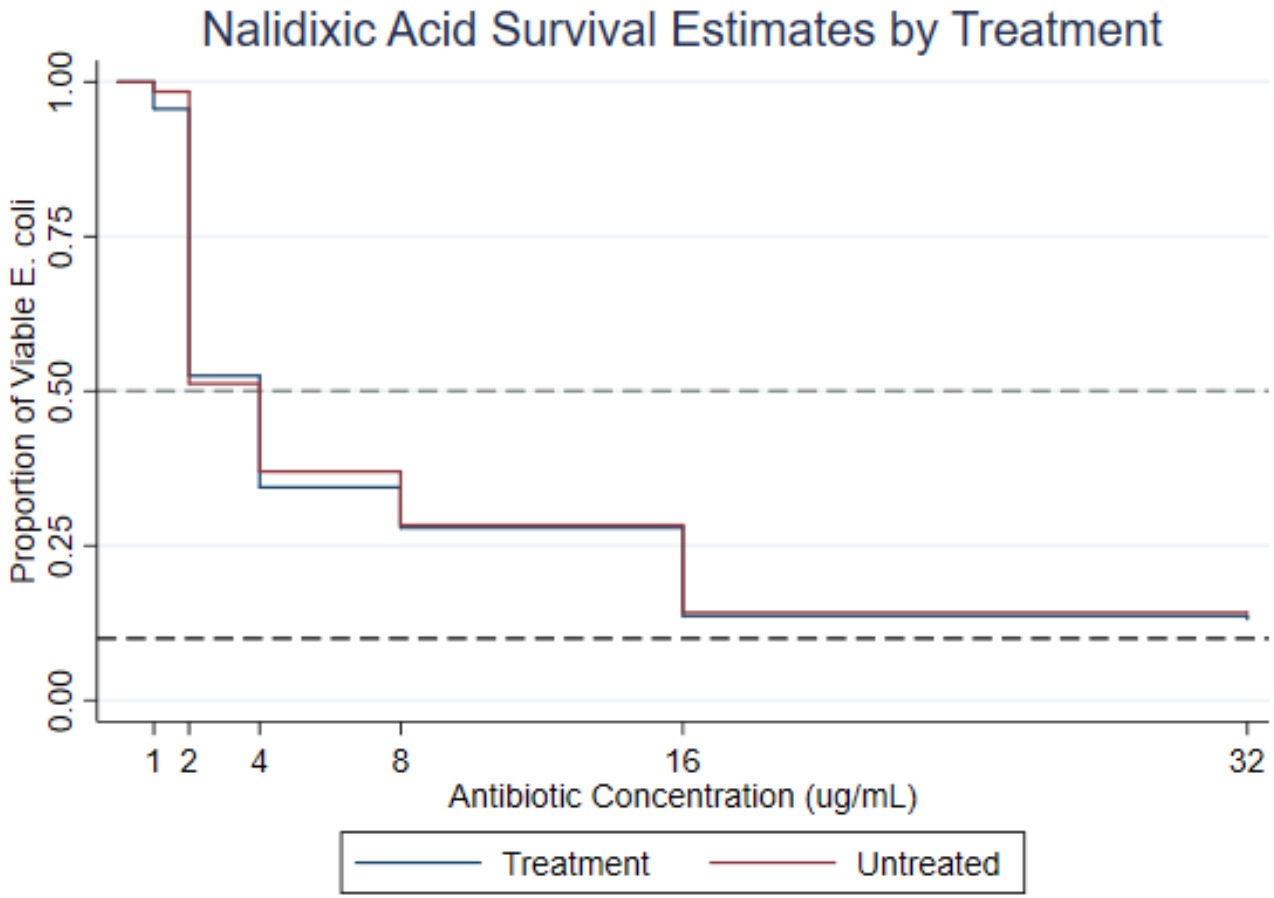

Figure 50: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to nalidixic acid. The treatment group is represented by the blue line and the untreated red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

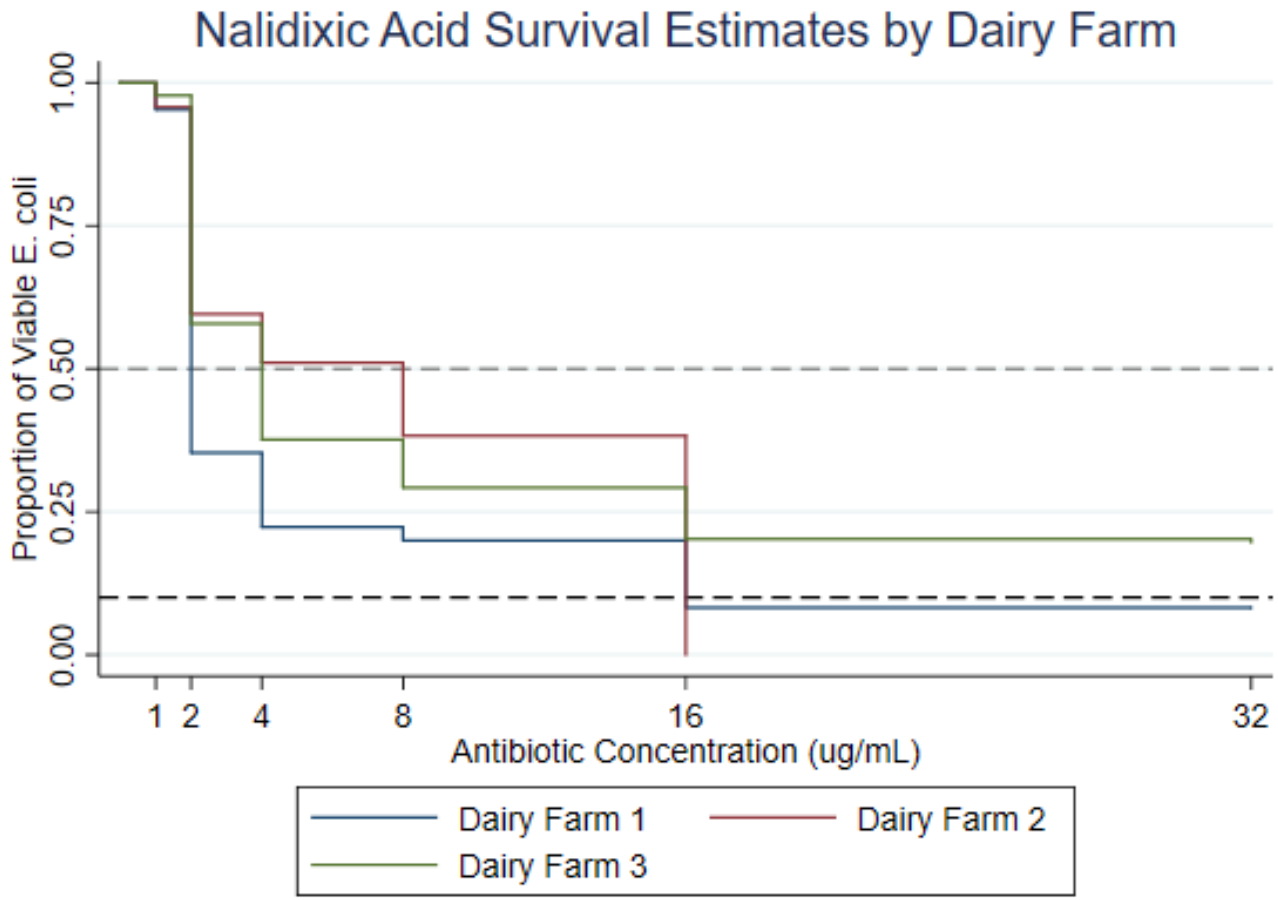

Figure 51: The Kaplan-Meier survival estimate is shown for isolates based on dairy farm regarding the phenotypic MIC to nalidixic acid. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

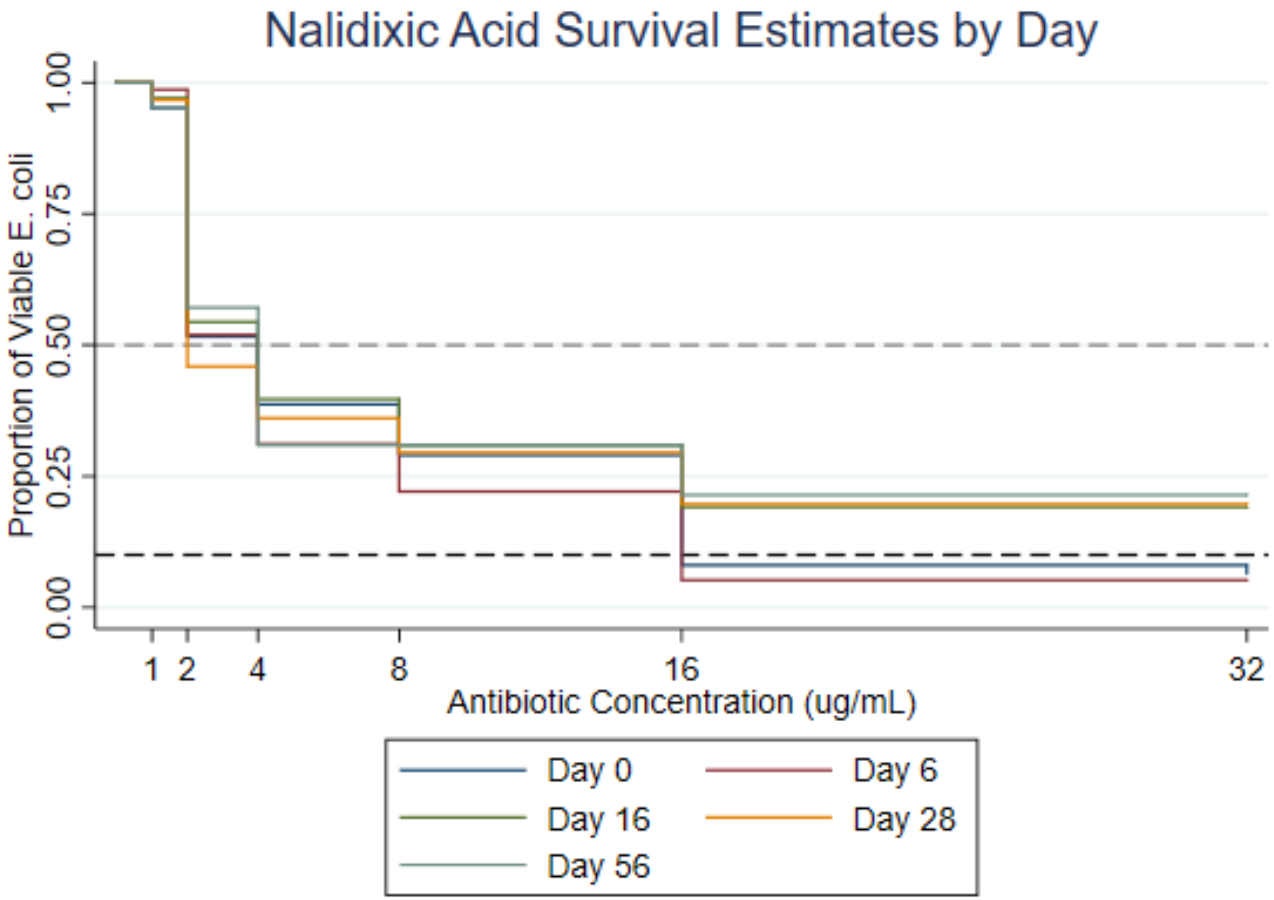

Figure 52: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to nalidixic acid. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

Isolates, regardless of grouping, showed an MIC 50 of 16 µg/mL and MIC 90 of 64 µg/mL to streptomycin (Figure 53). The rate ratio of the control group did not differ significantly from the treatment group (Rate ratio = 0.976, *P* = 0.8312, 95% CI: 0.778 – 1.224). The MIC 50 and 90s were both the same as isolates without a breakdown by group (Figure 54). The rate ratio decreased by 0.902 (*P* = 0.1394, 95% CI: 0.787 – 1.034) regarding isolates from Dairy Farm 1 to 2 and 2 to 3. The MIC 50 for isolates from Dairy Farm 1 was 8 µg/mL, 16 µg/mL on Dairy Farm 3, and 64 µg/mL on Dairy Farm 2 (Figure 55). This pattern was observed from the concentration of 8 µg/mL with isolates from Dairy Farm 2 reaching their MIC at lower rates and higher concentrations followed by Dairy Farm 3 and Dairy Farm 1. The rate ratio maintained the same as day increased (Rate ratio = 1.005, *P* = 0.1259, 95% CI: 0.998 – 1.012) due in part to the offsetting nature of isolates from days 28 and 56 (Figure 56). The MIC 50 among the groups was variable with isolates from day 56 having the lowest MIC 50 at 8 µg/mL, day 28 with the highest at 64 µg/mL, and days 0 (32 µg/mL), 6 (16 µg/mL), and 16 (32 µg/mL) falling in between.

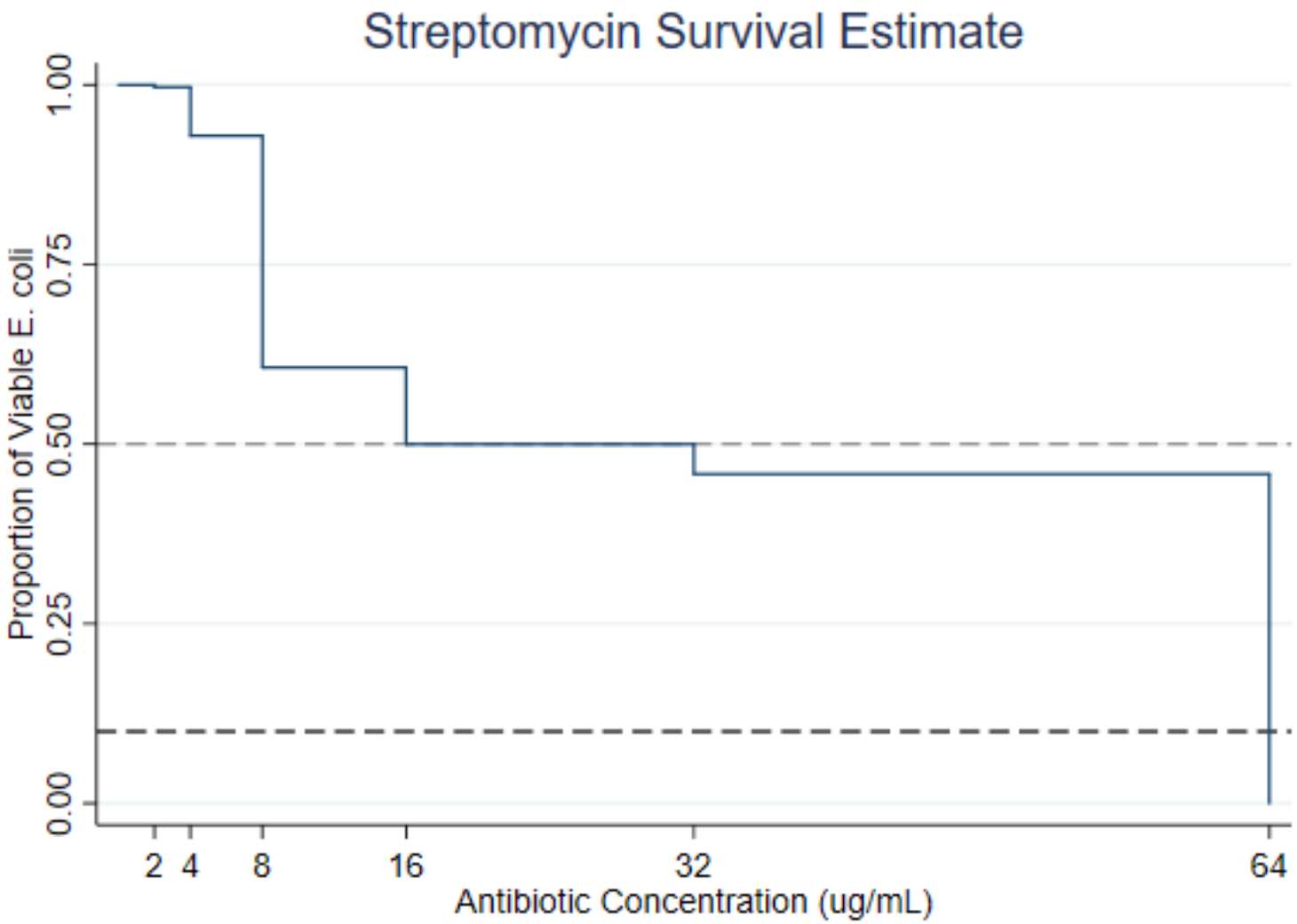

Figure 53: The Kaplan-Meier survival estimate is shown for isolates regarding the phenotypic MIC to streptomycin. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

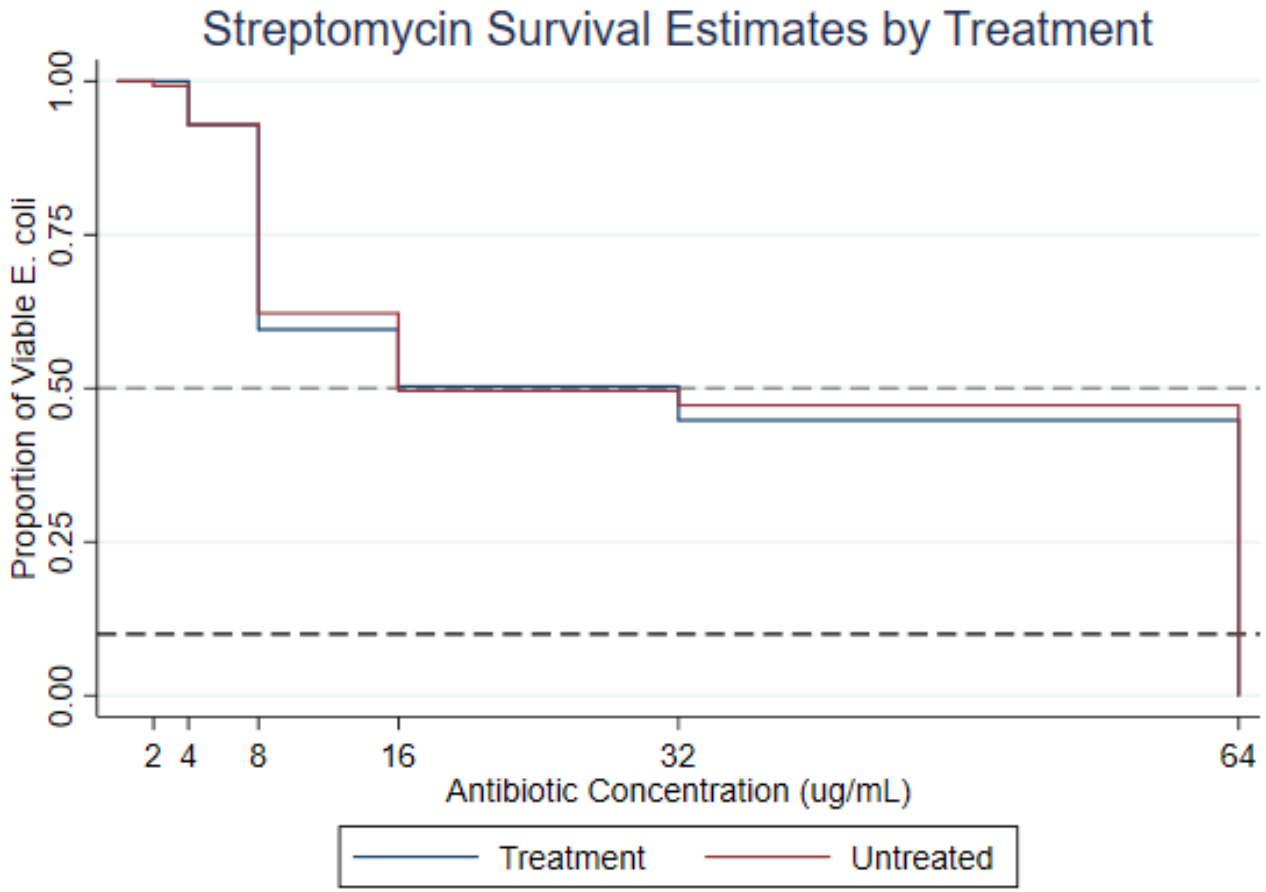

Figure 54: The Kaplan-Meier survival estimate is shown for all isolates based on treatment regarding the phenotypic MIC to streptomycin. The treatment group is represented by the blue line and the untreated red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

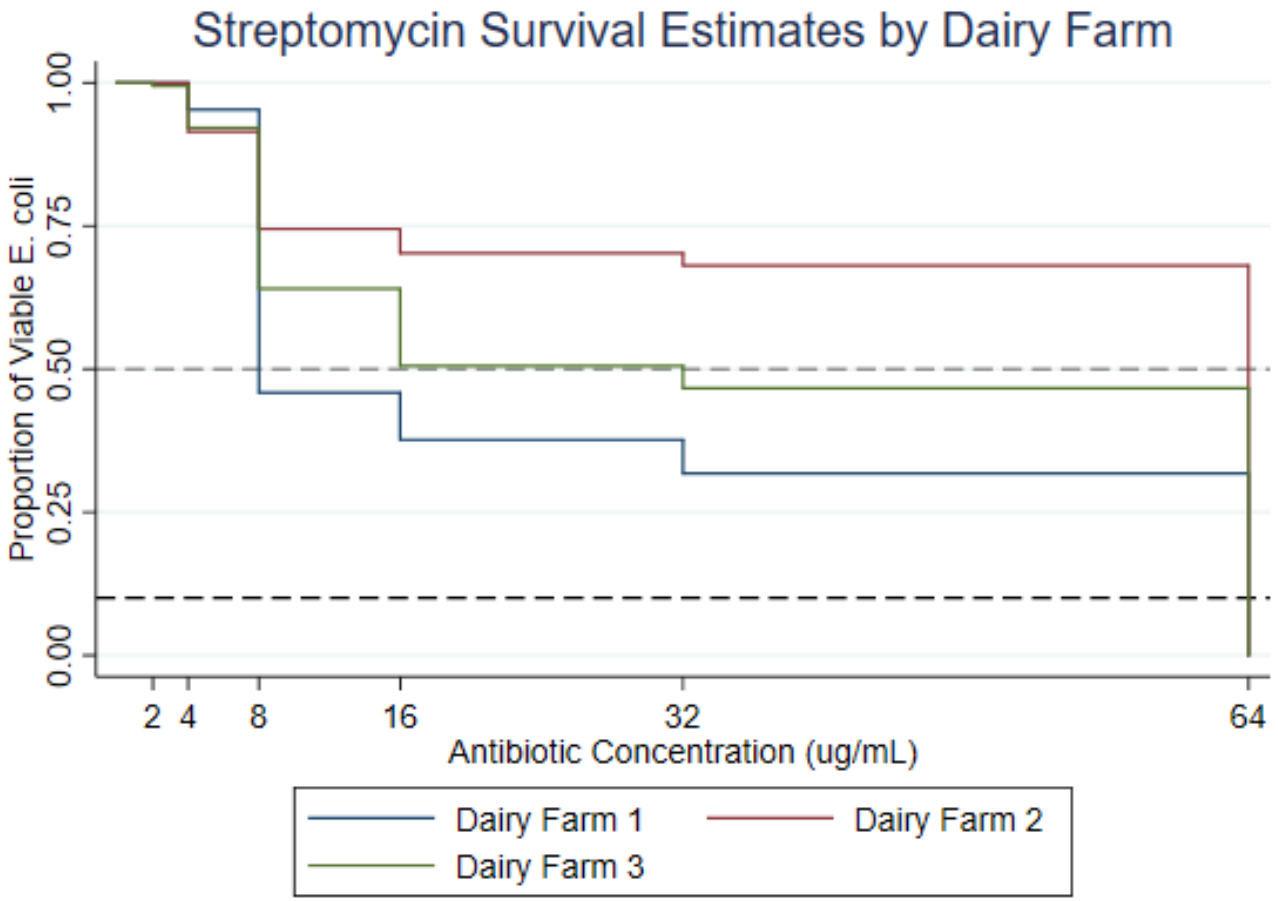

Figure 55: The Kaplan-Meier survival estimate is shown for isolates based on dairy farm regarding the phenotypic MIC to streptomycin. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

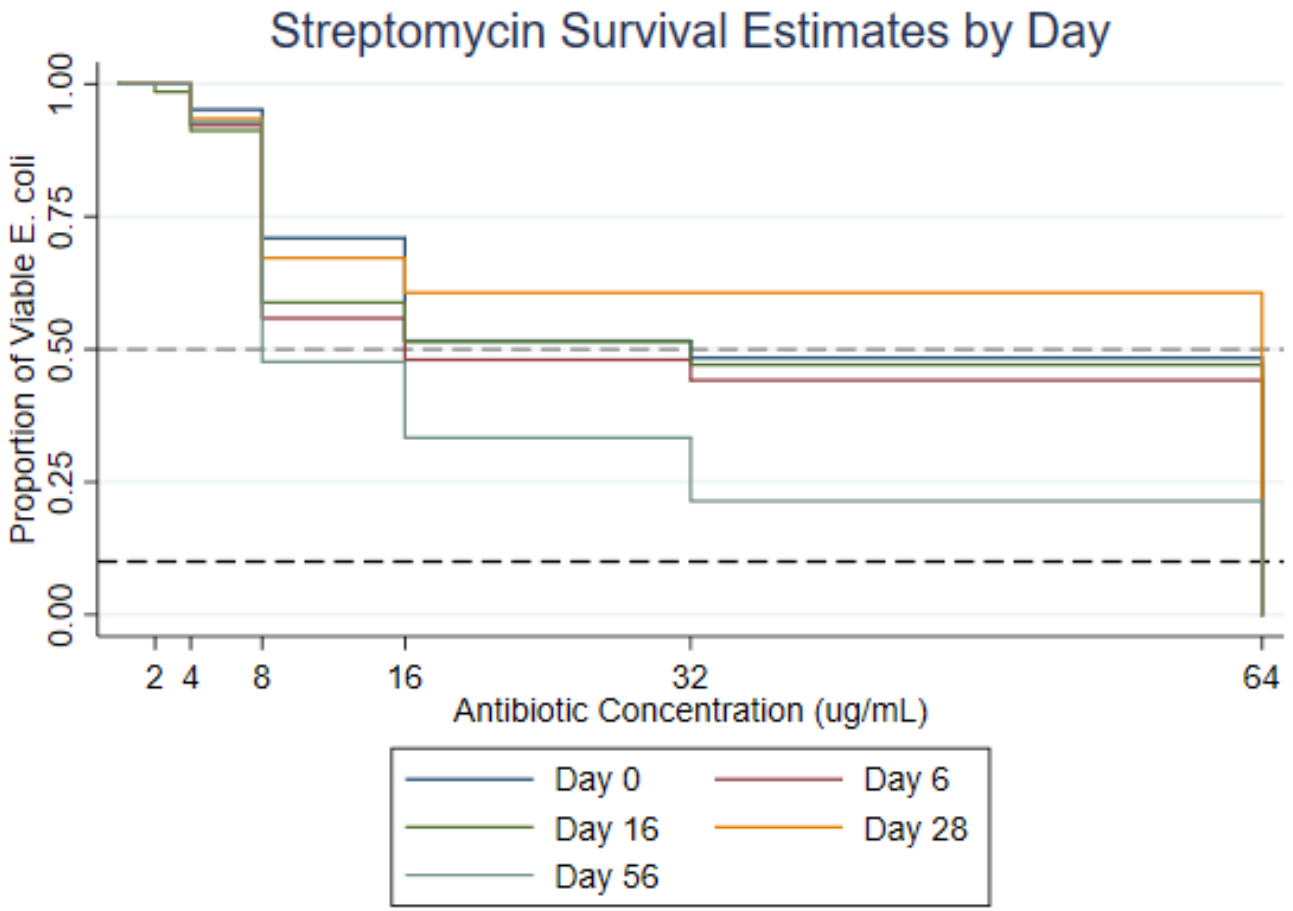

Figure 56: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to streptomycin. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

When tested with sulfisoxazole, both the MIC 50 and MIC 90 of isolates, regardless of grouping, was greater than the 256 µg/mL plate limit (Figure 57). The rate ratio slightly increased by 1.107 for isolates from the control group, but at a non-significant level (P = 0.5460, 95% CI: 0.796 – 1.541). The MIC 50 remained greater than 256 µg/mL among both groups (Figure 58). Following a similar pattern to other antibiotics to which susceptibility was tested, the rate ratio dropped 0.731 (P = 0.0019, 95% CI: 0.599 – 0.890) between Dairy Farms 1 and 2 and 2 and 3. While the pattern of MIC followed a similar trend among Dairy Farms 2 and 3 with MIC 50s greater than 256 µg/mL, Dairy Farm 1 had an MIC 50 of 16 µg/mL (Figure 59).There was, again, not a difference among the rate ratio regarding day (Rate ratio = 1.001, P = 0.7870, 95% CI: 0.992 – 1.001) most likely offset by isolates from day 56. The MIC 50 of isolates from days 0 and 56 were 32 µg/mL, while isolates from all other days had an MIC 50 greater than the 256 µg/mL plate maximum concentration (Figure 60). Isolates from day 28 tended to have the highest MICs followed by days 16, 6, 0, and 56.

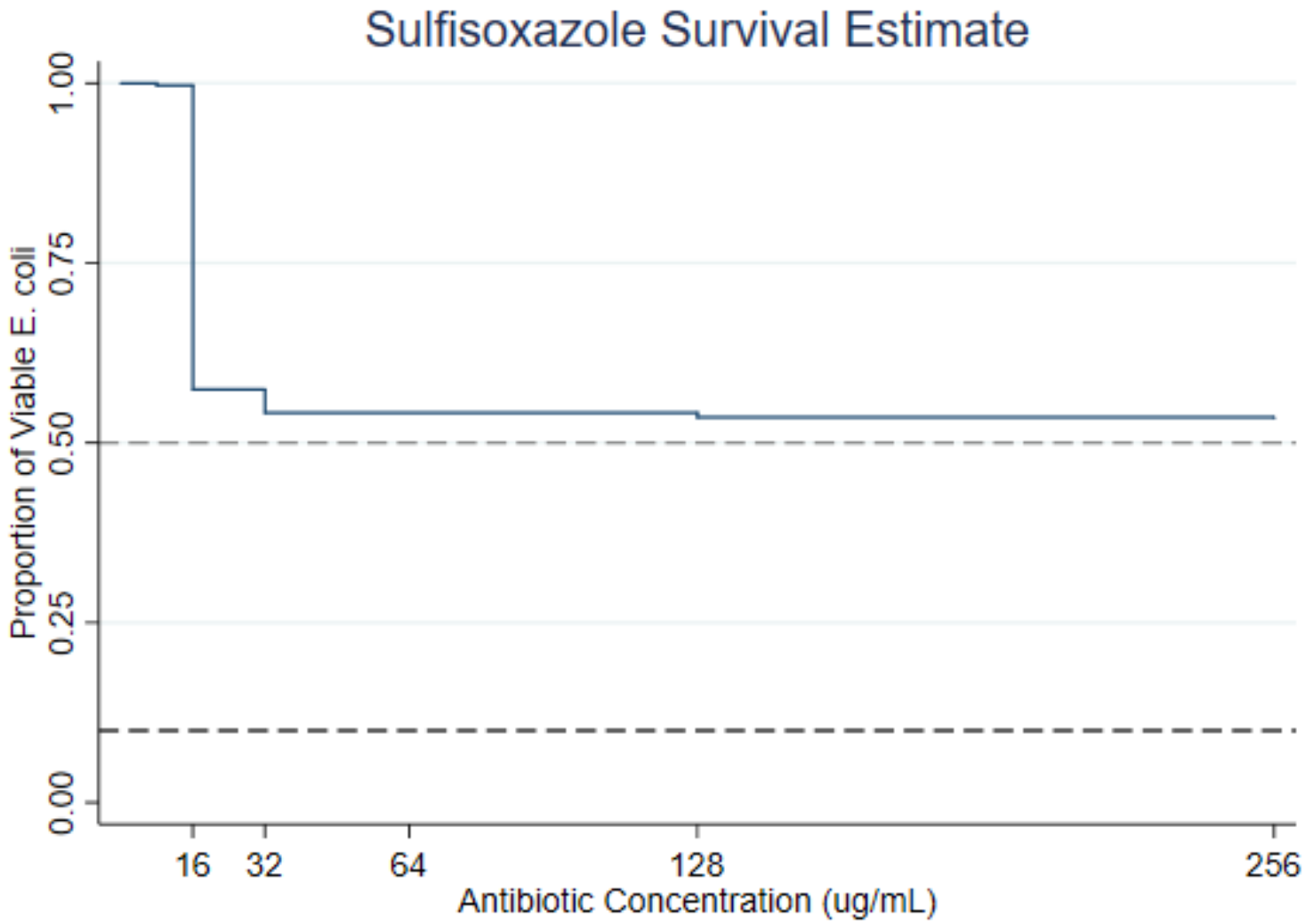

Figure 57: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to sulfisoxazole. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

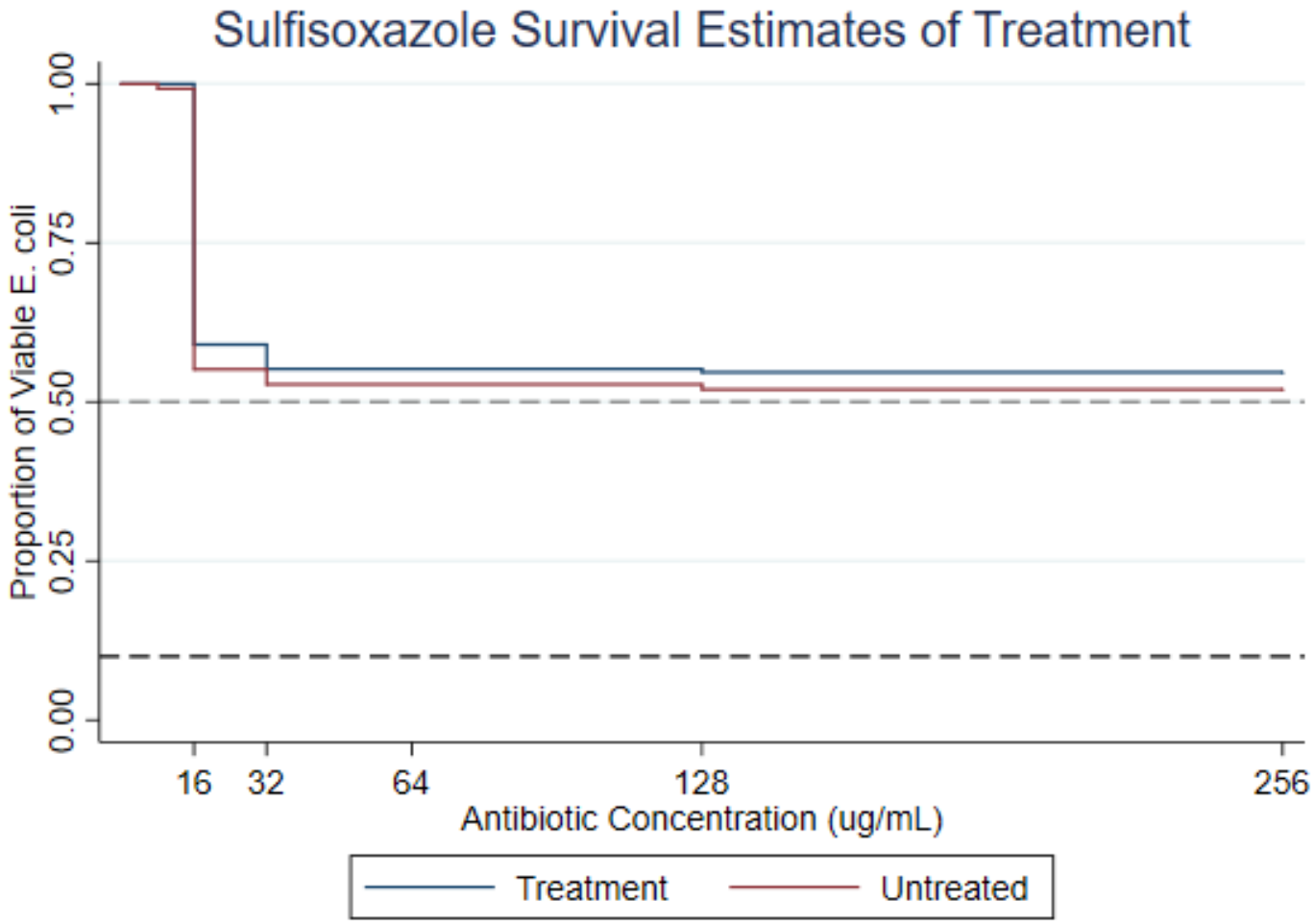

Figure 58: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to sulfisoxizole. The treatment group is represented by the blue line and the untreated red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

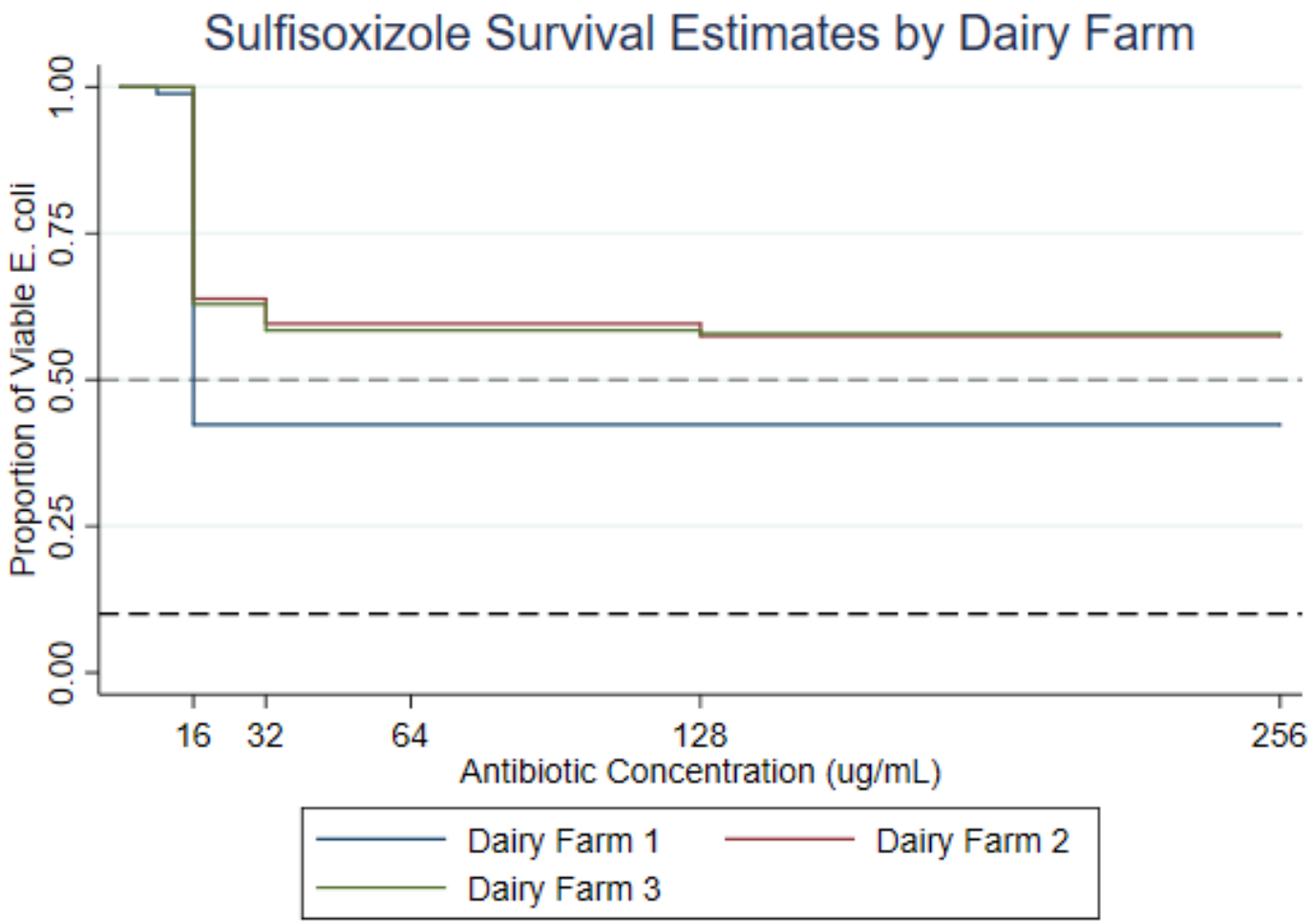

Figure 59: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to sulfisoxizole. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

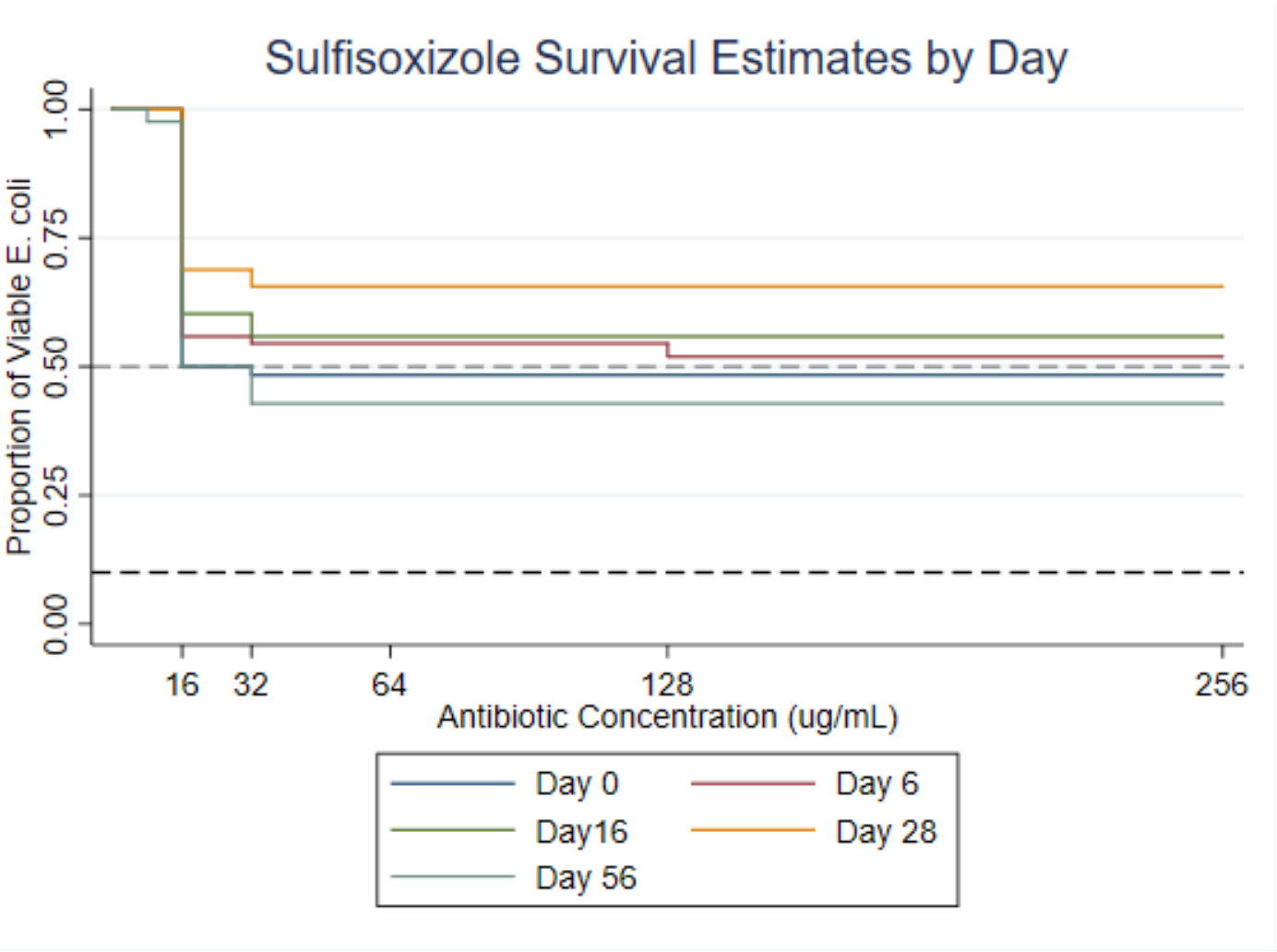

Figure 60: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to sulfisoxizole. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

All isolates from the CHROM-ESBL agar exhibited MICs 50 and 90 greater than the 32 µg/mL maximum plate concentration for tetracycline (Figure 61). While having a slightly reduced rate ratio not of statistical significance in the control group (Rate ratio = 0.923, *P* = 0.6860, 95% CI: 0.627 – 1.359), the MIC 50 of isolates from both treated and control animals maintained a concentration above 32 µg/mL (Figure 62). However, the rate ratio increased by 1.406 (*P* = 0.0017, 95% CI: 1.136 – 1.741) between isolates from Dairy Farm 1 to 2 and 2 to 3, which was the first instance of such a pattern among the antibiotics tested. While all isolate groups had MIC 50s greater than a concentration of 32 µg/mL, isolates from Dairy Farm 1 had the highest MIC concentrations followed by Dairy Farms 2 and 3 (Figure 63). Furthermore, the rate ratio was similar among sampling days (Rate ratio = 0.991, *P* = 0.0853, 95% CI: 0.980 – 1.001) with all isolate groups maintaining an MIC 50 about the maximum plate concentration (Figure 64).

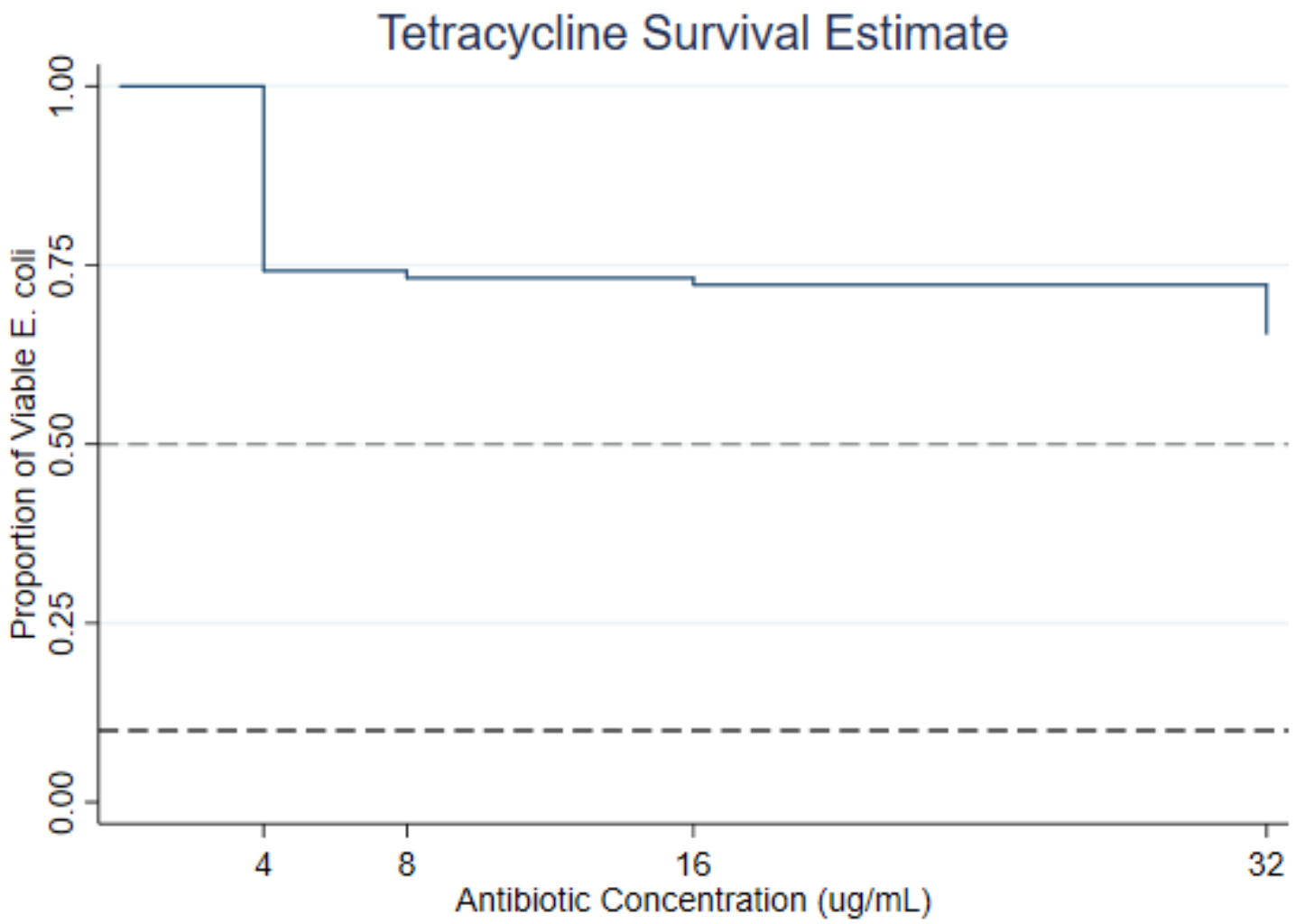

Figure 61: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to tetracycline. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

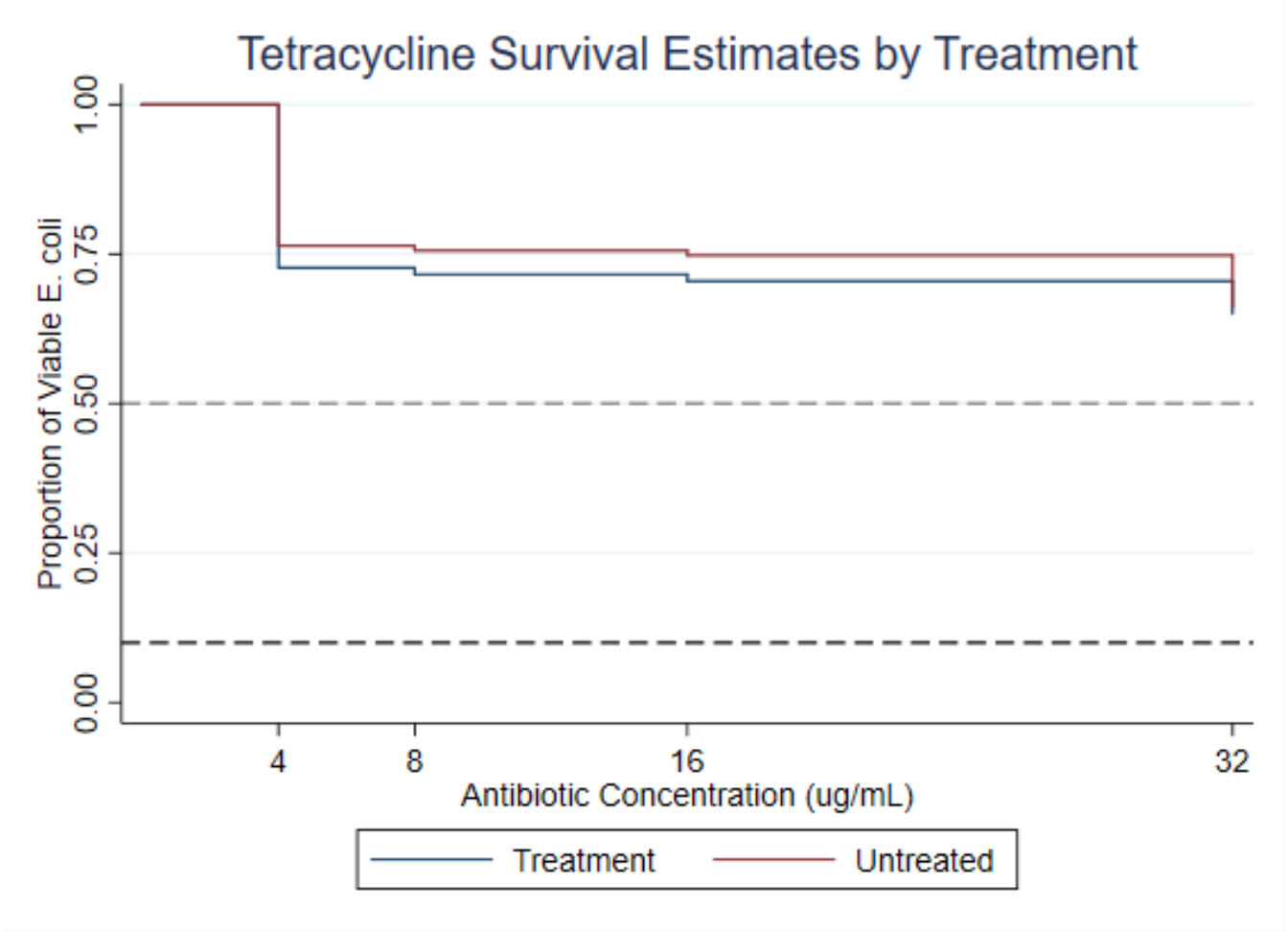

Figure 62: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to tetracycline. The treatment group is represented by the blue line and the untreated red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

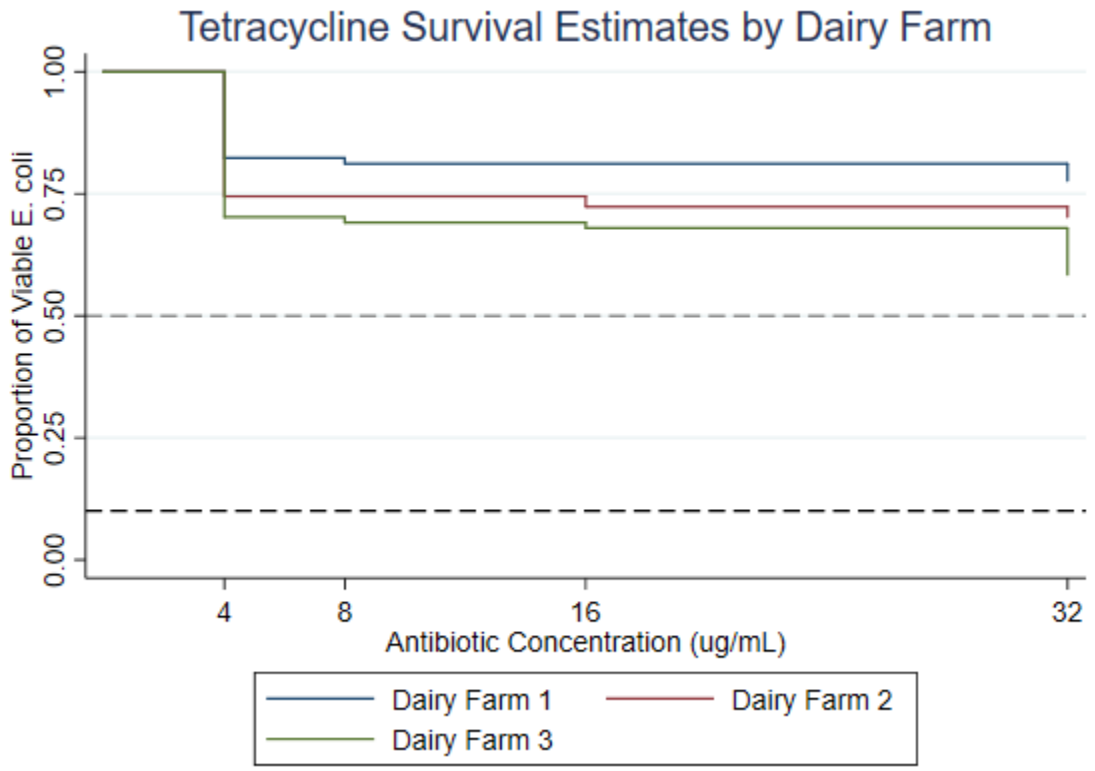

Figure 63: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to tetracycline. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

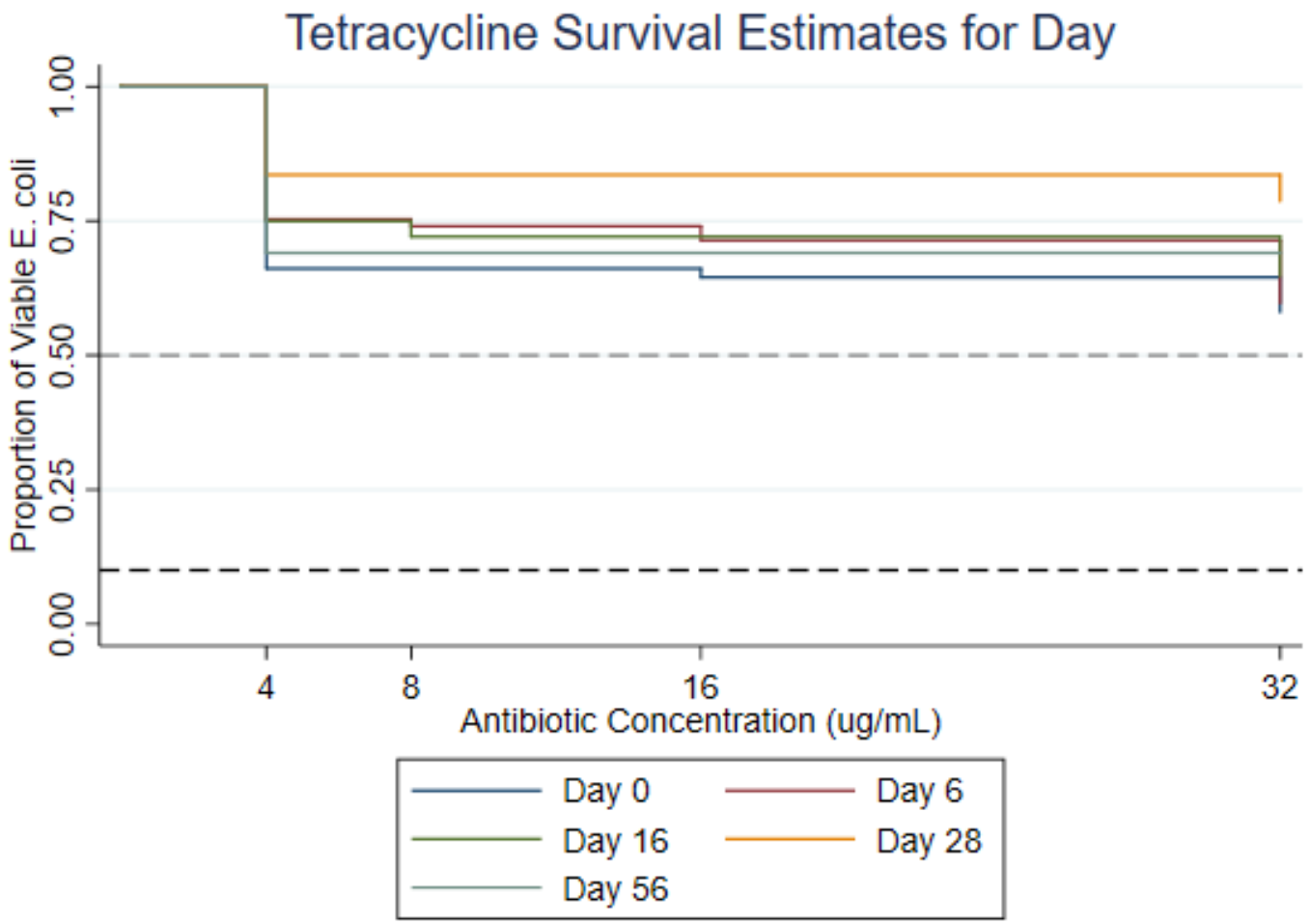

Figure 64: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to tetracycline. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

The MIC 50 of isolates to a trimethoprim/sulfamethoxazole combination was 0.12 µg/2.38 µg/mL with an MIC 90 greater than a concentration of 4 µg/76 µg/mL (Figure 65). With a rate ratio consistent among groups (Rate ratio = 0.997, *P* = 0.9838, 95% CI: 0.779 – 1.278), the MIC levels remained the same as the isolates without a breakdown by treatment group (Figure 66). The greatest discrepancy among the isolates was grouping by dairy farm. This variable was associated with a rate ratio of 0.232 (*P* = 0.0000, 95% CI: 0.190 – 0.284) between Dairy Farms 1, 2, and 3. This was reflected in the MIC 50 and 90 metrics, as well. The MIC 50 of isolates from Dairy Farms 1 and 2 was 0.12 µg/2.38 µg/mL and 0.25 µg/4.75 µg/mL on Dairy Farm 3 (Figure 67). A bigger impact was observed among the MIC 90, as isolates from Dairy Farm 1 had an MIC 90 of 0.25 µg/4.75 µg/mL, 2 µg/38 µg/mL for Dairy Farm 2, and greater than 4 µg/76 µg/mL among Dairy Farm 3 isolates. The MIC 50 for all sampling days, but day 28 (0.25 µg/ 4.75 µg/mL), was 0.12 µg/2.38 µg/mL (Figure 68). The MIC 90 for day 0 was 0.5 µg /9.5 µg/mL, but was elevated above the 4 µg /76 µg/mL trimethoprim/sulfamethoxazole combination for all other sampling days. Day 16 had the greatest proportion of isolates with elevated MICs followed by sampling days 28, 56, 6, and 0. The rate ratio decreased by 0.990 as day increased (*P* = 0.0058, 95% CI: 0.983 – 0.997).

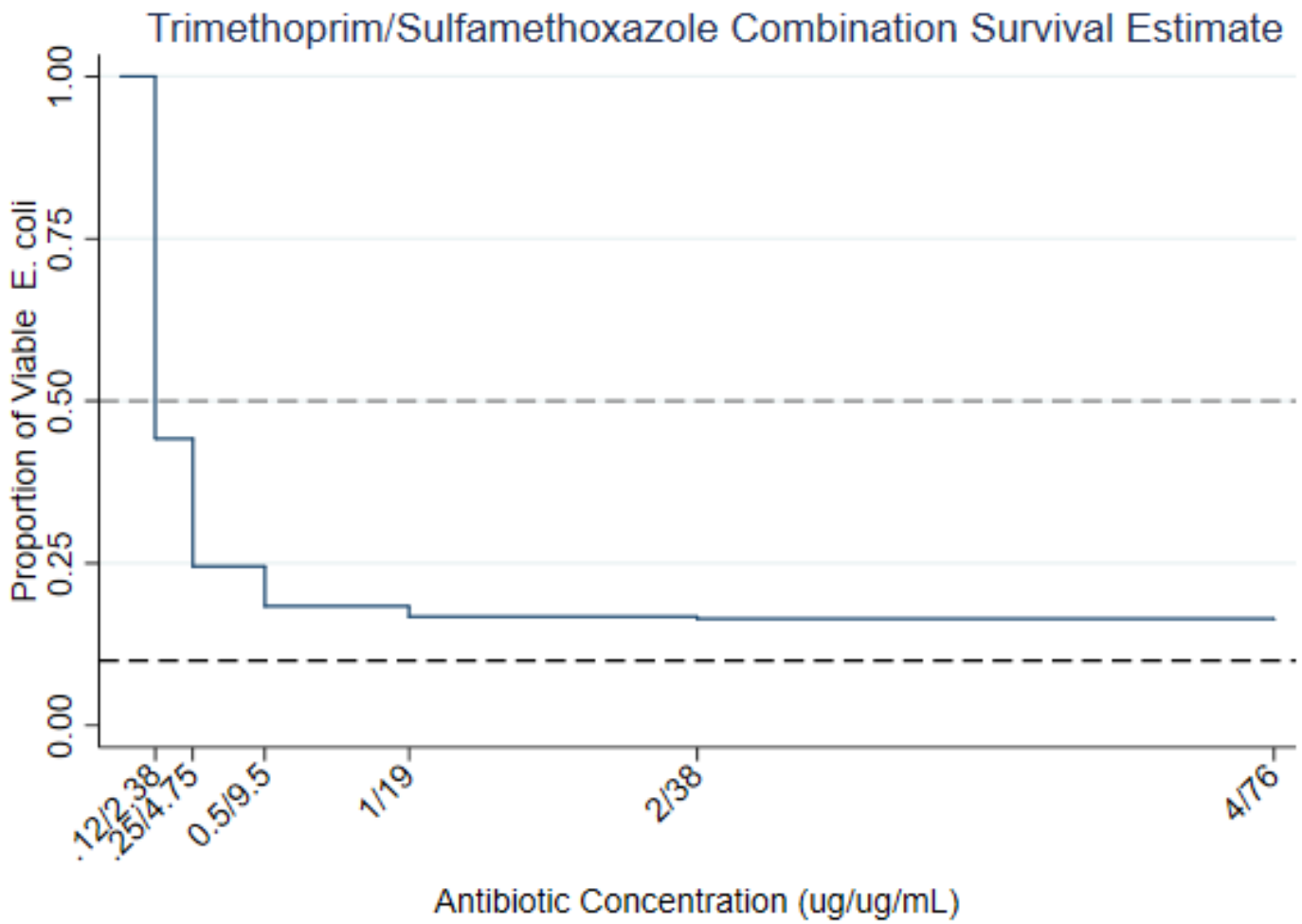

Figure 65: The Kaplan-Meier survival estimate is shown for all isolates regarding the phenotypic MIC to a trimethoprim/sulfamethoxazole combination. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

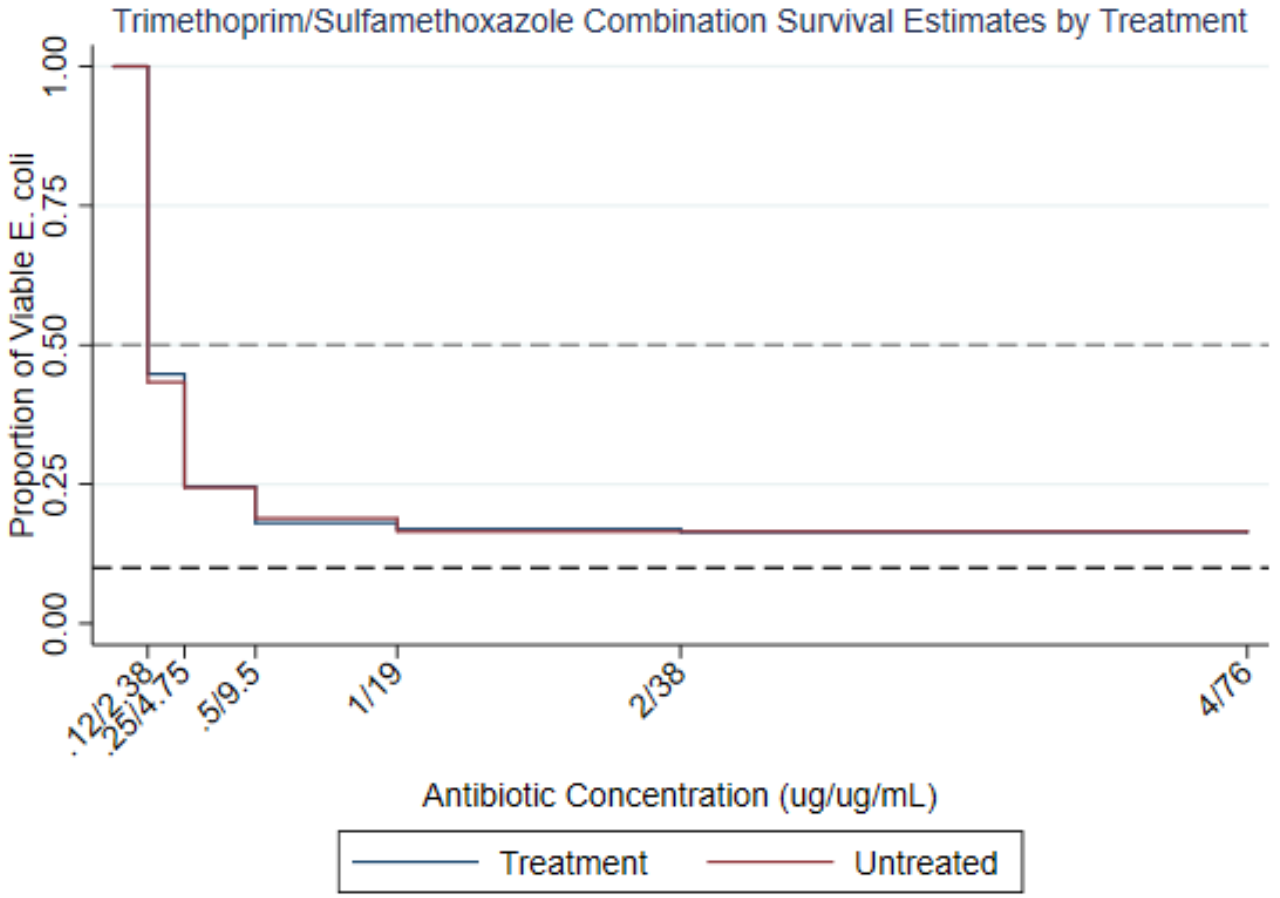

Figure 66: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to a trimethoprim and sulfamethoxazole combination. The treatment group is represented by the blue line and the untreated red. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

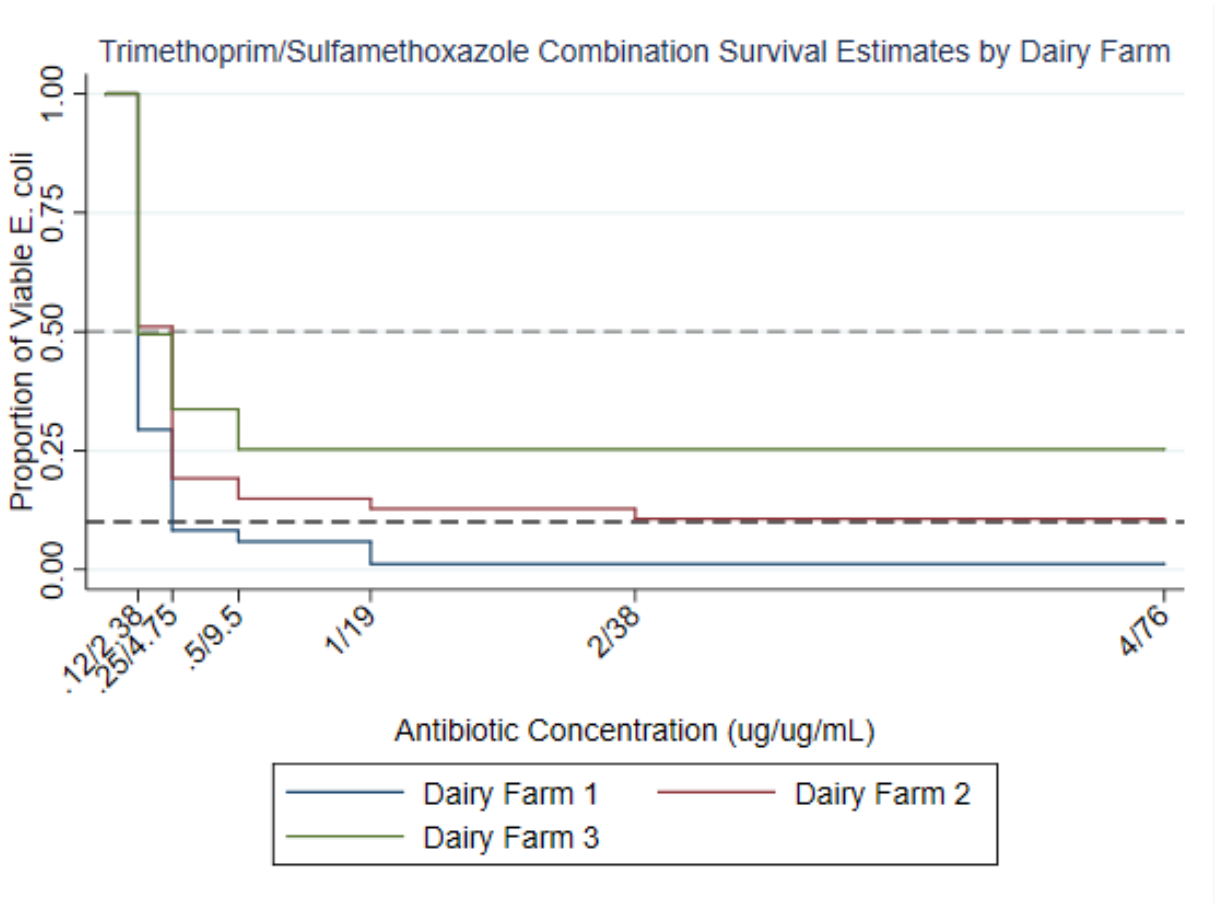

Figure 67: The Kaplan-Meier survival estimate is shown for isolates based on treatment regarding the phenotypic MIC to a trimethoprim and sulfamethoxazole combination. Dairy Farm 1 is represented by the blue line, Dairy Farm 2 the red, and Dairy Farm 3 the green line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

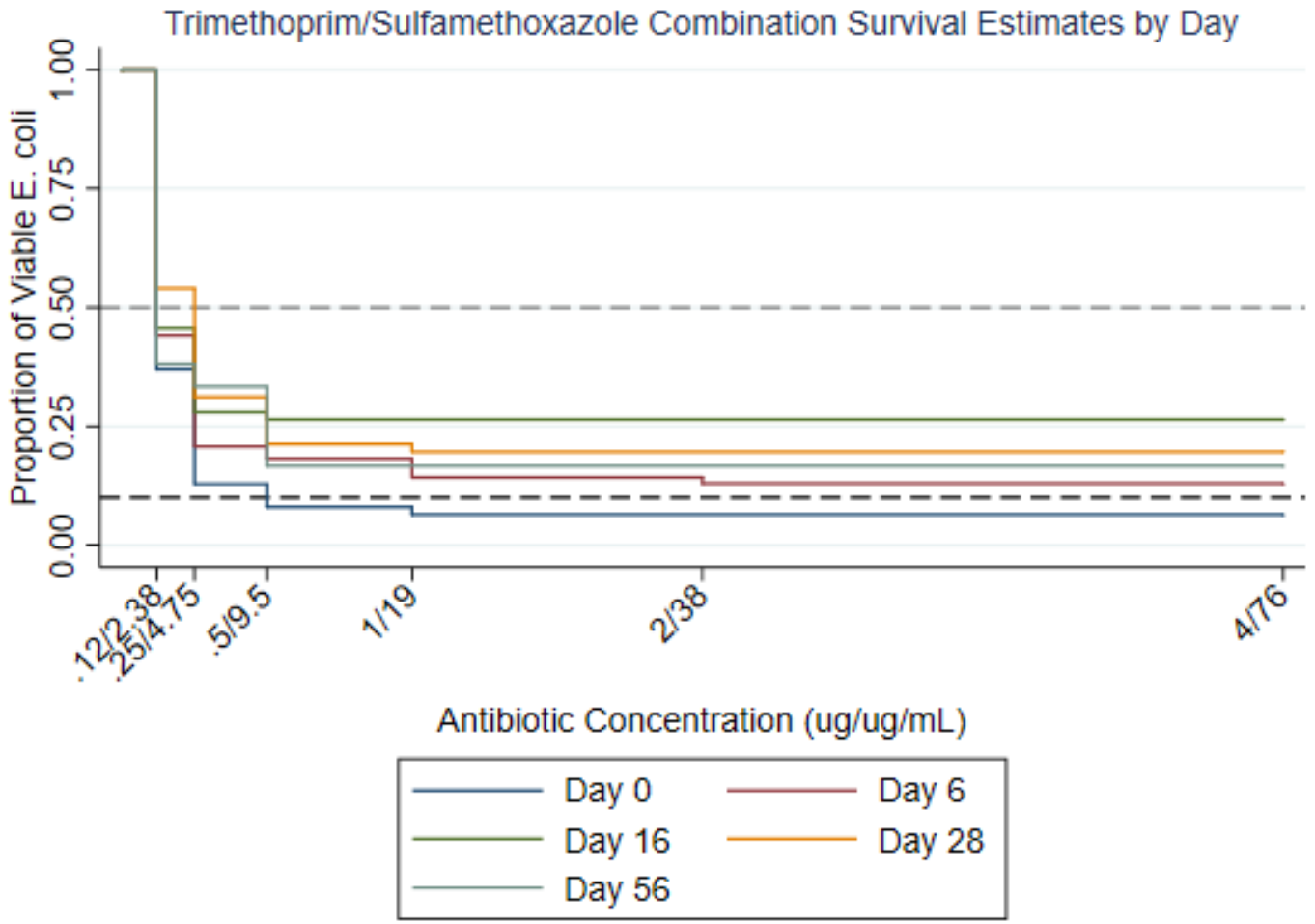

Figure 68: The Kaplan-Meier survival estimate is shown for isolates based on sample day regarding the phenotypic MIC to a trimethoprim and sulfamethoxazole combination. Day 0 is represented by blue, day 6 red, day 16 green, day 28 orange, and day 56 a gray line. The MIC 50 is shown by a dashed, grey line and the MIC 90 is represented by a dashed, black line.

4.4 *E. coli* WHOLE GENOME SEQUENCING

Select ESBL *E. coli* isolates were chosen for whole genome sequencing to explore the genotypic mechanisms for interesting resistance profiles and to gain insight into mechanisms of fluoroquinolone resistance. Of the 15 *E. coli* isolates, four were from Dairy Farm 1, 5 from Dairy Farm 2, and 6 from Dairy Farm 3. Isolates from treated animals accounted for 9 of the 15 isolates. Isolates were distributed by day with 3 from day 0, four from day 6, 3 from day 16, four from day 28, and 1 from day 56. Of selected isolates, 10 had an MIC of 0.5 µg/mL, three had an MIC of 1 µg/mL, and 2 had an MIC of 4 µg/mL to ciprofloxacin. Furthermore, 8 had an ESBL resistance profile, 1 had an AmpC resistance profile, 1 had an intermediate MIC to cefoxitin, 2 had an intermediate MIC to amoxicillin/clavulanic acid, had an intermediate MIC to both cefoxitin and amoxicillin/clavulanic acid, and 1 had resistance to cefoxitin but was susceptible to the amoxicillin/clavulanic acid. Isolates with intermediate susceptibility to cefoxitin or amoxicillin/clavulanic acid or with resistance to cefoxitin and susceptibility to clavulanic acid do not follow the typical AmpC or ESBL resistance profiles.

Resistance genes were determined via ResFinder (Zankari et al., 2012). These sequences had a mean contig length of 829 with a median of 185 and a range of 54 – 12,999. Additionally, the average N50 of sequenced *E. coli* isolates was 182,574 ranging from 991 – 507,721 and a median of 154,346. The mean genome size was 4,862,414 bp with a range of 4,627,288 bp – 5,354,275 bp with a median of 4,777,550 bp. One sequence was removed from analysis due to a genome size of 8,966,986 and suspected contamination. Of the isolates selected from MACFEP agar, four were from Dairy Farm 1 and 2 were from Dairy Farm 3. The $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-32}}$ genes were found among isolates from Dairy Farm 1 with 3 isolates also containing the *qnrB*19 gene encoding for reduced susceptibility to fluoroquinolones (Table 10). Suspected ESBL *E. coli*

isolates selected for sequencing based upon their ciprofloxacin MIC values displayed a wide range of *bla*$_{\text{CTX-M}}$ genes. *E. coli* isolates from Dairy Farm 1 contained *bla*$_{\text{CTX-M-1}}$ and $_{\text{-32}}$ within cluster 3 of the CTX-M gene family; isolates from Dairy Farm 2 contained *bla*$_{\text{CTX-M-1}}$, $_{\text{-15}}$, and $_{\text{-65}}$ among clusters 3 and 14 of the CTX-M gene family; and isolates from Dairy Farm 3 contained *bla*$_{\text{CTX-M-1}}$, $_{\text{-14, -15}}$, $_{\text{-27,-32}}$, $_{\text{-55}}$, and $_{\text{-65}}$ from clusters 3 and 14 of the CTX-M gene family (Zhao & Hu, 2013). One *E. coli* isolate from Dairy Farm 3 had both *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-32}}$, along with *bla*$_{\text{TEM-1A}}$ (the sequence from this isolate is thought to contain two bacteria due to an enlarged genome size noted above). In total, 2 *bla*$_{\text{CTX-M}}$ gene types were found in isolates from Dairy Farm 1, 3 from Dairy Farm 2, and 7 from Dairy Farm 3 among only 8, 5, and 8 *E. coli* isolates with a phenotypic ESBL resistance profile sequenced at each location.

Of the 6 isolates with a *bla*$_{\text{CTX-M-1}}$ gene, 5 of them also had a *bla*$_{\text{TEM-1A}}$ gene. Among isolates with *bla*$_{\text{CTX-M-14}}$ or higher (n = 14), 5 also contained *bla*$_{\text{TEM-1B}}$ with 1 containing *bla*$_{\text{OXA-10}}$. Uniquely, an isolate from Dairy Farm 1 with phenotypic cefoxitin resistance and ampicillin/clavulanic acid susceptibility contained a *bla*$_{\text{CTX-M-32}}$ gene, which does not fit the typical ESBL resistance profile. The only other resistance gene found in this isolate was *tet*(A). Each dairy farm had *E. coli* isolates with the fluoroquinolone reduced susceptibility gene *qnrB*19 with Dairy Farms 2 and 3 also having isolates with *qnrS*1. Furthermore, each farm had *E. coli* isolates with the *mph*(A) gene encoding for macrolide resistance (or, reduced susceptibility given the construct of the NARMS panel). Of the 8 isolates containing the *mph*(A) gene, 7 of them also contained the qnrB19 gene, and due to the selection criteria for the isolates, all had a *bla*$_{\text{CTX-M}}$ gene. Resistance genes for aminoglycosides, macrolides, phenicols, sulphonamides, rifampicin, tetracycline, and trimethoprim were also noted among the isolates.

In total, 13 different *E. coli* multi-locus sequence types were found among the sequenced isolates. Sequence types 10, 56, 301, 2325, and 5727 were found on Dairy Farm 1 with 10, 398, 683, 7588 on Dairy Farm 2, and 58, 69, 515, 2073, 2325, 5727, 7588, and an unknown sequence type on Dairy Farm 3. There were no dominant sequence types among the limited number of *E. coli* sequenced from the farms. However, sequence types 10, 2325, 5727, and 7588 were found on multiple farms.

Table 10: Resistance Genes of Interest among Sequenced *E. coli* Isolates.

Dairy Farm	Agar Type	Sequence Type	3GC Resistance Gene	Fluoroquinolone Gene	Macrolide Gene
1	CHROM-ESBL	*E. coli* [ST-10]	CTX-M-1	*qnr*B19	*mph*(A)
	MACFEP	*E. coli* [ST-5727]	CTX-M-1	N/A	*mph*(A)
	MACFEP	*E. coli* [ST-10]	CTX-M-1	*qnr*B19	*mph*(A)
	CHROM-ESBL	*E. coli* [ST-2325]	CTX-M-32	*qnr*B19	*mph*(A)
	CHROM-ESBL	*E. coli* [ST-301]	CTX-M-32	N/A	N/A
	CHROM-ESBL	*E. coli* [ST-301]	CTX-M-32	N/A	N/A
	MACFEP	*E. coli* [ST-56]	CTX-M-32	N/A	N/A
	MACFEP	*E. coli* [ST-10]	CTX-M-32	N/A	N/A
2	CHROM-ESBL	*E. coli* [ST-398]	CTX-M-1	*qnr*B19	*mph*(A)
	CHROM-ESBL	*E. coli* [ST-10]	CTX-M-1	*qnr*B19	*mph*(A)
	CHROM-ESBL	*E. coli* [ST-7588]	CTX-M-15	*qnr*S1	N/A
	CHROM-ESBL	*E. coli* [ST-683]	CTX-M-65	*qnr*S1	N/A
3	CHROM-ESBL	*E. coli* [ST-5727]	CTX-M-1	*qnr*B19	*mph*(A)
	CHROM-ESBL	*E. coli* [Unknown ST]	CTX-M-14 CTX-M-32	N/A	N/A
	CHROM-ESBL	*E. coli* [ST-69]	CTX-M-15	N/A	N/A
	CHROM-ESBL	*E. coli* [ST-7588]	CTX-M-15	*qnr*S1	N/A
	CHROM-ESBL	*E. coli* [ST-2325]	CTX-M-27	*qnrB19*	N/A
	CHROM-ESBL	*E. coli* [ST-515]	CTX-M-32	*qnr*B19	*mph*(A)
	MACFEP	*E. coli* [ST-58]	CTX-M-55	N/A	N/A
	MACFEP	*E. coli* [ST-2073]	CTX-M-65	N/A	N/A

Genes for 3GC and macrolide resistance and reduced susceptibility to fluoroquinolones are shown along with E. coli ST by dairy farm. CHROM-ESBL, CHROM-ESBL agar; MACFEP, MacConkey agar with 16 µg/mL of cefepime; 3GC, third-generation cephalosporin.

4.5 *SALMONELLA* SHEDDING

4.5.1 Salmonella Descriptive Statistics

The largest number of *Salmonella* positive samples were from the first sampling day (Table 11). Dairy Farm 3 had the lowest number of *Salmonella* positive samples with Dairy Farms 1 and 2 having nearly the same number of positive samples (Table 11). The number of *Salmonella* positive samples decreased in the subsequent days following day 0 (Table 11). At some point during the 3 sampling days, 50% of cattle from Dairy Farm 1, 47.6% of cattle from Dairy Farm 2, and 13.8% of cattle from Dairy Farm 3 tested positive for *Salmonella* shedding. *Salmonella* positive samples were found at similar levels among treated (17.1%) and untreated (18.7%) treatment groups (Figure 69). A higher percentage of samples were *Salmonella* positive in the trial beginning in the fall (18.64%) than the spring (12.69%).

Table 11: Frequency of *Salmonella* Growth by Dairy Farm and Sample Day

***Salmonella* Growth**	**Dairy Farm Number** Dairy Farm 1	Dairy Farm 2	Dairy Farm 3	**Total**
Sample Day 0				
Growth	34	32	7	73
No Growth	48	52	73	173
Total	82	84	80	246
Sample Day 6				
Growth	12	7	4	23
No Growth	72	69	75	216
Total	84	76	79	239
Sample Day 16				
Growth	7	12	4	23
No Growth	76	65	75	214
Total	83	77	79	239

The distribution of *Salmonella* growth on BGA is displayed by dairy farm and sampling day.

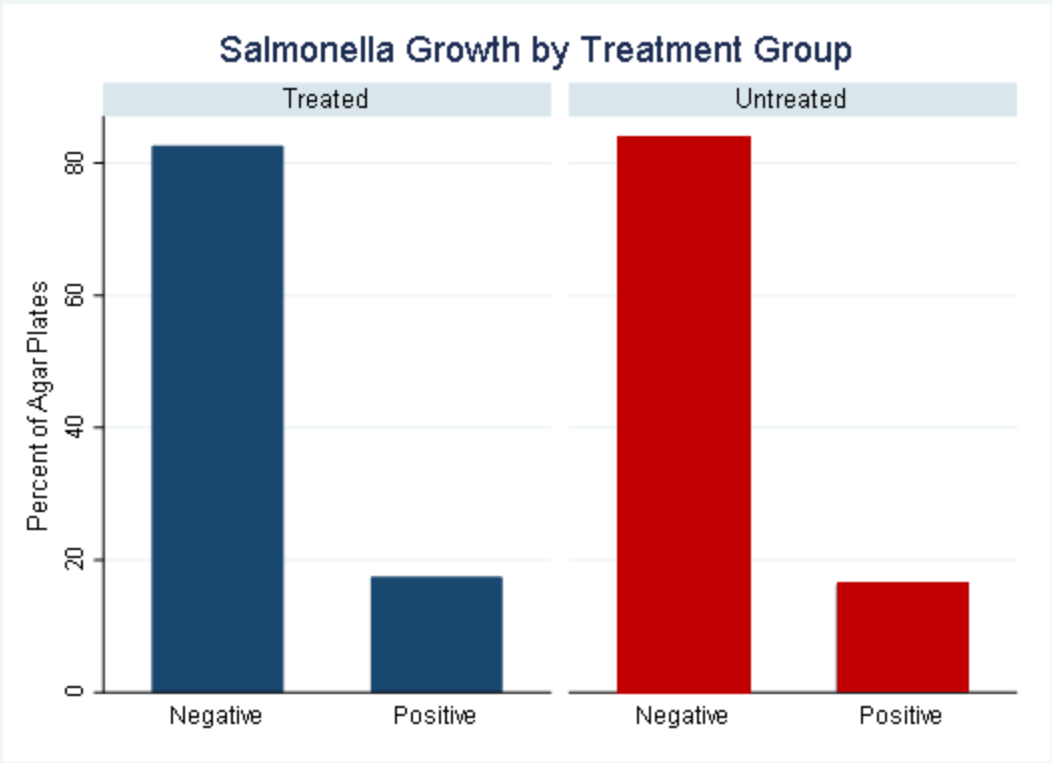

Figure 69: The percentage of samples testing positive and negative across the treated and untreated groups are shown above.

There were four samples positive for phenotypic 3GC resistant *Salmonella*. Dairy Farm 1 produced 1 3GC resistant *Salmonella* positive sample on day 0. Dairy Farm 2 did not test positive for any 3GC resistant *Salmonella* samples. Dairy Farm 3 tested positive for 1 3GC resistant *Salmonella* sample on day 0 and 2 on day 6. A positive sample from day 6 was from the same animal producing the 3GC resistant *Salmonella* on day 0. All 3GC resistant *Salmonella* positive samples were collected from treated animals; however, this was not significant based upon the Fisher's exact test ($P = 0.062$).

4.5.2 Prevalence of Salmonella Shedding

Salmonella prevalence via growth on BGA did not yield statistically significant differences between the treated and untreated groups across time (Figure 70). The treated group (cows with metritis) had a greater probability of being *Salmonella* positive than the untreated group on day 0 (P = 0.058, 95% CI = -0.037 – 2.140). The prevalence of *Salmonella* in the treated group decreased on day 6, but was not different from the untreated group. (P = 0.463, 95% CI = -1.812 – 0.824) and remained at similar levels on day 16, which was omitted for collinearity. These differences were not statistically significantly different from the treatment group for any of the 3 days due, in part, to robust marginal mean confidence intervals. A large amount of the variation in the model was attributed to the dairy farm (31.3%).

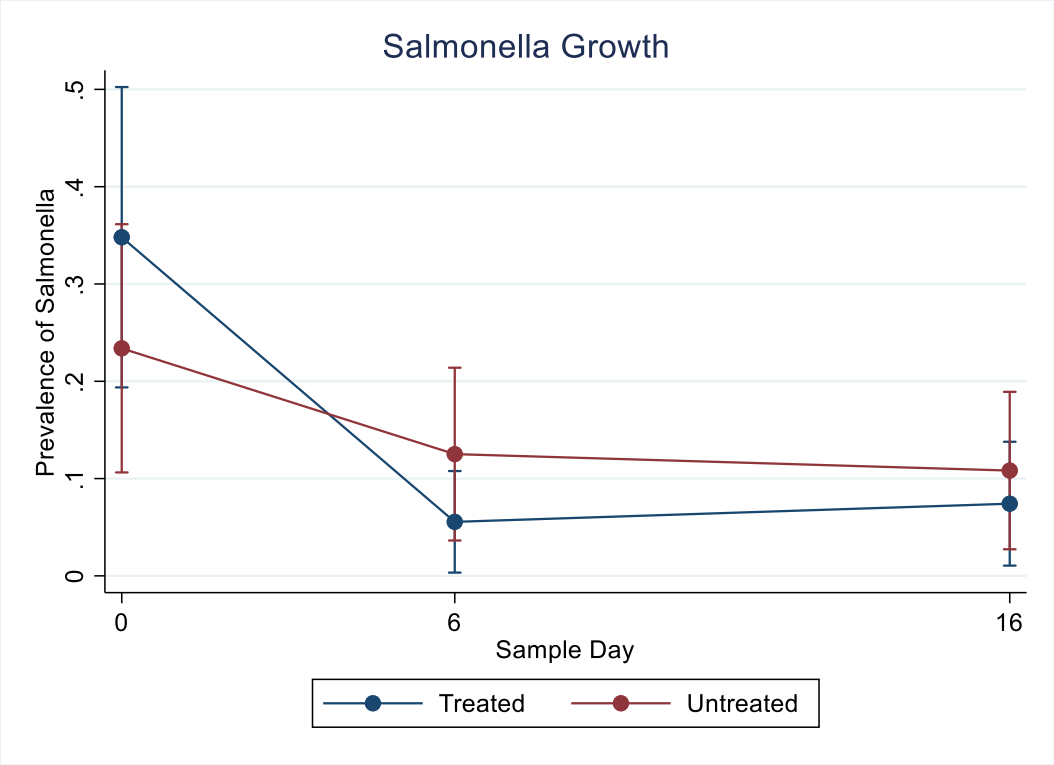

Figure 70: The probability of *Salmonella* growth across sampling day is shown above with the treated group represented by the blue line and the untreated group represented by the red line accompanied by 95% confidence intervals with marginal means.

Dairy Farms 1 and 2 had higher probability of samples from both treated and untreated groups testing *Salmonella* positive than those from Dairy Farm 3 (Figure 71). Both dairy farms displayed decreases in the probability of being *Salmonella* positive as time progressed; however, differences were not observed between treated and untreated groups. The prevalence observed in the treated group of Dairy Farm 1 started at 48.3% (95% CI = 32.7% – 63.8%) before decreasing to 9.4% (95% CI = 0.6% – 18.2%) on day 6 and 4.9% (95% CI = -1.7% – 11.4%) on day 16. The untreated group had a starting prevalence of 35.4% (95% CI = 20.7% – 50.0%) before decreasing

to 18.7% (95% CI = 6.9% – 30.5%), and 11.5% (95% CI = 2.0% – 20.9%). These numbers are similar to what was observed on Dairy Farm 2 where the treated group started with a prevalence of 49.6% (95% CI = 34.0% – 65.3%) prior to decreasing to 4.4% (95% CI = -1.6% – 10.5%) and increasing slightly to 14.5% (95% CI = 3.5% – 25.5%) on day 16. The untreated group on the same farm began with a prevalence of 28.9% (95% CI = 15.2% – 42.6%), then decreased to 12.9% (95% CI = 2.4% – 23.3%) on day 6 and was at 15.7% (95% CI = 04.2% – 27.2%) on day 16. The treated group of Dairy Farm 3 began with the lowest prevalence of the 3 groups at 6.5% (95% CI = -2.2% – 15.3%) prior to dropping to 3.3% (95% CI = -3.0% – 9.6%) and remaining at that level at day 16 (95% CI = -3.0% – 09.6%). The prevalence in the untreated group started at 12.0% (95% CI = 0.4% – 23.6%) before reducing to 6.2% (95% CI = -2.3% – 014.8%) on day 6 and dropping slightly to 6.1% (95% CI = -2.3% – 14.5%) on day 16.

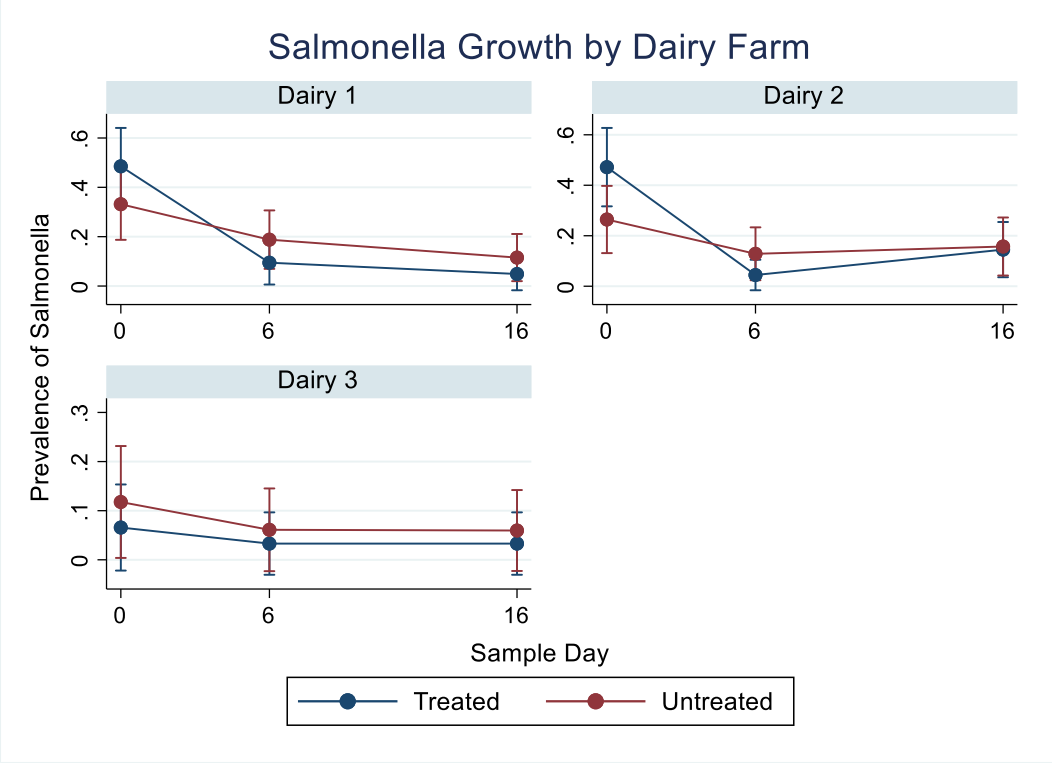

Figure 71: The probability of *Salmonella* growth is displayed by individual farm with the blue line representing treated animals and a red line representing untreated. 95% confidence intervals with marginal means are also shown.

4.6 *SALMONELLA* WHOLE GENOME SEQUENCING

To further evaluate the *Salmonella* isolates, all isolates displaying phenotypic 3GC resistance (n = 4) were sequenced and all (n = 119) but 1 isolate, selected from the BGA plates were sequenced (1 was not located in storage). SeqSero (http://www.denglab.info/SeqSero) was used to determine *Salmonella* serotypes and resistance genes were determined via ResFinder (Zankari et al., 2012). The average contig length was 230 base pairs (bp) with a median of 100 and range between 58 and 5154 base pairs. The average N50 of the sequences was 358,667 bp

with a median of 372,009 and a range of 3205 – 703,778 bp. The mean genome size was 4,809,808 with a range of 4,560,150 – 7,348,638 bp and a median of 4,760,431.

Each dairy farm contained multiple *Salmonella* serotypes, but different serotypes dominated within the general population (Table 12). Dairy Farm 1 had four serotypes and 1 unidentified, but serotypes Anatum and Montevideo accounted for 82.14% of the *Salmonella* sequenced on this farm. Dairy Farm 2 had many more serovars, as eleven were present with 4 samples having serotypes that were undetermined. Serovars Cerro, Meleagridis, and Muenster were found at a higher frequency. These serotypes accounted for 67.35% of the isolates sequenced from Dairy Farm 2. Only 12 serotypes were found within the general *Salmonella* population on Dairy Farm 3, although a third serovar was found upon evaluation of an isolate displaying phenotypic 3GC resistance. The dominant serovar on this dairy farm was Meleagridis, which accounted for 92.86% of the serovars within the general population.

Serovar dominance varied based upon the sampling season. Montevideo accounted for 85.0% of the 20 isolates in the spring, but only 44.4% of the four serovars isolated from fall samples on Dairy Farm 1. Similarly, serovar Cerro accounted for 85.71% of the 12 *Salmonella* serovars isolated from spring samples, while serovar Cerro only accounted for 22.9% of the 11 serovars isolated from fall samples followed by Meleagridis (20.0%) and Muenster (17.1%) on Dairy Farm 2. No samples tested positive for *Salmonella* during the spring on Dairy Farm 3, but serovar Meleagridis accounted for 92.9% of the *Salmonella* serovars isolated from fall and total *Salmonella* serovars on Dairy Farm 3. It is not surprising a greater percentage of total *Salmonella* was isolated from fall samples (Dairy Farm 1: 64.3%, Dairy Farm 2: 71.4%, and Dairy Farm 3: 100.0%) because more samples were taken in the fall sampling season; also, the literature supports a higher prevalence at the end of warmer months in summer.

While samples from Dairy Farm 2 had the highest number of serovars, Dairy Farms 1 and 2 had the highest number of animals testing positive for multiple serovars across the study days. Each of farms 1 and 2 had four and Dairy Farm 3 had 1. Only 1 animal was positive for 3 serovars and it was located on Dairy Farm 2. It is worth noting there may be more animals harboring multiple *Salmonella* serovars, as only 1 isolate from each positive sample was sequenced. Therefore, any animal determined to harbor multiple serotypes was because a different serotype was detected in either a sample from another sampling day in the study or because a different serotype was detected in an isolate from a plain BGA plate in comparison to an isolate from a BGACEF agar plate.

Table 12: Frequency of *Salmonella* Serotypes by Dairy Farm

Dairy Farm	Serovar	Spring Frequency (Percentage)	Fall Frequency (Percentage)	Overall Frequency (Percentage)
Dairy Farm 1	Anatum	0 (0.00%)	13 (36.11%)	13 (23.21%)
	Cerro	1 (5.00%)	2 (5.56%)	3 (5.36%)
	Meleagridis	2 (10.00%)	4 (11.11%)	6 (10.71%)
	Montevideo	17 (85.00%)	16 (44.44%)	33 (58.93%)
	N/A	0 (0.00%)	1 (2.78%)	1 (1.79%)
	Serovar Total: 4	*20 (35.71%)*	*36 (64.29%)*	*56 (47.06%)*
Dairy Farm 2	Agoueve	0 (0.00%)	1 (2.86%)	1 (2.04%)
	Cerro	12 (85.71%)	8 (22.86%)	20 (40.82%)
	Derby	0 (0.00%)	1 (2.86%)	1 (2.04%)
	Mbandaka	0 (0.00%)	1 (2.86%)	1 (2.04%)
	Meleagridis	0 (0.00%)	7 (20.00%)	7 (14.29%)
	Montevideo	0 (0.00%)	1 (2.86%)	1 (2.04%)
	Muenster	0 (0.00%)	6 (17.14%)	6 (12.24%)
	N/A	0 (0.00%)	4 (11.43%)	4 (8.16%)
	Oranienburg	0 (0.00%)	1 (2.86%)	1 (2.04%)
	Panama	1 (7.14%)	1 (2.86%)	2 (4.08%)
	Typhimurium	0 (0.00%)	1 (2.86%)	1 (2.04%)
	Virginia	1 (7.14%)	3 (8.57%)	4 (8.16%)
	Serovar Total:11	*14 (28.57%)*	*35 (71.43%)*	*49 (41.18%)*
Dairy Farm 3	Meleagridis	0 (0.00%)	13 (92.86%)	13 (92.86%)
	Montevideo	0 (0.00%)	1 (7.14%)	1 (7.14%)
	Serovar Total: 2	0 (0.00%)	*14 (100.00%)*	*14 (11.76%)*
	Serovar Total: 13	*34 (28.57%)*	*85 (71.43%)*	*119 (100%)*

The number of different *Salmonella* serovars in the general sample population per dairy farm are displayed, along with sevovar frequency.

Third-generation cephalosporin resistance was detected in 5 isolates from 5 samples in four animals. Isolates from the same sampling day showing beta-lactam resistance on both BGA and BGACEF agar are only included once in Table 13 to avoid overinflating the number of samples with 3GC resistance. All *Salmonella* isolates with 3GC resistance contained the $bla_{\text{CMY-2}}$

gene. Only one isolate was shown to possess the *bla*$_{\text{CMY-2}}$ gene in a sample not showing phenotypic resistance. This isolate also had genotypic resistance to aminoglycosides, sulfonamides, streptomycin, and trimethoprim. It is not surprising that the dairy farm with the highest usage of ceftiofur also harbored the highest number of samples with 3GC resistant *Salmonella*, whether phenotypic or genotypic resistance.

Table 13: Frequency of *Salmonella* Serotypes and Resistance Genes by Dairy Farm

Dairy Farm	Serotype	Frequency	Phenotypic Beta-Lactam Resistance (%)	Resistance Genes				
				Aminoglycoside Resistance Gene	Beta-Lactam Resistance Gene	Sulphonamide Resistance Gene	Streptomycin Resistance Gene	Trimethoprim Resistance Gene
Dairy Farm 1	Anatum	1	100%	N/A	*bla*$_{\text{CMY-2}}$	N/A	N/A	N/A
Dairy Farm 2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Dairy Farm 3	Meleagridis	3	66%	N/A	*bla*$_{\text{CMY-2}}$	N/A	N/A	N/A
				N/A	*bla*$_{\text{CMY-2}}$	N/A	N/A	N/A
				aadA2	*bla*$_{\text{CMY-2}}$	*sul*2	*str*A, *str*B	*dfr*A12
	Newport	1	100%	N/A	*bla*$_{\text{CMY-2}}$	N/A	N/A	N/A

Sequenced *Salmonella* displaying phenotypic or genotypic beta-lactam resistance are displayed, along with serotype, frequency, and resistance gene.

CHAPTER V

DISCUSSION

5.1 FECAL *E. coli*

The results of this study highlight the impact of a two-dose treatment of CCFA for metritis on the dynamics of total and 3GC resistant *E. coli* populations over time. The data have shown that a 13-day withholding period from the final administration of a two-dose treatment of CCFA for metritis may be insufficient to ensure that 3GC resistant *E. coli* populations return to baseline levels in all treated cattle and thus mitigate the risk of these organisms "escaping from the farm" as illustrated in the risk assessment framework of Hurd et al. (2004, 2006). It has previously been reported that at the aggregate level, dairy farms employing ceftiofur are 25 times more likely to have detectable *E. coli* with reduced susceptibility to ceftriaxone (Tragesser et al., 2006). The fact that all 3 dairy farms in our study harbored 3GC resistant *E. coli* is consistent with earlier work that found 92% of tested herds had ceftriaxone-resistant *E. coli* and 88% of tested herds used ceftiofur (Heider et al., 2009). While the number of samples containing resistant *E. coli* varied, each dairy farm had detectable *E. coli* with phenotypic resistance to ceftriaxone. As expected, based on work by Lowrance et al. (2007), Kanwar et al. (2008) and Singer et al. (2014), the total *E. coli* count in cattle decreased significantly following treatment with CCFA, but rebounded over time (Figure 6). The distribution of *E. coli* growth on MAC agar is likely right truncated to do restrictions regarding our colony counting methodologies.

Furthermore, 3GC resistant *E. coli* populations were significantly higher in treated groups than untreated groups on day 6 (Figure 8). This was observed even with large numbers of 0 counts and remained after the large 0 counts (Figure 4) were imputed to distribute those values

across levels below detection (Figure 4). It seemed implausible that the distribution contained true 0 values below the limit of quantification for the assay (i.e., that the 0 values were truly 0), given the cows are raised in common shared space. A number of techniques have been deployed and evaluated to deal with this problem (Boyer, Hanson, & Singer, 2013). While not significant ($P<0.05$) overall, with robust marginal mean confidence intervals, treated animals harbored higher levels of 3GC resistant *E. coli* than untreated groups on study day 16. While there were a few samples with 3GC resistance among control animals (75^{th} percentile = 0), there remained elevated levels in treated cows (75^{th} percentile = 2.603 $\log_{10}$ CFU). It has been suggested such increases in total 3GC resistant *E. coli* counts may be due to an increased ability to detect them due to the suppression of total growth (Singer, Patterson, & Wallace, 2008); however, at day 16 total *E. coli* CFU (MAC) did not differ significantly between groups. The probability of 3GC resistant *E. coli* increased in cows with parities above 3. This may be a result of these animals having been repeatedly treated with or had exposure to CCFA throughout their life.

When evaluating the arithmetic difference in total and 3GC resistant *E. coli* growth, higher proportions of colonies were resistant to 3GCs in the treated groups on days 6 and 16 than among untreated groups (Figure 10). However, the level of significance regarding these counts varied by dairy farm (Figure 11). This may be a result of increased 3GC resistant *E. coli* populations in environmental manure accumulating based on higher levels of historical ceftiofur usage. Due to the significance of historical usage in impacting levels of 3GC resistant *E. coli*, we hypothesize such historical dairy features play a role in expanding 3GC populations in the environment. This helps sustain a higher proportion of 3GC resistant *E. coli* in the gut of untreated animals, and leads to an increased time for resistant bacterial populations to return to pre-treatment levels following a two-dose CCFA treatment for metritis. However, due to our

small environmental sampling size, limited number of farms, and historical usage data consisting of only the year prior to the start of the study, further work is required to properly evaluate the role of historical usage.

It is also important to note the variation of systemic ceftiofur usage. A quarter of the historical usage on Dairy Farm 2 was intramammary, which would be less likely to affect levels of 3GC resistance among fecal *E. coli*. On the other hand, Dairy Farm 1 did not use any intramammary ceftiofur and a smaller portion of ceftiofur use on Dairy Farm 3 was intramammary which helps explain their elevated 3GC resistance among fecal bacteria. The currently observed differences in ceftiofur administration across multiple farms may be a result of other variables not considered for the purpose of our study, such as the incidence of foot rot and bovine respiratory disease, or the severity or form of metritis at the time of diagnosis. Additionally, since rates of treatment were collected from farm records and therefore were farm-level reported, actual treatment rates may differ from calculated values. Unfortunately, we were unable to aggregate historical usage data for additional antibiotics used on the farms in the previous year.

One might imagine farms using ceftiofur at elevated rates may be using greater amounts of other antibiotics, but this is far from certain and an important area to consider in future research. Across all dairy farms, the proportion of total and 3GC resistant populations returned to pre-treatment levels by days 28 and 56. It has been hypothesized that bacteria with 3GC resistance are less able to compete with general *E. coli* populations after the removal of the antibiotic selection pressure due to the fitness cost of harboring a functional AmpC $bla_{\text{CMY-2}}$ or another ESBL gene (Lowrance et al., 2007; Singer, Patterson, & Wallace, 2008), thereby explaining their decline post-treatment.

These results suggest a 13-day withholding period following CCFA treatment does not universally provide adequate time to allow for 3GC resistant populations of enteric *E. coli* to return to baseline levels. While these changes in 3GC resistant *E. coli* populations may seem inconsequential when evaluating a single gram of feces, they are not when considering the many kg of fecal matter produced by the animal. There are well-documented instances of beef being contaminated with AMR Enterobacteriaceae, based on meat samples taken in butcher shops (Singer, Patterson, & Wallace, 2008) and grocery stores (Schroeder et al., 2003; Johnson et al., 2005; Vogt & Dippold, 2005) and with potential transmission from such sources to the human population (Angulo, Nargund, & Chiller, 2004). We hypothesize, based upon our findings, there may be an increased risk posed to public health should an animal be culled at the current 13-day withholding period due to elevated levels of 3GC resistant fecal *E. coli*. Studies determining slaughter withholding periods tend to be performed in healthy animals (Witte et al., 2011); however, the pharmacokinetics and pharmacodynamics are likely to vary between healthy and clinically ill populations (Kissell et al., 2015) due to pathophysiological changes associated with illness. Our study provides insight into the fluctuation of bacterial populations in ill animals undergoing treatment versus their healthy untreated counterparts. While *E. coli* were not typed as commensal or pathogenic, because *E. coli* is an indicator species for gram-negative bacteria, these findings may model patterns that could be observed in other enteric gram-negative pathogens such as *Salmonella* spp. (Lowrance et al., 2007; Cummings, Aprea, & Altier, 2014).

Further studies should be performed to evaluate the effects of such treatment on 3GC resistance and shedding of *Salmonella*, along with quantitative polymerase chain reactions (qPCR) or shotgun metagenomics to evaluate the absolute and relative change in resistance genes found within samples across time. Based on these data, an additional withholding period

prior to slaughter would be advisable on farms with a long-standing history of cephalosporin use, or with known moderate to high levels of 3GC resistance, in order to reduce the risk to public health and improve antimicrobial stewardship. While a decade ago when ESBLs had not yet been reported in the U.S., Daniels et al. reported the frequency with which ceftiofur is administered does not impact levels of commensal *E. coli* containing the $bla_{\text{CMY-2}}$ gene at the herd-level (2009); however, discrepancies likely exist regarding genotypic and phenotypic AMR indicators based upon the level of antibiotic usage on dairy farm (Davis et al., 2011). Bacteria from dairy farms with low levels of antibiotic usage may still harbor bacteria with genetic elements encoding for resistance, but not at levels high enough to detect phenotypic resistance; meanwhile, the opposite may be observed on farms with high levels of antibiotic usage (Davis et al., 2011).

5.2 AmpC AND ESBL *E. coli* DIFFERENTIATION

The purpose of plating selected samples grown on MACCEF and CHROM-ESBL agar to the 5 different agar types (plain and agar containing ceftriaxone, cefoxitin, cefepime, or ceftriaxone with clavulanic acid at CLSI breakpoints) was to determine the proportion of isolates displaying phenotypic resistance to ceftriaxone had AmpC or ESBL phenotypic forms of resistance. Cefoxitin resistance has been shown to be highly correlated with the $bla_{\text{CMY-2}}$ AmpC gene while cefepime resistance highly correlated with the $bla_{\text{CTX-M}}$ ESBL gene (Davis et al., 2015). Unfortunately, even with a subset of samples that displayed growth on both MACCEF and CHROM-ESBL agars, we were unable to determine the proportion of 3GC resistance attributed to AmpC or ESBL resistance profiles via this method, regardless of enrichment.

Samples processed without incubation produced countable quantities of growth among all plates displaying growth at detectable limits; however, MACFEP plates did not yield detectable amounts of growth (Figure 12). When samples were enriched in MAC broth with 1 µg/mL of ceftriaxone for 18 hrs, 6 MACFEP plates yielded quantifiable amounts of *E. coli* growth, but a majority of growth on all other agar types yielded a growth of colonies that were too numerous to count (Figure 13). In parallel with the children's story "*Goldie Locks and the Three Bears*", if no enrichment is not enough and an extended period of enrichment is too much, some enrichment should be just right. This was not the case, as a 3 hour incubation in MAC broth with 2 µg/mL of ceftriaxone yielded MACFEP agar plates without detectable levels of *E. coli* growth, while other agar types yielded a majority of plates with countable levels of growth, as with the non-enriched samples.

The idea of increasing the concentration of ceftriaxone in the broth was brought about to remove bacteria with an MIC below 2 µg/mL. This was to reduce the competition for broth resources from undesired bacteria. The reduced enrichment time was to provide the bacteria in lower quantities ample time to divide without providing enough time for more populous bacteria to grow to levels beyond quantification, thereby reducing the chance of plates with colonies too numerous to count. Multivariate regression modeling determined there were no differences in the level or lack of growth among each of the agar plates without enrichment or with 3 hrs of enrichment ($P = 0.270$) (Figure 15). The extended enrichment period with 1 µg/mL of ceftriaxone displayed elevated levels of growth among each of the 5 agar plates ($P < 0.0001$).

We believe we are the first to attempt to determine the phenotypic proportion of AmpC and ESBL resistance mechanisms among a population with phenotypic 3GC resistance. Previous work by Mollenkopf et al. (2012) used MacConkey agar with cefepime and cefoxitin to estimate

the prevalence of ESBL and AmpC *E. coli* shedding with PCR to detect $bla_{\text{CTX-M}}$ or $bla_{\text{CMY-2}}$ genes. Davis et al. (2015) used plain MacConkey agar and MacConkey agar with ceftiofur, cefoxitin, or cefepime to screen for AmpC and ESBL *E. coli* in order to select isolates and use PCR to screen for $bla_{\text{CTX-M}}$ and $bla_{\text{CMY-2}}$ genes. However, neither of these studies sought to determine the phenotypic proportion of AmpC or ESBL *E. coli* through colony counting methodologies. This may be a result of *E. coli* housing the $bla_{\text{CTX-M}}$ gene for the ESBL resistance profile not being discovered in American agriculture until 2010 (Wittum et al., 2010). Another possible reason, as our results indicate, is ESBL *E. coli* are found in low levels requiring enrichment to coax to detectable levels. Each way in which ESBL *E. coli* were detected in our study involved an 18 hour enrichment period in MAC broth containing ceftriaxone. While proportions have not been determined based upon colony count data, microbroth dilution data from plates determining the phenotypic MIC of gram-negative bacteria can be utilized to determine the proportion of AmpC and ESBL *E. coli* within the selected isolates. This would be based upon the MIC of isolates to a second-generation cephalosporin (cefoxitin) and the addition of a beta-lactam inhibitor (clavulanic acid). Due to resistance to these compounds, it can be inferred the *E. coli* isolate has an AmpC resistance profile. Resistance to a third-generation cephalosporin without resistance to a second-generation cephalosporin or a pairing with a beta-lactamase inhibitor would indicate an ESBL. Unfortunately, a fourth-generation cephalosporin is not included on such gram-negative plates to further support the presence of an isolate containing an ESBL resistance profile. Additionally, whole genome sequencing could provide further insight into the genotypic mechanisms involved.

5.3 AmpC/ESBL FECAL *E. coli* SHEDDING

5.3.1 Descriptive Statistics

Through the CROM-ESBL agar selection process, nearly 26% of samples tested positive for *E. coli* that should have had an ESBL resistance profile; however, only 74.52% of those isolates showed a phenotypic ESBL *E. coli* resistance profile (resistance to ampicillin and ceftriaxone but susceptible to cefoxitin and amoxicillin/clavulanic acid) with the others showing an AmpC phenotypic resistance profile (resistance to ampicillin, ceftriaxone, cefoxitin, and amoxicillin/clavulanic acid combination). Resistance to cefoxitin is highly correlated with an AmpC resistance genotype (Davis et al., 2015). While nearly 2/3 of phenotypic ESBL *E. coli* isolates were from treated cows, ESBL versus AmpC *E. coli* was not dependent on the animal being treated. This is most likely a result of our enriching the samples to make sure we identified as many ESBL *E. coli* positive samples as possible and not counting the CHROM-ESBL agar plates. While we previously tried to perform this with selective MacConkey agar, but were unable to do so due to low populations of ESBL *E. coli*, and were further unable to accomplish this due to CHROM-ESBL agar not exclusively identifying the ESBL resistance profile.

5.3.2 E. coli Antibiotic Class Resistance

Co-selection of antimicrobial resistance can happen due to resistance genes being contained on the same mobile genetic elements. These genes are then spread via the horizontal transfer of plasmids, transposons, and integrons between bacteria (Bennett, 2008; Tacão et al., 2014). Therefore, selection for resistance to an antibiotic can occur without the use of that antibiotic. Mechanisms for the ESBL resistance profile are not an exception, as the *bla*$_{\text{CTX-M}}$ gene

is commonly plasmid-mediated (Livermore et al., 2007). It is important to remember characterized isolates in our study were selected through a selective process using CHROM-ESBL agar, are not representative of the total population, and samples with plates displaying no growth were specified as negative.

The isolates characterized to generate microbroth dilution data were not those of the general population, but of a subpopulation of *E. coli* representing a resistance profile found in smaller numbers within the overall population. The number of antibiotic classes to which an isolate was resistant in the presented model was first dependent upon a sample producing an isolate on the selective agar, which automatically classified the isolate to have resistance to 2 or more antibiotic classes. Selecting isolates from the general *E. coli* population may yield different results.

In addition to the typical ESBL resistance profile, co-resistance has been observed to aminoglycosides, tetracycline, quinolones, and sulfamethoxazole/trimethoprim in *E. coli* isolates (Cantón and Coque, 2006) from medical centers (Winokur et al., 2001[b]; Morosini et al., 2006), aquatic systems (Tacão et al., 2014; Bajaj et al., 2015), companion animals (Moreno et al., 2008), and dairy cows (Schwaber et al., 2005; Mann et al., 2011). While our study found co-resistance among aminoglycosides and tetracycline, co-selection was also observed among phenicols and sulfonamides, but not quinolones or sulfamethoxazole/trimethoprim. However, there was a connection regarding reduced susceptibility to quinolones. It has been shown ESBL *E. coli* isolates from beef farms were less likely to have resistance to aminoglycosides, quinolones, and trimethoprim/sulfamethoxazole in comparison to those on farms with dairy and beef cattle (Schmid et al., 2013). Alternatively, while there were differences among co-resistance of ESBL *E. coli* from dairy and beef cattle, isolates with AmpC resistance profiles were co-

resistant to streptomycin, sulfamethoxazole, and tetracycline from both groups, although isolates from beef sources were resistant to ampicillin and chloramphenicol (Tragesser et al., 2006; Lowrance et al., 2007).

Our results based upon Kaplan-Meier curves and Mantel-Haenszel rate ratios, gave insight into how the variables of treatment, farm, and day impact the MIC values of isolates selected from CHROM-ESBL agar, regardless of AmpC and ESBL resistance phenotype, and the concentration at which isolates from those groups reach their MIC. The MIC of isolates was significantly higher as for Dairy Farm 1 in comparison to Dairy Farm 2, which was lower than Dairy Farm 3 for amoxicillin/clavulanic acid (Figures 17-20), cefoxitin (Figures 25-28), ceftiofur (Figures 29-32), chloramphenicol (Figures 37-40), ciprofloxacin (Figures 41-44), gentamicin (Figures 45-48), streptomycin (Figures 53-56), and trimethoprim/sulfamethoxazole (Figures 65-68) while moving upward for all other tested antibiotics (Figures 21-24, 33-36, 49-52, 57-60), but tetracycline Figures (61-64). Tetracycline was significant in the opposite direction. This may be due to Dairy Farm 1 using lower order antibiotics, like tetracycline, more frequently than the other 2. The MIC of isolates increased each time day increased for amoxicillin/clavulanic acid, cefoxitin, and trimethoprim/sulfamethoxazole (Figures 20, 28, and 68), but rates decreased as day increased for naladixic acid (Figure 52). The only statistical difference among the antibiotics with regards to treatment group was the control group had a greater proportion of isolates with smaller MICs to gentamicin than the treated group (Figure 46).

Concerning trends were observed regarding MICs of the fluoroquinolone, ciprofloxacin, with multiple isolates (n = 36) testing as resistant. Isolates from later sampling days tended to have higher MICs (Figure 44), while isolates from Dairy Farm 2 had a top MIC of 0.5 µg/mL, Dairy Farms 1 and 3 had isolates with MICs greater than 4 µg/mL. Particularly interesting,

reduced susceptibility to quinolones as conferred by the *qnr* genes typically allows the bacteria greater opportunity to develop the gyrase mutation under reduced antibiotic concentration. This was observed on Dairy Farms 1 and 3 where there were several isolates with resistance and reduced susceptibility. However, Dairy Farm 3 did not have any isolates with an MIC about 0.5 µg/mL (of the isolates tested); meaning they likely had fewer bacteria with *qnr* genes. Seeing quinolone resistance in isolates from this setting is concerning due to the fluoroquinolones not being used in dairy cows past 20 months of age. If this resistance form were to persist within animals and the environment and be co-selected for with ESBL isolates, as previous studies have shown, this could create a dangerous resistance combination of 2 already serious forms of resistance. Both third (and higher) generation cephalosporins and fluoroquinolones are deemed critically important of highest priority to human medicine (WHO, 2019). Furthermore, the finding of macrolide resistance/reduced susceptibility via the *mph*A gene is concerning, especially when found in conjunction with quinolone reduced susceptibility and 3GC resistance genes. Macrolides are commonly used in feedlot production settings, but not in dairy production. Bacteria with such resistance may be coming from nearby feedlot settings in the region. Unfortunately, if now found in dairy farm settings in the region and associated with a 3GC resistance gene, macrolide resistance may become inadvertently selected for through 3GC use.

One should resist drawing too much from the results of treatment and day because of the inability to interact variables in these latter analyses. The interaction of treatment and day is biologically significant due to the rate at which the antibiotic is absorbed, metabolized, and excreted by the animal. However, the results relating to dairy farm further provide insight into 1 of the themes spanning this project. Isolates tested for their phenotypic MIC to all antibiotics, with exception to tetracycline, saw greater proportions of isolates with higher MICs on Dairy

Farm 3 followed by Dairy Farm 2 and Dairy Farm 1. This means Dairy Farm 3 tended to have a higher proportion of isolates with elevated MICs followed by Dairy Farm 2 and Dairy Farm 1. The exception of tetracycline could mean it is a preferred drug of use on Dairy Farm 1 whereas other dairy farms may use higher order antibiotics more frequently. This may simply be due to the selection bias of the CHROM-ESBL agar selection protocol. Using isolates from the general *E. coli* population may show Dairy Farm 2 is still the best performer and our results are a characteristic of the isolates specific to the CHROM-ESBL isolation process. Nonetheless, this highlights the continued importance of the dairy farm environment and farm management in the mitigation of antimicrobial resistance and stewardship of antimicrobial usage.

5.4 *E. coli* WHOLE GENOME SEQUENCING

The variant of the ESBL $bla_{\text{CTX-M}}$ gene differs in dominance depending on geographical location. Studies in both the Netherlands (Gonggrijp et al., 2016) and the Nile Delta region of Egypt (Braun et al., 2016) identified $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$ as the dominant ESBL genes, while a study in the Republic of Korea (Tamang et al, 2013) identified $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-32}}$ as the dominant ESBL genes. While $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-32}}$ made up 60% of the $bla_{\text{CTX-M}}$ genes found in our study, we also found 5 other types of $bla_{\text{CTX-M}}$ genes. However, we cannot say these are the dominant $bla_{\text{CTX-M}}$ genes in our study due to only sequencing 20 *E. coli* isolates. The $bla_{\text{CTX-M-32}}$ gene was the first $bla_{\text{CTX-M}}$ gene found in the panhandle of Texas (Cottell et al., 2013). Our study identified 7 different $bla_{\text{CTX-M}}$ genes in the region with each location testing positive for multiple types. However, not long after ESBL *E. coli* harboring a $bla_{\text{CTX-M}}$ gene was discovered in Ohio, each of 5 farms testing positive for $bla_{\text{CTX-M}}$ genes tested positive for only 1 form of the gene suggesting a herd homogeneity (Mollenkopf et al., 2012). This team also noted $bla_{\text{CTX-M-1}}$, $_{\text{-14}}$, and

$_{-15}$ were the most common (Mollenkopf et al., 2012), all of which were found on our sequenced samples. Similar results were recently observed in Pennsylvania with clonal *E. coli* with 3GC resistance being indistinguishable within the region (Salaheen et al., 2019). However, our results indicate the broader dissemination of $bla_{\text{CTX-M}}$ genes since first discovered in American agriculture in 2010 (Wittum et al., 2010). Additionally, with a small number of *E. coli* sequenced, the high diversity among sequence types, along with resistance genes, does not suggest 1 or 2 clonal genes within the region or on a farm. This may also be a result of animal movement on and between large farms in this region allowing for easier spread of $bla_{\text{CTX-M}}$ genes throughout the region.

Consistent with elevated levels of 3GC resistant *E. coli* counts and increased prevalence of phenotypic ESBL and MDR *E. coli*, Dairy Farm 3 had the greatest variety of $bla_{\text{CTX-M}}$ genes in the study. Furthermore, a large proportion of isolates containing the $bla_{\text{CTX-M-1}}$ gene also contained a $bla_{\text{TEM-1A}}$ gene, which has been previously observed (Mollenkopf et al., 2012). Other $bla_{\text{CTX-M}}$ genes also possessed a $bla_{\text{TEM-1B}}$ gene. These genes encode for resistance to penicillins and earlier generation cephalosporins (Salverda, de Visser, & Barlow, 2010), which is also coded for by $bla_{\text{CTX-M}}$ genes.

Fluoroquinolone resistance can be selected for through a selection pressure of third-generation cephalosporin usage, as referenced throughout the document. Fluoroquinolone resistance can be facilitated via genes, such as *qnr*A or *qnr*B, and topoisomerase mutations (Robicsek et al., 2006). Whether the *qnr*A or *qnr*B gene is selected for depends on the $bla_{\text{CTX-M}}$ gene with which it is associated (Nordmann & Poirel, 2005; Jacoby et al., 2006). Our selection of isolates with phenotypic MICs below a level of resistance was supported by the work of Bajaj et al. (2016) suggesting molecular characterization of only those isolates with phenotypic resistance

could inhibit further understanding of co-resistance or multi-drug resistance. All 3 isolates containing *qnr*S1 had a ciprofloxacin MIC of 0.5 µg/mL, which is consistent with previous work highlighting isolates with this gene do not show high levels of fluoroquinolone resistance (Bajaj et al. 2016).

A Swedish study found 60% of calf, 44% of environmental, and 28% of cow samples tested positive for quinolone-resistant *E. coli* (Duse et al., 2016). Areas associated with calves tended to be more highly associated with quinolone-resistant *E. coli*, suggesting intervention in these areas may result in a decreased prevalence of quinolone-resistant *E. coli* on the farm (Duse et al., 2016). This area of intervention makes sense given fluoroquinolones are used to treat disease in calves. Along with co-selection from cephalosporin usage entering adulthood, these resistance genes may persist in low levels in the calf gut through maturation until the application of 3GC selection pressures leading to the resistance observed in the animals in our study given a majority of cows enrolled were first-lactation. Another generated hypothesis is that fluoroquinolone resistance is introduced through factors outside of the farm's control (Duse et al., 2015). Only 2 types of plasmid-mediated fluoroquinolone genes were found in our study with 1 dairy farm only having 1 type of gene, supporting a previous hypothesis that clonal fluoroquinolone strains tend to move about the farm (Duse et al., 2016). Overall, fluoroquinolone resistance mechanisms were found in *E. coli* isolates with ESBL resistance mechanisms. While there are many hypotheses surrounding why this may be so, more research into fluoroquinolone resistance and the factors leading to such resistance on dairy farms is needed to better understand and design interventions for this problem. Similarly, further research is needed into the introduction and selection of *E. coli* with an *mph*(A) in dairy cows, as macrolides are not used in dairy production. Unfortunately, with *E. coli* containing this gene associated with plasmids

containing a $bla_{CTX\text{-}M}$ gene in dairy cows in the region, the use of 3GC may also select for co-resistance to macrolides.

5.5 *SALMONELLA* SHEDDING

A 1996 study representing 83% of dairy cows in the United States found the prevalence of *Salmonella* shedding among dairy cows was 27.5% (Wells et al., 2001). The southern market (consisting of Texas and New Mexico) had the highest shedding among farms with milking cows at 45.0% (Wells et al., 2001). Studies during the 2000s found *Salmonella* prevalence rates among dairy farms between 44% and 56% (Warnick et al., 2003; Callaway et al., 2005; & Heider et al., 2009). While our study only contained 3 dairy farms, all farms had cows testing positive for *Salmonella*, although the number of *Salmonella* positive animals varied by farm. Similarly, Loneragan et al. (2012) found at least 1 culled cow from each of 9 study dairy farms in the northern Panhandle of Texas tested positive for shedding *Salmonella*.

Fecal sample-level prevalence among milking cows was 5.4% in 1996 (Wells et al., 2001), but had increased to 9.96% in 2002 (Callaway et al., 2005) and has been maintained at 9.9% through the decade (Heider et al., 2009) before increasing to 30.0% in 2012 (Loneragan et al., 2012). The prevalence of *Salmonella* shedding among the samples from our study was 16.85% (Table 12); however, 37.5% of study animals tested positive for *Salmonella* shedding at some point during the 3 sampling days we analyzed (days 0, 6, and 16). This may be elevated based upon sample collection taking place in the morning (Fitzgerald et al., 2003).

Interestingly, while the team of Edrington et al. (2004) found a lower prevalence of *Salmonella* shedding in the winter sampling period, our study showed an approximate 6 percent

increase in *Salmonella* shedding prevalence in the trial extending from the fall into the winter seasons. Sample-level prevalence of *Salmonella* shedding varied based upon dairy farm. Dairy Farm 1 had a prevalence of 21.8%, the prevalence on Dairy Farm 2 was 22.3%, and Dairy Farm 3 samples had a prevalence of 6.3% (Table 12). These results are consistent with those of Callaway et al. (2005) in which the prevalence within herd varied from 0 to 37%. However, the prevalence of *Salmonella* shedding animals at any time of the study was elevated above that 37% mark on 2 of the 3 locations.

Regression analysis showed there were no differences among treated and untreated groups regarding *Salmonella* shedding; however, shedding varied by sampling day (Figure 70). The prevalence on day 0 was 30.9% and decreased to 9.5% and 9.7% on study days 6 and 16 (Table 12). This was earlier observed in Muñoz-Vargas et al. (2018) where cows were more likely to shed *Salmonella* in the week after calving than in the time leading to calving. Similar trends were observed in a study by Fitzgerald et al. (2003) in which cows less than 60 days in milk had a higher percentage testing positive for *Salmonella* shedding. Further study by Hume et al. (2004) found no effect regarding genotypic *Salmonella* shedding among lactating and non-lactating dairy cows, but days in milk at enrollment was not mentioned. The level of *Salmonella* shedding decreased in our treatment group and remained at decreased levels on study days 6 and 16. Meanwhile, Ohta et al. (2017) found the prevalence of *Salmonella* in feedlot cattle decreased after CCFA treatment, but rebounded over time when measured out to 26 days post treatment with CCFA.

5.6 *SALMONELLA* WHOLE GENOME SEQUENCING

The highest number of serotypes found on dairy farms enrolled in our study was eleven with an additional four isolates not having a determinable serotype. However, there have been as many as 30 *Salmonella* serotypes associated with on-farm dairy cows (Wells et al., 2001), but this diversity tends to remain between 15 – 17 serotypes spread among the cattle population (Fitzgerald et al., 2003; Callaway et al., 2005; & Loneragan et al., 2012). Even with such diversity of serotypes, dairy farms tend to have dominant serotypes across the animal population with those serotypes changing based upon farm location (Callaway et al., 2005), sample type (Loneragan et al., 2012), and on-farm versus at-market sampling (Wells et al., 2001). This was true of our study too, as 3 or fewer serotypes made up greater than 65% of the serotypes found at each location. Much like the findings of Callaway et al., (2005), each of our 3 dairy farms had animals shedding multiple *Salmonella* serotypes.

Results from four Southwestern dairy farms in 2001 – 2002 found, while Montevideo, Senftenberg, Mbandaka, and Kentucky were the dominant serovars among their study locations, the dominant serovar varied based upon the season in which samples were collected (Edrington et al., 2004). The dominant *Salmonella* serotypes among Southwestern dairy farms in 2003 were Senftenberg, Newport, and Anatum (Fitzgerald et al., 2003). In 2012, dairy farms in the north Panhandle of Texas had higher percentages of Salmonella serotypes Anatum, Kentucky, and Cerro overall, but serotypes Cerro and Muenster were the overwhelmingly dominant serotypes in fecal samples (Loneragan et al., 2012).

Interestingly, the dominant serovars among our dairy farms were Montevideo, Meleagridis, and Cerro, which are the dominant serotypes of an older study (Wells et al., 2001). Wells et al., (2001) found serotypes Montevideo, Melegridis, and Cerro were the dominant

serotypes among on-farm fecal *Salmonella* from a wide geographic range consisting of the Northwestern, Midwestern, Northeastern, and Southern (including Texas and New Mexico) dairy farms. Samples from milking cows consisted of serotypes Montevideo, Kentucky, and Menhaden representing the dominant *Salmonella* serotypes (Wells et al., 2001). Another multi-state study found the dominant serovar varied based upon the dairy region (Callaway et al., 2005).

Levels of antimicrobial resistance among *Salmonella* isolates from agricultural settings remain consistently low, particularly regarding resistance to ceftriaxone. Antimicrobial resistance among *Salmonella* isolates collected from a broad range of dairy farms in 1996 remained low with no isolates having resistance to ceftriaxone (Wells et al., 2001). The same was true of a study in the southwestern United States a decade later (Fitzgerald et al., 2005). Additionally, a study evaluating antimicrobial susceptibility to thousands of *Salmonella* isolates collected from diagnostics, farms (regardless of species), slaughter facilities, and others from 1999 to 2003 found resistance to ceftriaxone was consistent at 0.3% throughout the study period (Frye & Fedorka-Cray, 2007). However, ceftiofur resistance rose from 4.0% to 18.8% over the four-year period (Frye & Fedorka-Cray, 2007).

Similarly, 1,320 *Salmonella* isolates from dairy farms in Wisconsin, Michigan, and Minnesota did not possess resistance to ceftriaxone, even as 76% of dairy farms possessed at least 1 isolate with resistance to 5 antimicrobials and 9.4% of farms with at least 1 isolate with resistance to 9 antimicrobials (Ray et al., 2007). Furthermore, an Australian study determined none of the *Salmonella* isolates from the 75 positive dairy cows had resistance to any of the tested antimicrobials (Barlow et al., 2015). Additional studies in feedlot cattle identified no ceftiofur resistance within the general population (Khaitsa et al., 2007) and low levels of multi-drug resistant *Salmonella* among animals treated with CCFA (Ohta et al., 2017). Our results are

consistent with those of the aforementioned studies, as only *Salmonella* isolates from four samples from 3 animals tested as phenotypically ceftriaxone-resistant. An additional sample from another animal tested as genotypically positive for beta-lactam resistance.

Previous work has identified *Salmonella* serotypes Give (Fitzgerald et al., 2005), Typhimurium, Dublin (Wells et al., 2001), and Anatum, Kentucky, and Newport (Loneragan et al., 2012) as serotypes from fecal dairy cow sample isolates exhibiting multi-drug resistance. Of the *Salmonella* isolates from each of those studies, only *Salmonella enterica* serovar Newport contained an AmpC resistance profile (Loneragan et al., 2012). In our study, 3 serotypes (Anatum, Meleagridis, & Newport) were found to contain the $bla_{\text{CMY-2}}$ gene conferring an AmpC resistance profile. Based on NARMS and Foodborne Diseases Active Surveillance Network data from 1996 to 2013, serotype Newport is the most likely *Salmonella* serotype to carry ceftriaxone resistance in humans with ground beef accounting for 13.5% of ceftriaxone resistant *Salmonella* from 2002 to 2013 (Iwamoto et al., 2017). Meleagridis most frequently contained the gene, but also accounted for nearly a quarter of the serotyped isolates. In our study, Meleagridis was the only serotype found to harbor genotypic multi-drug resistance. The isolate contained genes for resistance to aminoglycosides, beta-lactams, sulfonamides, streptomycin, and trimethoprim.

5.7 LIMITATIONS

Limitations of this study include its small geographic region. Our study included only 3 dairy farms from western Texas and eastern New Mexico, which tend to have warmer, drier climates, versus other dairy production regions, such as Wisconsin and New York. Differences in climate may impact bacterial survival outside the host, affecting the oral-fecal transmission

dynamics within the farm, bacterial fitness in the environment, and the ability of such bacteria to further distribute resistance genes in the environment. In addition to climate, environment and resource availability could impact 3GC resistant bacterial populations or else herd management techniques resulting from those factors (Berge et al., 2010). Studies across diverse regions of the U.S. with high levels of dairy production would aid in providing additional insight into the generalizability of our findings. It is beneficial to sample animals from a dairy farm multiple times to evaluate temporal dynamics; however, due to the resources required for repeated intensive temporal sampling only 3 dairy farms could be enrolled in the study. As a result, the generalizability of our data is restricted. Our use of multiple imputations to complete the truncated left tail of the empirical distribution and assist in meeting model assumptions may have introduced bias into the models.

Furthermore, phenotypically evaluating antimicrobial resistance susceptibility to the general *E. coli* population could provide better insight into the levels and forms of antimicrobial resistance within this population. We were unable to obtain phenotypic count data differentiating the AmpC and ESBL resistance profiles. Developing a method to do so would provide an opportunity to understand the changes and interactions of bacteria with such resistance profiles across time and treatment. We sequenced a small number of *E. coli* isolates. Expanding the number of sequenced isolates based upon their 3GC and fluoroquinolone resistance profiles would strengthen any hypotheses regarding the mechanisms bringing bacteria with reduced susceptibility fluoroquinolone to dairy farms.

CHAPTER VI

CONCLUSION

The findings of this study suggest that the currently labeled 13-day slaughter-withholding period following a second dose of CCFA is generally inadequate to allow levels of 3GC resistant bacteria to return to baseline levels in treated dairy cows housed on dairy farms with elevated levels of resistance to third-generation cephalosporins. Existing slaughter withholding times designed to avoid pharmaceutical residues in meat products provide no microbial safety assurances once bacterial resistance establishes in agricultural environments. A longer stakeholder-initiated voluntary slaughter-withholding period could be deployed to reduce the risk of fecal contamination at slaughter harboring antimicrobial resistant enteric bacteria. Dairy-specific stewardship approaches targeting practices with higher risk of selecting for antimicrobial resistance would be beneficial in mitigating resistance. Further research into the genetic diversity of resistance will provide more information into the mechanisms at play and the added potential for 3GC resistance co-selection via alternative antimicrobial classes that may be employed instead of cephalosporins.

While there were no statistically significant differences between our treatment and control groups, general patterns were observed based on the prevalence of phenotypic ESBL *E. coli* and MDR *E. coli* and by sampling day. With increases observed in each prevalence type, it is hypothesized there are environmental factors at play as the animals move throughout the dairy system. This is further supported by differences in baseline prevalence among both phenotypic ESBL *E. coli* and MDR *E. coli* prevalence by individual dairy farm showing an animal's initial risk is associated with the environment of its farm as a whole and changes through each stage of

production. The role of the dairy farm is further shown via the Mantel-Haenzel rate ratios and Kaplan-Meier survival plots. While similar patterns were observed within each location, the initial risk varied by farm. This shows stewardship tools tailored to each dairy farm's individual needs could have positive impacts in mitigating the spread of enteric bacteria with 3GC and multi-drug resistance.

Our results indicate *Salmonella* shedding is not impacted by metritis or a two-dose CCFA treatment for metritis, as there were no differences between either the treated or untreated group on any of the sampling days evaluated. There was a higher prevalence of *Salmonella* shedding on day 0 than the other days suggesting the stress of calving may disrupt the gastrointestinal flora resulting in increased *Salmonella* shedding. As with many other studies, each study location was positive for multiple *Salmonella* serotypes with a few serotypes dominating each farm. On a positive note, few samples were found to possess *Salmonella* with 3GC resistance mechanisms and, although all positive samples were from treated cows, significance was not determined between resistance and treatment.

In final conclusion, the current slaughter withholding period after a two-dose CCFA treatment is not adequate time to allow for 3GC resistance to decrease back to baseline and voluntary slaughter withholding periods should extend up to 28 days after the first treatment. Furthermore, stewardship tools should be tailored toward individual dairy farms, as different locations tend to have different baseline risks of 3GC, phenotypic ESBL, and MDR *E. coli*. By addressing antimicrobial stewardship on dairy farms, improvements can be made in controlling AMR development and spread in the farm environment and extending the volunteer slaughter withholding period reduces the risk of fecal slaughter contamination harboring 3GC resistant enteric bacteria.

CHAPTER VII

FUTURE WORK

A common theme throughout our study was the difference among the farms regarding baseline levels of 3GC resistant *E. coli*, levels of 3GC resistant *E. coli* in the environment of those locations, and baseline *Salmonella* shedding. Unfortunately, due to a small number of environmental samples from a small number of dairy farms, we were unable to properly evaluate the role of the dairy farm environment in our models. Furthermore, we only evaluated the historical usage of CCFA on the farm since that was the selection pressure we were evaluating. Future work needs to involve enrolling a larger number of dairy farms with frequent environmental sampling and the evaluation of the historic usage of multiple antibiotics at both cow and herd levels, along with other management factors to better evaluate the factors behind differing baseline levels of AMR on farms.

Additionally, our study had low levels of *Salmonella* shedding and phenotypic ESBL *E. coli*. This provided resulted in large confidence intervals among our data for each animal group on each sampling day, so only patterns could be observed. Future work should focus on generating counts for *Salmonella* and a more specific test for doing the same for phenotypic ESBL *E. coli* as another way of evaluating these pathogens and resistance profile beyond prevalence.

While we explored phenotypic MICs of *E. coli* with an expected ESBL resistance profile to evaluate co-resistance, future work should evaluate the phenotypic MICs of *E. coli* from the general (non-selective) population to evaluate how the treatment impacts the resistance profile of the broader population. Further research should be done into the drivers of fluoroquinolone and macrolide resistance in dairy cattle. Our results show MICs from sampling days later in the trial

were higher than those from earlier days and a difference in MIC by location. However, this class of antibiotic should not be used in dairy cattle above 20 months of age. Understanding the mechanisms and drivers of this resistance, along with why it is seen more in isolates from sampling days farthest from CCFA treatment, is important in curbing its spread.

Lastly, future work should include metagenomics to evaluate the changes of bacterial populations within the ecosystem of the intestine and inferred from the fecal environment. Little is known about how the administration of an antibiotic affects the microbiome of the gut environment sequentially over time in the post-partum dairy cow. The changes in the fecal microbiome from baseline, to shortly after the treatment administration, and during population recovery should be evaluated. The selection of these days will allow for the observation of how other bacterial populations are affected by a two-dose CCFA treatment, what bacterial populations expand (or contract) after treatment, and if those populations remain in such states as time moves forward. Through this additional work, we can continue to build upon the impacts of the CCFA selection pressure, the role of dairy farm environment and management in baseline levels of AMR, and the selection pressures for bacterial resistance to fluoroquinolones in multi-parity cattle when antimicrobials are utilized in calves and heifers.

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