EFFECTS OF ANTIBIOTIC ALTERNATIVES ON ANTIMICROBIAL RESISTANCE AMONG FECAL BACTERIA IN BEEF CATTLE

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2020

Major Subject: Biomedical Sciences

ABSTRACT

Antimicrobial resistance (AMR) represents a growing crisis in both human and veterinary medicine. We evaluated the use of two antibiotic alternatives – heavy metals and essential oils – in beef cattle and their effects on gram-negative and gram-positive bacteria. Further, we explored whether direct-fed microbials and a tylosin voluntary withdrawal period would decrease macrolide resistance among enterococci.

In the first randomized and controlled field trial, we measured the impact of supplemental zinc and menthol on antimicrobial resistance among commensal enteric bacteria of feeder cattle. Fecal suspensions were plated onto plain- and antibiotic-supplemented MacConkey and m-*Enterococcus* agar for quantification of total and antimicrobial-resistant *Escherichia coli* and *Enterococcus* spp., respectively. Temporal effects on overall *E. coli* growth were significant ($p<0.05$); however, there were no significant effects on antibiotic-supplemented agar. Zinc was associated with significant increases in growth on erythromycin-supplemented m-*Enterococcus* agar. Cattle fed zinc had significantly higher macrolide resistance among fecal enterococci isolates.

In the second field trial, within two sequential replicates (n=90 and n=96 finisher cattle, respectively) we measured the impact of an *Enterococcus faecium*-based probiotic (DFM) and an altered pen environment on antimicrobial resistance among fecal enterococci in cattle fed tylosin. Diluted fecal samples were spiral-plated on plain and antibiotic supplemented m-*Enterococcus* agar. In the first replicate, tylosin significantly ($p<0.05$) increased the relative quantity of erythromycin-resistant enterococci. This effect

was diminished in cattle fed the DFM in conjunction with tylosin. This effect was not statistically significant ($P > 0.05$) in the second replicate. Isolates were speciated and resistance phenotypes were obtained for *E. faecium* and *E. hirae*. *E. faecium* isolates were sequenced, which yielded sequence types (ST), resistance genes and phylogeny. Samples of the DFM were sequenced and found to contain *E. faecium* ST296, which was not present on Day 0 of either replicate. This DFM sequence type was found in fecal samples after Day 0, the majority of which were isolated from cattle in one of the DFM-fed pens. Increased prevalence of ST296 occurred with a concomitant decrease in ST240; of note, the latter typically harbored both *ermB* and *tet*(M).

We also explored the effects of the same trial factors on the fecal microbiome using 16S rRNA metagenomics. Bacterial taxonomic analyses based on alpha- (Shannon index) and beta-diversity (Bray-Curtis dissimilarity index), were not different ($P>0.05$) across treatment groups, suggesting there was no effect of the DFM or tylosin on the microbiome. Period effects, independent of trial factors, differed at $P < 0.05$.

In conclusion, zinc and menthol were not associated with significant changes in antibiotic resistance among *Escherichia coli*; however, excess dietary zinc was associated with increased macrolide resistance among enterococci. Tylosin exhibited a significant ($P<0.05$) – though inconsistent – effect on macrolide resistance. An *E. faecium*-based probiotic mitigated macrolide resistance, with an increase in the relative prevalence of ST296 (i.e., probiotic strain) accompanying a decrease in multi-drug resistant ST240 strains. Of importance, the addition of tylosin, a probiotic, or both did not affect the more abundant components of the fecal microbiome of finisher steers.

DEDICATION

Thanks to my parents, especially my mom, for always driving me to never be content with the current *status quo*, and always showing me that further education is possible no matter how long it takes. Your work ethic was instilled in me at a young age, and has served me well. Y'all have always been proud of me, and have supported my achievements, but through your inspiration and ambition I've been able to push myself, setting higher goals and going further than I ever would have thought. To my undergraduate research advisor Dr. Crosby Jones, who started me on this path, I appreciate your guidance. You took a chance letting an 'ag girl' into your microbiology class without the prerequisite course, and after a semester you even allowed me to be a research student. Through your microbiology and immunology courses, and subsequently as a research pupil, I found a subject I love and was able to turn it into a career. Lastly, to Drew, you have been my rock through the majority of this degree. I can't express how much I appreciate being able to lean on you when I'm mentally exhausted, stressed, and sleep deprived. I am so grateful for you trying to take as many burdens off of me as possible, and for your unwavering encouragement and support.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Morgan Scott, he has inspired me to think in a multi-dimensional way. I will miss attending conferences with him, as he always asked unique and thought-provoking questions that expanded my own thought process. I can only hope to grow and expand similarly in my own philosophical nature. I appreciate his guidance, and feel fortunate to work under his expertise. Many thanks to Dr. Keri Norman, who was always willing to help me answer molecular questions, and provided guidance in sequencing. Thanks to Dr. Lawhon for her guidance in clinical microbiology, helping me troubleshoot answers when I had questions about *Enterococcus* or *E. coli*. Also, thanks to Dr. Sawyer, who provided intellectually stimulating conversation on our many trips to and from McGregor. I do miss our conversations about microbiology and its relation to animal science. I have much appreciation for Javier Vinasco and Roberta Pugh, without their assistance throughout the course of this research, I would be lost. Between all the help in the lab when needed, to sorting out order placements, I am grateful for their support. I also appreciate Raghavendra Amachawadi, T.G. Nagaraja, and Jim Drouillard at Kansas State for their assistance and sample collection during the zinc and menthol trial. Lastly, thanks also go to my lab mates, colleagues, and friends in the ME^2 lab and in College Station, for making my time at Texas A&M University a great experience.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professor H. Morgan Scott of the Department of Veterinary Pathobiology, primary advisor, as well as Keri N. Norman of the Department of Veterinary Integrative Biosciences, Sara D. Lawhon of the Department of Veterinary Pathobiology, and Jason E. Sawyer of the Department of Animal Science. All work performed for the dissertation was completed by the student independently, with the exception of fecal sample collection for which the Animal Use Protocol relied on approved McGregor feedlot staff. The Animal Use Protocol was submitted to the Texas A&M AgriLife Research Agriculture Animal Care & Use Committee (AACUC), with approval #2015-026A.

Funding Sources

The zinc and menthol trial work was supported by the USDA-NIFA-AFRI Food Safety Challenge Grant project 2013-68003-21257. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the USDA or NIFA.

The DFM and tylosin trial work was made possible by USDA-NIFA-AFRI under Grant Number (2016-68003-24607). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the USDA or NIFA.

NOMENCLATURE

AMR	Antimicrobial Resistance
CDC	Centers for Disease Control and Prevention
CGE	Centre for Genomic Epidemiology
FAO	Food and Agriculture Organization of the United Nations
FDA-CVM	Food and Drug Administration – Center for Veterinary Medicine
HPRC	High Performance Research Computing laboratory
MDR	Multi-drug Resistance
MIC	Minimum Inhibitory Concentration
MLST	Multi-locus Sequence Type
NARMS	National Antimicrobial Resistance Monitoring System
NGS	Next Generation Sequencing
OIE	World Organization for Animal Health
PCoA	Principal Coordinates Analysis
PBS	Phosphate-buffered Saline
TAMU	Texas A&M University
USDA	United States Department of Agriculture
WGS	Whole Genome Sequencing
WHO	World Health Organization

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CHAPTER I

INTRODUCTION

Background

Antimicrobials have a long history of use in both animals and humans for prevention, control and treatment of infectious diseases (Dibner and Richards, 2005; Levy and Marshall, 2004; Podolsky, 2018). In addition, in the 1940s, sulfonamides were found to increase growth in chicks leading to almost seven decades of antimicrobial use for growth promotion in food animals; by 1951, a combination vitamin B_{12}/low-dose chlortetracycline was officially licensed for use in food animals (Gustafson and Bowen, 1997; Kirchhelle, 2018; Moore *et al.*, 1946). Antibiotics are no longer labeled for growth promotion uses in the United States, prompting research into effective alternatives (Allen *et al.*, 2013; Stanton, 2013). Some of these alternatives include antimicrobial peptides, probiotics, heavy metals, clay minerals, egg yolk antibodies, essential oils, and recombinant enzymes (Huyghebaert *et al.*, 2011; Verstegen and Williams, 2002). However, a review by Thacker *et al.* concluded that no current compounds are equal to antibiotics in efficacy, and most give inconsistent results (Thacker, 2013). Additionally, there is great concern for the possibility of co-selection of antibiotic resistance among antimicrobial alternatives (Wales and Davies, 2015).

Antimicrobials remain important for the prevention, control, and treatment of disease and are necessary to maintain healthy cattle in the feedlot. Metaphylactic (control) and prophylactic (prevention) uses of antimicrobials are two of the primary methods to proactively manage infectious diseases in animals. One major concern for feedlot beef cattle health is liver abscesses, commonly accepted to be caused by ruminal

acidosis associated with high grain rations (Nagaraja and Chengappa, 1998). Liver abscesses are a major economic cost, with the most severe liver abscesses having the greatest effect. In such cases, severe abscessation may reduce the value of beef carcasses by $38 per animal through decreased carcass quality and yield (Brown and Lawrence, 2010).

Additionally, severe liver abscesses may reduce feed efficiency (Brink *et al.*, 1990; Brown *et al.*, 1975; Reinhardt and Hubbert, 2015). Control of these liver abscesses using labeled in-feed antibiotics may reduce their prevalence at slaughter by 40% to 70% (Nagaraja and Lechtenberg, 2007). There are currently five antibiotics approved for the prevention of liver abscesses; these include bacitracin, chlortetracycline, oxytetracycline, tylosin, and virginiamycin. Tylosin has consistently been found to control liver abscesses most efficiently (Brown *et al.*, 1975; Nagaraja and Chengappa, 1998). However, tylosin use has been associated with macrolide resistance among enterococci, prompting research into alternatives to its use (Chen *et al.*, 2008; Jackson *et al.*, 2004).

Study objectives

Using samples derived from two prospectively designed field studies in beef feedlot cattle, we address the two main topics introduced above. First, we explore the potential for co-selection of antibiotic resistance among gram-negative and gram-positive bacteria through the single- and combined-use of two non-antibiotic antimicrobials: zinc and menthol. Second, we explore the potential for exogenous sources of antimicrobial-susceptible bacteria – in this case, *Enterococcus faecium* – to attenuate the selection for resistant bacteria during a course of disease prevention antibiotics (tylosin); further, we explore the potential benefits of moving tylosin-fed

cattle into pen environments in which antibiotics have never been administered during the last 28 days pre-slaughter. In this dissertation we took a three-pronged approach to studying the effects of antibiotic alternatives on antimicrobial resistance. First, we determined the changes in prevalence and quantity of susceptible and resistant fecal commensal bacteria (*Enterococcus* spp., and in one trial *E. coli*). Second, we determined the phenotypic and genotypic characteristics of several dominant strains of enterococci; that is, before, during, and after interventions with antibiotic alternatives. Third, we examined changes in the microbial diversity in beef cattle based on time (age/period effects) and trial factors such as probiotics, antibiotics, and changes in pen environment. This latter objective was undertaken using 16 rRNA metagenomic sequencing using pen-pooled fecal samples.

CHAPTER II

CRITICAL REVIEW OF THE LITERATURE

Antibiotic uses in beef cattle and their impacts

Antimicrobial use in food animals is often regarded as a double-edged sword, with the weight of benefits versus risks presenting moral and ethical challenges (Hao *et al.*, 2014). The occurrence and spread of antimicrobial resistant bacteria in food producing animals given antibiotics has been shown to have impacts on bacterial resistance to critically important antibiotics in human medicine; most notably, associated with the use of avoparcin and selection for vancomycin resistance in countries not including the United States (Aarestrup, 2000; Bager *et al.*, 1997). Additionally, resistance elements such as genes and plasmids that are acquired by commensal bacteria can be shared with more pathogenic species, causing a detrimental effect on effective therapy in human medicine (O'Brien, 2002). While the link between using antimicrobials in food producing animals and detrimental effects on human health is contested by some, effectively arguing that the risk to human health is minimal, others argue in favor of the precautionary principle, especially of eliminating non-therapeutic uses of antibiotics (Marshall and Levy, 2011; Phillips *et al.*, 2004). Many efforts have been and are being made to continue to promote prudent and judicious use of antibiotics wherever they are used (Hoelzer *et al.*, 2017).

In both human and veterinary medicine, antimicrobial resistance is rapidly becoming a worldwide crisis. In 2019, the Centers for Disease Control and Prevention (CDC) released an updated document called: "*Antibiotic Resistance Threats in the United States*", largely developed from their original 2013 document, which compiled a

comprehensive list of the top AMR threats (CDC, 2013, 2019). This updated CDC report stated that at least 2.8 million cases occur annually in the United States, resulting in over 35,000 deaths. In 2015, the World Health Organization (WHO) released its Global Action Plan to combat antibiotic resistance, to be implemented in collaboration with the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) under a "Tripartite" agreement (WHO, 2017). The second objective in the WHO plan includes: "*...research to identify alternatives to nontherapeutic uses of antimicrobial agents in agriculture and aquaculture, including their use for growth promotion and crop protection*". The Global Action Plan complements both the 2012 and 2013 Food and Drug Administration Center for Veterinary Medicine (FDA-CVM) Guidance for Industry #209 and #213, respectively, which outlined judicious use of antibiotics (including restricting growth-promotion uses) and recommendations for new product uses (FDA, 2012, 2013). Additionally, in 2019 FDA-CVM released a draft Guidance for Industry #263, which outlined implementation of veterinary oversight for all medically-important antimicrobial use in animals, effectively ending the last loophole for non-veterinary oversight sales as over-the-counter drugs for parenteral and systemic use (FDA, 2019).

While growth promotion use is no longer allowed in the United States since 2016, antibiotics are still widely used for prevention, control, and treatment of disease. Antibiotics, including tylosin and chlortetracycline, are used in feed for the prevention and control of liver abscesses in cattle. High grain diets are widely accepted as the main cause of ruminal acidosis, rumenitis, and subsequent liver abscess formation (Amachawadi and Nagaraja, 2016; Nagaraja and Chengappa, 1998). Though it remains

unknown as to any single specific causative agent and pathogenesis for liver abscesses, *Fusobacterium necrophorum* (previously known as *Sphaerophorus necrophorus*, *Fusiformis necrophorus*, and *Bacillus necrophorus)*is commonly isolated from abscessed livers, as is *Trueperella pyogenes* (previously known as *Actinomyces pyogenes*). It has been suggested that these two organisms initiate a synergistic reaction in causing the formation of liver abscesses (Nagaraja *et al.*, 1999). Recently, *Salmonella enterica* has been isolated through anaerobic culture from a liver abscess (Amachawadi and Nagaraja, 2015; Amachawadi *et al.*, 2016).

Liver abscesses in cattle result in diminished carcass value and are responsible for large economic losses in the fed beef cattle industry (Brown and Lawrence, 2010; Nagaraja and Lechtenberg, 2007). Cattle with severe liver abscesses may require more carcass trimming because of adhesion of the abscesses to the diaphragm and surrounding organs. In some instances, condemnation of the entire viscera may be necessary. Abscesses are the leading cause of liver condemnation, ranking second in the top 10 concerns of packers (Nagaraja and Chengappa, 1998). Additionally, the accidental rupture of an abscess and contamination of a carcass with pus causes interruption in the flow of carcasses along the chain on the slaughter floor, thus costing time and labor.

That said, likely the greatest economic impact of liver abscesses is from reduced animal performance and carcass yield. Cattle with abscessed livers have reduced feed intake, reduced weight gain, decreased feed efficiency, and decreased carcass dressing percentage (Brink *et al.*, 1990). Feed intake and feed conversion are impacted by severe liver abscesses, reducing intake by 5% and gain-to-feed by 14% (Brink *et al.*, 1990). A study by Brown *et al.* also reported that adhesions increased the loss in HCW (hot

carcass weight) by 3 kg in one comparison, and by 8.7 kg in a second comparison (Brown *et al.*, 1975). They also reported a reduction in marbling score, in addition to reductions in yield grade, fat depth, and percent of kidney-pelvis-heart (KPH) fat in cattle with severe liver abscesses versus cattle with normal livers (Brown and Lawrence, 2010). Additionally, a meta-analysis on liver abscess risks of cattle receiving tylosin versus cattle not receiving tylosin in conventional feeding systems showed that the feeding of tylosin reduced the risk of liver abscesses from 30% to 8% (Wileman *et al.*, 2009).

Five antimicrobials are approved for the prevention of liver abscesses, including: bacitracin, chlortetracycline, oxytetracycline, tylosin, and virginiamycin. Bacitracin has been noted as the least effective and tylosin as the most effective of the five antibiotics. Chlortetracycline, fed at 70 mg per animal per day, reduces the prevalence of liver abscesses by 21% versus negative controls and reduces the prevalence of severe liver abscesses by 35%; in contrast, the inclusion of tylosin in the feed to provide 75 mg per animal per day reduced total and severe liver abscess prevalence by 67 and 85%, respectively (Brown *et al.*, 1975; Nagaraja *et al.*, 1999).

Meanwhile, tylosin has been shown to select for macrolide resistance when used as a growth promoter in swine (Aarestrup and Carnstensen, 1998). In cattle, tylosin has also been associated with increased resistance to macrolides among enterococci (Jacob *et al.*, 2008; Zaheer *et al.*, 2013). A systematic review of tylosin use in cattle determined that when fed at approved dosages for typical durations, tylosin tended to increase the proportion of macrolide-resistant enterococci in cattle, and suggested a zoonotic risk to human beef consumers (Cazer *et al.*, 2020). Additionally, in a recent surveillance study

of all enterococci across the One Health continuum, *Enterococcus hirae* was the most common species isolated from cattle, followed by *Enterococcus villorum* and *Enterococcus faecium*. Furthermore, resistance to tetracycline and macrolides appeared abundant among the majority of enterococcal species, likely due to the common use of antibiotics in both human and veterinary medicine.

This is of importance because *Enterococcus faecium*, though a common commensal bacterium, is recognized as a leading opportunistic cause of nosocomial infections in intensive human health care settings (Emori and Gaynes, 1993). Additionally, *E. faecium* has been noted as the second most prevalent nosocomial pathogen (Arias and Murray, 2008, 2012). In the 2019 updated version of the Antimicrobial Resistance Threats Report by the Centers for Disease Control and Prevention, *E. faecium* was indentified as the most common cause of central line associated bloodstream infections (CDC, 2019). While *E. faecium* is less likely to possess virulence factors than *E. faecalis*, it is more likely to bear a multi-drug resistance geno- and phenotype (Huycke *et al.*, 1998). Remarkably, when the phylogeny of multidrug-resistant *E. faecium* is traced, it was found that while the emergence of the hospital-adapted lineage occurred in association with the beginning use of antibiotics, the bacterial population at that time consisted of a majority of animal strains and was not associated with human commensals (Lebreton *et al.*, 2013).

Therefore, consideration of the possibility and impact of shared mobile genetic elements must be made. For example, the plasmid pRE25 has been shown to be widely shared between geographically diverse isolates of both animal and human clinical origin (Rosvoll *et al.*, 2010). Additionally, there is the opportunity for transference of mobile

genetic elements with other bacteria; most notably, between enterococci and *Staphylococcus aureus* through mutual production of the peptide cAM373 (Clewell *et al.*, 1985). A problem arises when examining resistance to erythromycin in association with feeding tylosin, because *erm* family genes provide resistance to a wide variety of macrolides, lincosamides, and streptogramin B. Additionally, there is the possibility of co-selection of resistance to tetracycline when feeding tylosin, and *vice versa* (Chen *et al.*, 2008). Furthermore, erythromycin is a critically important antibiotic, deemed to be of the highest priority critically important macrolide class by the World Health Organization (WHO, 2019).

Proposed antibiotic alternatives and co-selection

It is important to note that while research to identify innovative alternatives to antibiotics is necessary given the current global AMR situation, it is equally important to explore potential undesired effects of those alternatives. Heavy metals, including copper and zinc have been suggested as alternatives to antibiotics used for growth promotion and disease prevention and control. Zinc oxide (ZnO) at supra-nutritional levels has been shown to influence the gut microbiota of weaned piglets in a manner similar to growth-promoting antibiotics, though differences in average daily gain were not statistically significant between treated and untreated groups (Højberg *et al.*, 2005). It is important to be aware of co-selection potential between tolerance/resistance to heavy metals and antibiotic resistance (Sabry *et al.*, 1997). Specifically, heavy metal tolerance/resistance and antibiotic resistance genes are often carried on the same mobile genetic elements (Partridge *et al.*, 2018; Summers, 2006; Wales and Davies, 2015). It has been documented that bacterial populations subjected to high levels of copper become more

resistant, and copper resistance has been reported in both gram-negative, and gram-positive bacteria (Brown *et al.*, 1995; Duncan *et al.*, 1985; Hasman and Aarestrup, 2002; Stoyanov *et al.*, 2003; Tetaz and Luke, 1983). The link between metal tolerance and antimicrobial resistance, such as for zinc and methicillin in staphylococci (MRSA), or copper and macrolides in enterococci has also been reported in Norway (Yazdankhah *et al.*, 2014) and other countries. The same effect was seen in Danish swine, with tolerance to zinc associated with resistance to methicillin via the *mec*A gene, and described with the *czr* gene cluster (Aarestrup *et al.*, 2010; Hassan *et al.*, 1999). This phenomenon is not restricted to Europe, as swine receiving supra-nutritional zinc in Kansas showed the same co-selection of MRSA (Amachawadi *et al.*, 2015). Unfortunately, there are currently no studies on the effect of supranutritional zinc supplemented in the feeder stage of cattle in relation to phenotypic antimicrobial resistance among *E. coli* and *Enterococcus* spp. By studying if zinc has a similar effect on *E. coli* and enterococci compared *Staphylococcus aureus* we can determine if it could be a viable antibiotic alternative in beef cattle.

Another antibiotic alternative suggested is the use of an essential oil such as menthol, origanum, or thymol, among many others. These have been shown to be effective against both gram-negative and gram-positive bacteria (Prabuseenivasan *et al.*, 2006). In a study by Li *et al.*, piglets fed a combination of thymol and cinnamaldehyde had similar weight gain and feed efficiency as piglets fed antibiotics (Li *et al.*, 2012). Additionally, menthol has been shown to increase weight gains in poultry (Ocak and Sivri, 2008). Cargill has produced a proprietary blend of essential oils, including those derived from thyme, cinnamon, and oregano, for supplementation in poultry in order to

reduce antibiotic use (Cargill, 2016). Menthol has also been suggested as an antibiotic alternative in cattle, with equivocal results suggesting that it has no effect on total coliform counts in cattle feces, and no increase in resistance to many antibiotics for *Escherichia coli* isolates. However, it also was shown to yield increased prevalence of tetracycline resistant *Escherichia coli* after 30 days in feed (Aperce *et al.*, 2016). While essential oils have also been suggested as alternatives to tylosin used to prevent and control liver abscesses, Meyer *et al.* found no difference in weight gain between cattle fed an essential oil blend and those fed tylosin; however, the total number of liver abscesses was reduced for steers fed tylosin, while cattle which received both essential oils and tylosin had a statistically significant increase in calculated yield grade (Meyer *et al.*, 2009; Weissend *et al.*, 2017). However, at the time of writing there were no published studies on the effects of the interaction of zinc with essential oils, such as menthol. By studying both the independent effects of zinc and menthol on antimicrobial resistance, and their interaction when used in combination, we can report on their usefulness and validity as alternatives.

A 2015 study by Beukers *et al.* suggested that tylosin withdrawal prior to slaughter contributed to a reduction in the proportion of macrolide resistant enterococci (Beukers *et al.*, 2015). In accordance with this, Walter *et al.* found that cattle which were fed tylosin during the first 84 days had fewer severe liver abscesses than those fed in the last 84 days, suggesting that the vulnerable time for abscess formation lies during the beginning of the feeding period (Walter *et al.*, 2018). This was not supported by Davedow *et al.*, who found a marginal difference in severe liver abscessation between cattle fed tylosin for the entire feeding duration versus those fed tylosin only during the

last 75% of days on feed; in contrast, cattle that were given tylosin for the first 78% of the feeding period had significantly more severe liver abscessation (Davedow *et al.*, 2020). Additionally, a 2018 publication by Muller et. al. showed no difference in antimicrobial resistance levels when comparing cattle fed intermittent tylosin supplementation versus continuous treatment, also suggesting environmental factors may be important in carrying over resistance from one lot of cattle to the next; that is, negating any contemporary effects of tylosin reductions on AMR bacteria ((Müller *et al.*, 2018). By combining these two factors, we can determine if withdrawal of tylosin, combined with introduction to new pens, each have a significant effect on the reduction of macrolide resistant enterococci, or combine to achieve a synergistic effect.

Recently, *Saccharomyces cerevisiae* fermentation products (SCFP) have been suggested for use to prevent liver abscesses, though there have been no statistically significant differences in treatment groups reported with respect to abscess prevalence or severity (Huebner *et al.*, 2019). *Enterococcus faecium* has also been suggested for use as a probiotic due to its tolerance of bile acids and its antagonistic effects towards intestinal pathogens; specifically, it harbors bacteriocins active against gram-positive foodborne pathogens, such as *Listeria monocytogenes* (Izquierdo *et al.*, 2009). However, care must be taken when choosing probiotic products, because some *E. faecium* strains from commercially available products may exhibit resistance to medically important antibiotics, or multi-drug resistance (Amachawadi *et al.*, 2018). Through supplementation with a macrolide-susceptible probiotic, we can posit the effects on reducing the prevalence of macrolide-resistant enterococci, when the probiotic is used

alone, when combined with tylosin, and when combined with tylosin withdrawal and new antibiotic-free pens.

Effects on bacterial diversity and microbial ecology

While antibiotics have been proven to increase feed efficiency and decrease the prevalence and severity of liver abscesses, their effects on cattle ruminal and fecal microbiome are not well understood. However, in humans, antibiotics are known to upset the natural intestinal (especially hindgut) microbiome, allowing for propagation of antibiotic-resistant pathogens including vancomycin-resistant enterococci, and *Clostridioides difficile* (previously known as *Clostridium difficile*). This dysbiosis, especially for those patients with recurring *C. difficile* colitis, has been effectively treated with transplantation of a healthy fecal microbiota (Bakken *et al.*, 2011). Robust and diverse commensal bacterial populations and their by-products can suppress intestinal pathogens through competitive exclusion, in addition to enhancing host immune defenses (Buffie and Pamer, 2013).

It is widely accepted that commensal gut bacteria can affect many aspects of mammalian health, including through contributions to nutrition. Additionally, diet has been shown to have an impact on microbial community composition (Flint *et al.*, 2012). Changes in the gut bacteria of ruminants due to diet and antibiotics have been studied previously, primarily using real-time quantitative polymerase chain reaction (qPCR), or via anaerobic culture (Goad *et al.*, 1998; Tajima *et al.*, 2001; Weimer *et al.*, 2008). However, these methods do not provide a full picture of all microbial communities, and next-generation sequencing using either 16S rRNA amplicon sequencing or shotgun metagenomic sequencing (or, both) has begun to provide a more complete approach to

determining the effects of diet and antimicrobials on the cattle enteric microbiome (de Menezes *et al.*, 2011; Delgado *et al.*, 2019; Pitta *et al.*, 2016).

Using both these methods, Weinroth *et al.* investigated the effects of tylosin on fecal and soil microbiomes and on the resistome of feedlot cattle, in addition to its effects on liver abscess prevalence. The data gathered suggest that tylosin has little effect on cattle resistomes or microbiomes; meanwhile, the pen environment and feedlot location both seemed to have a stronger impact on both the resistome and the microbiome of feeder cattle (Weinroth *et al.*, 2019). Similarly, in cattle fed tylosin and ractopamine when compared to a control group with no fed antibiotic, there were no differences in the presence of antimicrobial resistance genes in the feces, and there were no observed differences at the phylum level; however, treated cattle had reduced abundance of gram-positive bacteria at the genus level (Thomas *et al.*, 2017). While 16S metagenomic data cannot determine impacts on the resistome, they can be used to determine if tylosin or the supplemented probiotic are correlated with dysbiosis in cattle. Additionally, 16S metagenomic data can be used to determine any temporal effects.

Meanwhile, there remains a clear lack of data in several key areas. Evaluation of the use of heavy metals in conjunction with use of essential oils as feed additives in beef cattle concerning their phenotypic effects on both gram-negative and gram-positive bacterial resistance to antibiotics is needed. In addition, assessment of the effects of a *Saccharomyces cerevisiae* and *Enterococcus faecium* based probiotic, in combination with tylosin withdrawal at day 84 and an environmental change reflecting movement to an unused pen, on phenotypic and genotypic antibiotic resistance outcomes in

Enterococcus spp. is needed, in addition to an evaluation of those same factors on overall bacterial diversity and microbial ecology.

CHAPTER III

EFFECTS OF ZINC AND MENTHOL ON PHENOTYPIC ANTIMICROBIAL RESISTANCE OF *E. COLI* AND *ENTEROCOCCUS* SPP.

Background

Heavy metals, including copper and zinc, have been suggested as alternatives to antibiotics for growth promotion uses. A study in 2005 by Hojberg *et al.* suggested that zinc oxide could influence the gut microbiota of weaned piglets in a similar manner to antibiotics, including the suppression of gram-positive commensal bacteria. However, the ever-present risk of antimicrobial resistance remains a factor when using supra-nutritional metal feed supplementation, since bacterial tolerance (or, resistance) to zinc has been associated with resistance to methicillin via the co-located *czr* and *mecA* genes, respectively, in the SCCmec cassette among staphylococci in Danish swine, and also has been associated with the *czr* gene cluster in *Pseudomonas aeruginosa* (Aarestrup *et al.*, 2010; Hasman and Aarestrup, 2002). Additionally, supplementation of copper in swine diets has been associated with macrolide and glycopeptide resistance among enterococci, encoded via the *tcrB* gene (Hasman and Aarestrup, 2002). However, it remains unknown how zinc affects *E. coli* and *Enterococcus* spp. when added to cattle diets, and if any co-selection for resistance to other antibiotics occurs.

Other alternatives to antibiotics include essential oils, such as menthol, cinnamaldehyde, or thymol. In poultry, a feed additive consisting of menthol, anethol and eugenol increased feed conversion in chicks, and menthol alone has been shown to increase weight gain in poultry (Ocak and Sivri, 2008; Paraskeuas *et al.*, 2017). In cattle, results from previous studies suggested there was no significant effect on total coliform

counts; however, there was an increased prevalence of tetracycline-resistant *E. coli* (Aperce *et al.*, 2016). At the time of writing, there were no studies concerning resistance of *Enterococcus* spp. to antimicrobials when essential oils were fed, nor were there studies reporting the effects of essential oils fed in conjunction with other alternatives such as metals. We explored the potential for co-selection of antibiotic resistance among gram-negative and gram-positive bacteria through the independent and combined-use of feedgrade zinc and menthol using *E. coli* and *Enterococcus* spp.

Materials and methods

Experimental design

A randomized controlled trial in a 2x2 factorial design was previously conducted at Kansas State University (KSU) at the Beef Cattle Research Center and approved by the KSU Institutional Animal Care and Use Committee (Animal Use Protocol #3334). In total, 80 steers were placed in individual pens, stratified by weight and then randomly assigned by weight block to a treatment group. Treatments were: 1) supra-nutritional zinc fed at elevated feed concentrations (300 ppm; n=20 steers), menthol (fed as 0.3% of dry matter; n=20 steers), a combination of supra-nutritional zinc and menthol (n=20 steers), and a control group fed neither zinc nor menthol (n=20 steers). Animals were allowed to acclimate to their pens for two weeks to ensure proper equilibration of bacterial flora with neighboring cattle and the environment prior to the onset of the trial.

Fecal samples were collected *per rectum* from each steer using a new rectal palpation sleeve weekly for 5 weeks, starting with Day 0 prior to initiating the experimental regimens. Animals were fed their respective treatment diet for three weeks, with the peak of treatment effect expected at Day 21. The treatments were then

discontinued, to allow for a 2-week washout period. Samples were processed in the laboratory into two 5 ml tubes; that is, preparing one tube without glycerol and one tube with 50% sterile glycerol at a 1:1 ratio of glycerol to feces. Tubes were then stored at -80°C until later use.

Quantification, isolation, speciation

Samples from Day 0 were used as the baseline, and samples from Day 21 were considered the maximum treatment effect time point for analysis. Samples preserved with glycerol were thawed on ice and mixed thoroughly with Phosphate-buffered saline (PBS) (Gibco Life Technologies, Thermo Scientific Microbiology, Oakwood Village, OH) in a 1:10 dilution, using 9 milliliters of PBS and 1 gram of feces. An aliquot of 50 µL of this dilution was spiral plated using an EddyJet® 2 Spiral Plater (Neutec Group Inc, Farmingdale, NY) on to MacConkey agar for quantification of fecal coliforms; specifically, magenta-colored colonies indicating lactose fermentation and therefore presumptive *Escherichia coli*. This dilution also was spiral plated to MacConkey supplemented with tetracycline at 16 milligrams per liter (mg/L) and MacConkey supplemented with ceftriaxone at 4 mg/L. This same dilution also was spiral plated to m-*Enterococcus* agar for quantification of purple to red colonies indicating enterococci, and to m-*Enterococcus* agar supplemented with tetracycline at 16 mg/L and to m-*Enterococcus* agar supplemented with erythromycin at 8 mg/L. MacConkey agar plates were incubated at 37°C for 18 hours; in contrast, m-*Enterococcus* plates were incubated at 42°C for 48 hours. All plates were counted using the Flash & Go® System (Neutec Group Inc, Farmingdale, NY).

Two colonies from each plain (non-antibiotic) agar plate were selected and streaked to Tryptic Soy Agar (TSA) agar with 5% sheep blood for confirmation of species using Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF). Employing a single-use sterilized wooden toothpick, a single isolate of presumptive *Escherichia coli*, or *Enterococcus spp.*, was spread onto two wells of a reusable 96-well target plate (Bruker Daltonik GmbH., Billerica, MA). Once dry, one microliter (µl) of 70% formic acid was added to the first well of each sample spot pair only for *Enterococcus spp.* (gram-positive) isolates and to one empty well to serve as a negative control. Formic acid was restricted in use to gram-positive isolates, as it is unnecessary for gram-negative bacteria such as *E. coli*. One µl of the bacterial test standard (BTS) solution (Bruker Daltonik GmbH., Billerica, MA) was applied to the first and second wells on the plate as a positive control. After drying of all wells, one µl of HCCA matrix solution (Bruker Daltonik GmbH., Billerica, MA) was added to each well, including all the sample wells, BTS wells, formic acid negative control well, and an additional empty well serving as a secondary negative control. The target plate was then transferred to the MALDI-TOF Microflex LT/SH for reading, using MBT Compass v1.4 software. After confirmation of genus and species, these same confirmed isolates were then used for phenotypic susceptibility testing.

Phenotypic susceptibility testing

Susceptibility testing for all *Enterococcus* spp. and *Escherichia coli* isolates was performed using broth microdilution via the Sensititre® system (TREK, Thermo Scientific Microbiology, Oakwood Village, OH) to determine minimum inhibitory concentration (MIC). Isolates were freshly plated to TSA with 5% sheep blood agar and

incubated at 37°C for 18-24 hours. Subsequently, a bacterial dilution equivalent to a 0.5 McFarland standard was made using 11 ml of sterilized water. Next, 50 µl of the culture suspension was transferred to 11 ml of sterile Mueller-Hinton broth; finally, 50 µl of the broth culture was inoculated to each well of the NARMS gram-positive CMV3AGPF plate for *Enterococcus* spp. and the gram-negative CMV3AGNF plate for *Escherichia coli* using the Sensititre® automated inoculation delivery system (TREK, Thermo Scientific Microbiology, Oakwood Village, OH). Antibiotics on the CMV3AGPF plate included: chloramphenicol, ciprofloxacin, daptomycin, erythromycin, gentamicin, kanamycin, lincomycin, linezolid, nitrofurantoin, penicillin, quinupristin/dalfopristin, streptomycin, tetracycline, tigecycline, tylosin, and vancomycin (Table 1). Antibiotics on the CMV3AGNF plate included amoxicillin/clavulanic acid, ampicillin, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulfamethoxazole (Table 2). Three positive and one negative control wells also were included on each plate. Plates were incubated at 37°C for 18 hours for the CMV3AGNF plate and 24 hours for the CMV3AGPF plate, with *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, *Pseudomonas aeroginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 serving as quality control strains run along with each new serial number or batch of plates. Plates were read using a Sensititre OptiRead™ (TREK, Thermo Scientific Microbiology, Oakwood Village, OH) device. The results were interpreted as susceptible, intermediate, or resistant in accordance with CLSI M100 document guidelines, using NARMS breakpoints when a CLSI breakpoint was unavailable via SWIN software (TREK,

Thermo Scientific Microbiology, Oakwood Village, OH) (Table 1, Table 2); later, intermediate results were recoded as susceptible for binary variable statistical analysis purposes. Minimum inhibitory concentrations (MICs) were plotted using Excel and a 95% exact confidence interval for the proportion of resistance isolates was calculated using Stata® version 16.1 (StataCorp LLC, College Station, TX) to create an integrated table of data and an illustrative figure, affectionately known as a "squashtogram".

Antibiotic	Class	Range	Breakpoint
Gentamicin	Aminoglycoside	128-	≥500
Kanamycin	Aminoglycoside	128-	≥1024
Streptomycin	Aminoglycoside	512-	>1000
Vancomycin	Glycopeptide	0.25-	≥32
Tigecycline	Glycylcycline	0.015-	≥0.5
Lincomycin	Lincosamide	1-8	≥8
Daptomycin	Lipopeptide	0.25-	≥8
Erythromycin	Macrolide	0.25-8	≥8
Tylosin	Macrolide	0.25-	≥32
Nitrofurantoin	Nitrofuran	2-64	≥128
Linezolid	Oxazolidinone	0.5-8	≥8
Penicillin	Penicillin	0.25-	≥16
Chloramphenicol	Phenicol	2-32	≥32
Ciprofloxacin	Quinolone	0.12-4	≥4
Quinupristin/dalfopristin	Streptogramin	0.5-32	≥4
Tetracycline	Tetracycline	1-32	≥16

Table 1 Antibiotics ordered by class, concentration ranges (mg/L), and interpretive breakpoints for CMV3AGPF plate, using CLSI criteria, and NARMS interpretive human breakpoints when a CLSI equivalent was unavailable

Antibiotic	Class	Range	Breakpoint
Gentamicin	Aminoglycoside	0.25-16	≥16
Streptomycin	Aminoglycoside	2-64	≥64
Cefoxitin	Cephem	0.5-32	≥32
Ceftiofur	Cephem	0.12-8	≥32
Ceftriaxone	Cephem	0.25-64	>4
Sulfisoxazole	Folate Pathway Inhibitor	16-256	≥512
Trimethoprim/sulfamethoxazole	Folate Pathway Inhibitor	0.12/2.38-4.75/76	≥4/76
Azithromycin	Macrolide	0.12-16	≥32
Ampicillin	Penicillin	1-32	≥32
Chloramphenicol	Phenicol	2-32	≥32
Ciprofloxacin	Quinolone	0.015-4	≥1
Nalidixic Acid	Quinolone	0.5-32	≥32
Tetracycline	Tetracycline	4-32	≥16
Amoxicillin/clavulanic acid	β-Lactam/β-Lactamase inhibitoer	1/0.5-32/16	≥32/16

Table 2 Antibiotics ordered by class, concentration ranges (mg/L), and interpretive breakpoints for CMV3AGNF plate, using CLSI criteria, and NARMS interpretive human breakpoints when a CLSI equivalent was unavailable

Statistical analyses

All statistical analyses were performed using Stata® v.16.1 (StataCorp LLC, College Station, TX). To achieve normalized distributions, colony count derived quantities (CFU/g feces) were transformed to log base 10 ($\log_{10}$ CFU per gram of feces) for use as dependent variables in multi-level mixed effects linear regression. To determine the relative quantity of antibiotic resistant $\log_{10}$ CFU per gram of feces to total $\log_{10}$ CFU per gram of feces, a new variable was created by subtracting the $\log_{10}$ CFU per gram of feces grown on antibiotic-supplemented agar from the $\log_{10}$ CFU per gram of feces of the corresponding plain agar plate. These differences were then also used as a dependent variable in multi-level mixed effects linear regression. A 3-way full factorial

model was constructed, factors being zinc (binary), menthol (binary) and sample day (2-level factor for Day 0 and Day 21). Full models were retained in all cases for biological reasons, regardless of the statistical significance of the interaction terms, because the treatments had not been applied before Day 0 sampling.

For statistical analysis of phenotypic susceptibility of isolates, resistance to each antibiotic class (antibiotic class as defined by CLSI) was graphed by day and treatment group. The gram-negative plate consisted of nine classes of antibiotics, and the gram-positive plate consisted of 13 classes of antibiotics. Additionally, binary resistance to each class of antibiotic was summed for each isolate to create a new variable representing multidrug resistance count (an integer variable), which also was graphed by day and treatment group. This variable was then used to determine multidrug resistance as a binary variable (MDR, defined as resistance to ≥ 3 classes of antibiotics) for each isolate. A 3-way full factorial multi-level mixed effects logistic regression model was then used to determine the effect of sample day, zinc and/or menthol on the relative odds of multi-drug resistance (a binary variable). For each statistical model, marginal means were calculated and plotted by sample day with a 95% confidence interval.

Results

Descriptive statistics

A total of 160 samples were plated to previously described agars. A total of 320 presumptive *E. coli* isolated from plain MacConkey agar from Day 0 and Day 21 were subjected to MALDI-TOF. From Day 0, 158 isolates (98.75%) were confirmed as *E. coli*. The two isolates that were not *E. coli* were identified as *Proteus mirabilis* and

Citrobacter sedlakii. From Day 21, 159 isolates (99.40%) were confirmed as *E. coli*. The single non-*E. coli* isolate was identified as *Pseudomonas chlororaphis*.

A total of 320 presumptive *Enterococcus* spp. isolated from plain m-*Enterococcus* agar from Day 0 and Day 21 were subjected to MALDI-TOF. From Day 0, 95 (59.40%) enterococcal isolates were *E. faecium*, 33 (20.63%) were *E. hirae*, 17 (10.63%) were *E. mundtii*, three (1.88%) were *E. casseliflavus*, two (1.25%) were *E. durans*, one (0.63%) was *E thailandicus* and one (0.63%) was *E. avium*. Five out of the 160 (3.13%) isolates were not *Enterococcus* spp.; one was *Aerococcus viridans* and four could not be identified using MALDI-TOF. From Day 21, 81 (50.63%) of the enterococci were *E. faecium*, 30 (18.75%) were *E. hirae*, 21 (13.13%) were *E. mundtii*, 11 (6.88%) were *E. casseliflavus*, 9 (5.63%) were *E. faecalis*, four (2.5%) were *E. thailandicus*, and one (0.63%) was *E. durans*. A total of three (1.88%) isolates were not *Enterococcus* spp., one (0.63%) was *Aerococcus viridans*, one was *Streptococcus lutetiensis* and one isolate could not be identified using MALDI-TOF.

Multi-level mixed effects linear regression modeling of plate quantification

All samples (n=160) were quantifiable on plain MacConkey agar, while 99.37% (n=159) were quantifiable on tetracycline-supplemented MacConkey, and 73.12% (n=117) of samples were quantifiable on ceftriaxone-supplemented agar. For plain MacConkey agar (Figure 1A), sampling period significantly ($p<0.05$) affected $\log_{10}$ CFU per gram of feces, while treatment group did not influence the outcome independent of period effects. The lowest and highest CFU per gram of feces collected from Day 0 ranged from $\log_{10}$ 5.31 to 7.50, with a mean of 6.74 from 80 samples. In contrast, the range in $\log_{10}$ CFU per gram of feces from Day 21 was from a minimum of 3.44 to a

maximum of 6.67, and with a mean of 5.82 from 80 samples. The control group decreased significantly ($P < 0.05$) from a mean $\log_{10}$ CFU per gram of feces of 6.79 (95% CI of 6.52 to 7.07) on Day 0 to a mean $\log_{10}$ CFU per gram of feces of 6.05 (95% CI of 5.81 to 6.36) on Day 21. The menthol group decreased from a mean $\log_{10}$ 6.812 (95% CI of 6.53 to 7.08) on Day 0 to a mean $\log_{10}$ 5.71 (95% CI of 5.43 to 5.98) on Day 21. Zinc was also decreased from a mean $\log_{10}$ 6.69 (95% CI of 6.41 to 6.97) on Day 0 to a mean $\log_{10}$ of 5.86 (95% CI of 5.59 to 6.14) on Day 21. Lastly, the combined zinc and menthol decreased from a $\log_{10}$ 6.68 (95% CI of 6.40 to 6.95) on Day 0 to $\log_{10}$ 5.63 (95% CI of (95% CI of 5.36 to 5.91) on Day 21. These decreases in $\log_{10}$ CFU per gram of feces were significant ($p<0.05$) for period effects alone, treatment group and interactions of treatment with period did not have a significant effect.

In comparison, concerning the results of growth on tetracycline-supplemented MacConkey agar both the menthol and the combined menthol and zinc groups exhibited statistically significantly ($P < 0.05$) decreased $\log_{10}$ CFU per gram of feces from Day 0 to Day 21. The range in $\log_{10}$ CFU per gram of feces from Day 0 was a minimum of 4.04, and a maximum of 7.38 with a mean of 5.98, from a total of 80 samples. The range in $\log_{10}$ CFU per gram of feces from Day 21 was a minimum of 2.60, and a maximum of 6.59 with a mean of 5.17, from a total of 79 samples. The single sample which exhibited no growth on tetracycline-supplemented agar was from the combined zinc and menthol group on Day 21. The menthol group significantly decreased from a mean $\log_{10}$ CFU 6.06 (95% CI of 5.69 to 6.43) per gram of feces on Day 0, to a mean $\log_{10}$ CFU of 5.04 (95% CI of 4.67to 5.41) per gram of feces on Day 21. The combined zinc and menthol group significantly decreased from a mean $\log_{10}$ CFU of 6.05 (95% CI of 5.68 to 6.42)

per gram of feces on Day 0 to a mean $\log_{10}$ CFU of 4.84 on Day 21 (95% CI of 5.68 to 6.42). Additionally, both the control and zinc group decreased, though not significantly ($P > 0.05$) (Figure 1B). The zinc group decreased from a mean $\log_{10}$ CFU of 5.85 (95% CI of 5.48 to 6.22) per gram of feces on Day 0 to a mean $\log_{10}$ CFU of 5.24 (95% CI of 4.87 to 5.61) on Day 21. The control group was least affected, with a decrease from a mean $\log_{10}$ CFU of 5.97 (95% CI of 5.60 to 6.34) on Day 0, to a mean $\log_{10}$ CFU of 5.55 (95% CI of 5.18 to 5.92) on Day 21.

By subtracting the $\log_{10}$ growth on tetracycline-supplemented agar from corresponding growth on plain MacConkey agar the difference is presented, generally as a positive integer. Using this difference in $\log_{10}$ counts (x), and expressed as 10^x, a difference of 1 expressed as $10^{-1} = 0.1$, a difference of 2 expressed as $10^{-2} = 0.01$, each serving as an estimate of the prevalence of tetracycline resistance among coliforms. Therefore, the difference in growth between plain and antibiotic supplemented agar with respect to resistance is inversely related, and a decrease in the difference is interpreted as an increase in the resistance.

When looking at the difference between growth on plain and tetracycline-supplemented MacConkey agar, neither sample day nor treatment had a significant effect (Figure 1C). A tendency existed for the control, menthol, and zinc groups to exhibit a decreased difference in the $\log_{10}$ CFU per gram of feces between plain and tetracycline-supplemented agar. For the control, this was a decrease from a mean difference of 0.822 (95% CI of 0.54 to 1.10) on Day 0 to 0.531 (95% CI of 0.25 to 0.80) on Day 21. For the zinc group, this was a decrease from a mean of 0.842 (95% CI of 0.47 to 1.02) to 0.668 (95% CI of 0.39 to 0.94) from Day 0 to Day 21. For the menthol

group, this was a decrease from a mean difference of 0.751 (95% CI of 0.56 to 1.12) on Day 0 to 0.622 (95% CI of 0.34 to 0.90) on Day 21. These decreases in the difference between plain and tetracycline supplemented agar indicate an increase in resistance. However, these differences were not significant for sample day or treatment ($p>0.05$). The combined zinc and menthol group showed an increase in the difference between plain and tetracycline-supplemented agar, from a mean difference of 0.623 (95% CI of 0.34 to 0.90) on Day 0 to 0.772 (95% CI of 0.49 to 1.05) on Day 21, suggesting a decrease in resistance, however these results were also not significant ($p>0.05$).Additionally, growth on ceftriaxone-supplemented MacConkey agar exhibited no significant effects for day or treatment group. All $\log_{10}$ CFU per gram of feces tended to decrease from Day 0 to Day 21, with a mean $\log_{10}$ CFU of 2.90 per gram of feces on Day 0 to a mean $\log_{10}$ CFU of 2.41 on Day 21. The range of $\log_{10}$ CFU per gram of feces on Day 0 was from $\log_{10}$ 2.61 to 5.18 in 64 samples. The samples which did not grow on ceftriaxone supplemented agar were spread equitably across treatments, with 4 samples each from the control and menthol group, 3 samples from the zinc group, and 5 samples from the combined zinc and menthol group. The range of $\log_{10}$ CFU per gram of feces on Day 21 was from $\log_{10}$ 2.60 to 5.49 from 53 samples. On Day 21 the samples which did not grow on ceftriaxone-supplemented MacConkey remained distributed across the treatment groups, with 5 samples from the control group, 7 samples from the menthol group, 6 samples from the zinc group, and 9 samples from the combined zinc and menthol group. The control group mean $\log_{10}$ CFU per gram of feces decreased from 2.94 (95% CI of 2.20 to 3.68) on Day 0 to 2.71 (95% CI of 1.97 to 3.45) on Day 21. The menthol group mean $\log_{10}$ CFU per gram of feces decreased from 2.86 (95% CI of 2.12

to 3.60) on Day 0 to 2.35 (95% CI of 1.61 to 3.09) on Day 21. The zinc group mean $\log_{10}$ CFU per gram of feces decreased from 3.16 (95% CI of 2.42 to 3.90) on Day 0 to 2.46 (95% CI of 1.73 to 3.20) on Day 21. The combined zinc and menthol group decreased from a mean $\log_{10}$ CFU of 2.64 (95% CI of 1.90 to 3.38) per gram of feces on Day 0 to 2.09 (95% CI of 1.35 to 2.82) on Day 21. However, these decreases in $\log_{10}$ CFU from Day 0 to Day 21 were not significant (Figure 1D). As previously mentioned, by subtracting the $\log_{10}$ growth on ceftriaxone-supplemented agar from corresponding growth on plain MacConkey agar, the result is a proportion which is inversely related to resistance, i.e., a decrease in the difference between plain and ceftriaxone-supplemented agar is interpreted as an increase in resistance. Neither day nor treatment group significantly impacted the difference in $\log_{10}$ CFU per gram of feces growth on plain versus ceftriaxone-supplemented MacConkey agar (Figure 1E). However, all treatment group differences tended to decrease between sampling days to varying degrees. The control group decreased from a mean difference of 3.85 (95% CI of 3.12 to 4.58) on Day 0 to 3.37 (95% CI of 2.64 to 4.10) on Day 21. The menthol group decreased from a mean difference of 3.94 (95% CI of 3.21 to 4.68) on Day 0 to 3.35 (95% CI of 2.62 to 4.08) on Day 21. The zinc group decreased from a mean difference of 3.53 (95% CI of 2.80 to 4.26) on Day 0 to 3.39 (95% CI of 2.66 to 4.12) on Day 21. The combined zinc and menthol group decreased from a mean difference of 4.03 (95% CI of 3.30 to 4.76) on Day 0 to 3.54 (95% CI of 2.81 to 4.27) on Day 21. However, these decreases in the difference in $\log_{10}$ CFU per gram of feces growth between plain and ceftriaxone-supplemented MacConkey from Day 0 to Day 21 were not significant ($p>0.05$).

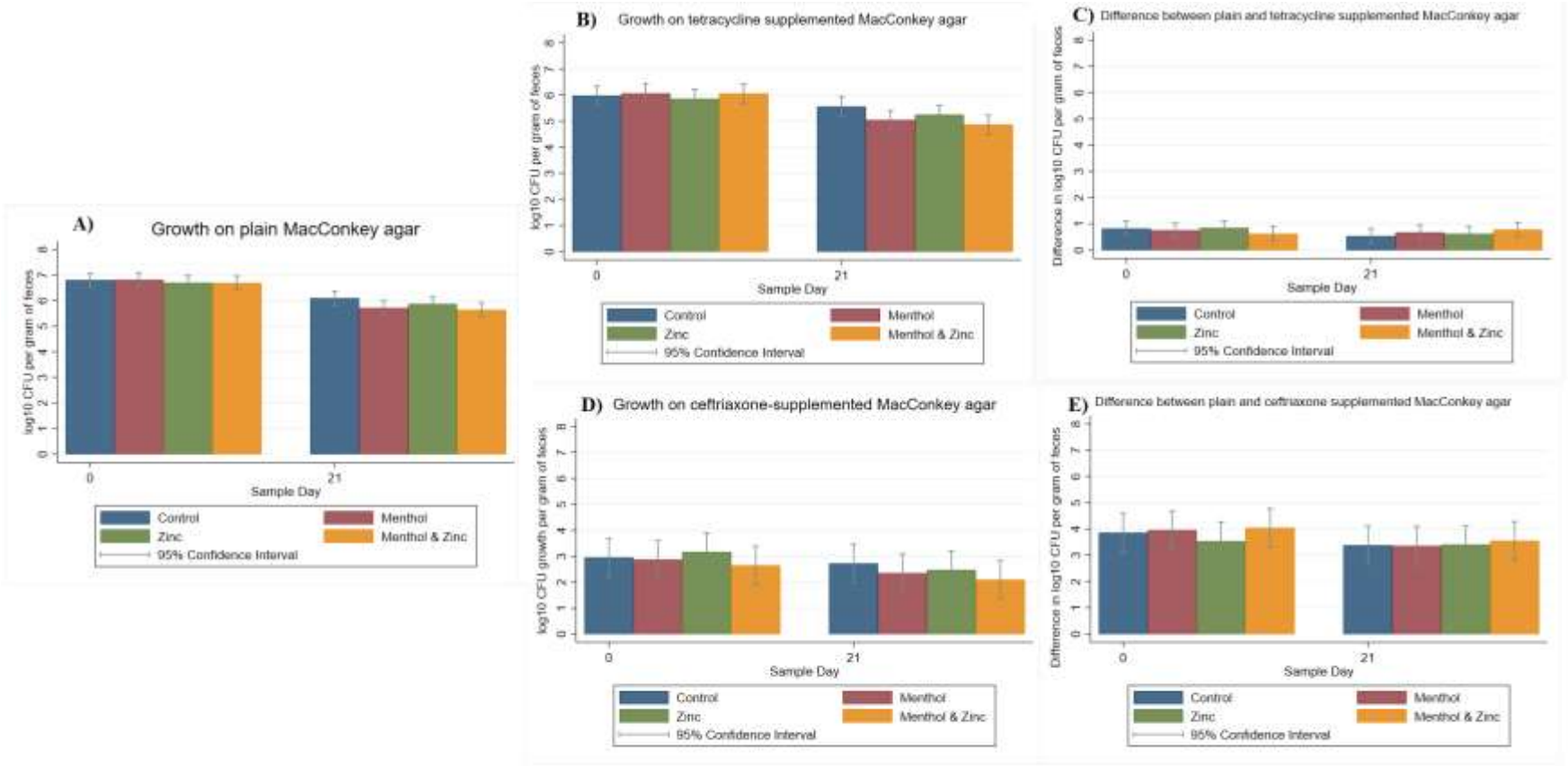


Figure 1 A) log_{10} CFU per gram of feces on plain MacConkey agar, B) log_{10} CFU per gram of feces on tetracycline-supplemented (16 mg/L) MacConkey agar, C) Difference in log_{10} CFU between plain and tetracycline-supplemented (16 mg/L) MacConkey agar, D) log_{10} CFU per gram of feces on ceftriaxone-supplemented (4 mg/L) MacConkey agar, and E) Difference in log_{10} CFU between plain and ceftriaxonesupplemented (4 mg/L) MacConkey supplemented agar.

For enterococci, all samples (n=160) were quantifiable on both m-*Enterococcus* agar, and m-*Enterococcus* agar supplemented with tetracycline, while 96.87% (n=155) were quantifiable on m-*Enterococcus* agar supplemented with erythromycin. For growth on plain m-*Enterococcus* agar, neither treatment nor sample day had a significant effect ($P > 0.05$). The $\log_{10}$ CFU per gram of feces range on Day 0 was 3.83 to 6.55, with a mean of 5.81, from 80 samples. The menthol group, started on day 0 at a higher $\log_{10}$ CFU per gram of feces than the control group with a mean of 6.10 (95% CI of 5.89 to 6.31) $\log_{10}$ CFU per gram of feces compared to a mean of 5.85 (95% CI of 5.65 to 6.06), respectively. This was significantly higher than for the zinc group, and the combination zinc and menthol group, which had a mean $\log_{10}$ CFU per gram of feces of 5.62 (95% CI of 5.42 to 5.83) and 5.67 (95% CI of 5.46 to 5.88), respectively, at Day 0. These significant differences were observed on Day 0, despite the randomization process (Figure 2A). There were no significant differences among treatment groups by Day 21, which overall had a $\log_{10}$ CFU per gram of feces range on Day 21 of 4.31 to 6.52, with a mean of 5.79. The treatment groups at Day 21 were not significantly different than their baselines at Day 0. The $\log_{10}$ CFU per gram of feces growth on tetracycline supplemented agar on Day 0 ranged from 2.60 to 6.51, with a mean of 5.25 from 80 samples. The menthol group tended to have higher $\log_{10}$ CFU per gram of feces growth on tetracycline-supplemented agar on Day 0, with a mean $\log_{10}$ CFU of 5.63 (95% CI of 5.31 to 5.95) per gram of feces, compared to 5.11 (95% CI of 4.79 to 5.43) for the

control group, 5.03 (95% CI of 4.71 to 5.35) for the zinc group, and 5.24 (95% CI of 4.92 to 5.57) for the combined zinc and menthol group; however, this was not significantly different ($P > 0.05$) from the other treatment groups (Figure 2B).

On Day 21, there were still no significant differences among treatment groups, and no significant differences compared to Day 0. The $\log_{10}$ CFU per gram of feces growth on tetracycline supplemented agar on Day 21 ranged from 3.38 to 6.46, with a mean of 5.25 from 80 samples. The control group mean $\log_{10}$ CFU per gram of feces on Day 21 was 5.27 (95% CI of 4.95 to 5.59), compared to 5.11 (95% CI of 4.79 to 5.43) on Day 0. The menthol group mean $\log_{10}$ CFU per gram of feces on Day 21 was 5.22 (95% CI of 4.91 to 5.54), compared to 5.63 (95% CI of 5.31 to 5.95) on Day 0. The zinc group mean $\log_{10}$ CFU per gram of feces on Day 21 was 5.20 (95% CI of 4.88 to 5.52), compared to 5.03 (95% CI of 4.71 to 5.35) on Day 0. The combined zinc and menthol group mean $\log_{10}$ CFU per gram of feces on Day 21 was 5.30 (95% CI of 4.98 to 5.62), compared to 5.24 (95% CI of 4.92 to 5.57) on Day 0.There was a tendency for the difference between plain and tetracycline supplemented agar to decrease from Day 0 to Day 21, with a mean difference of 0.741 on Day 0 and a mean difference of 0.54 on Day 21 (hence, proportion of resistance increased). The menthol group tended to increase, from a mean difference of 0.46 (95% CI of 0.26 to 0.68) at Day 0 to a mean difference of 0.67 (95% CI of 0.47 to 0.89) at Day 21 (Figure 2C), suggesting less tetracycline resistance. However, the changes observed were not significant ($p>0.05$). The zinc group

had a mean difference of 0.59 (95% CI of 0.38 to 0.80) on Day 0 and a mean difference of 0.54 (95% CI of 0.34 to 0.76) on Day 21. The combined zinc and menthol group had a mean of 0.421 (95% CI of 0.21 to 0.63) on Day 0 and a mean of 0.41 (95% CI of 0.20 to 0.62) on Day 21.The zinc group was significantly ($P < 0.05$) lower in $\log_{10}$ CFU per gram of feces on erythromycin supplemented agar compared to the other treatment groups on Day 0 (Figure 2D) with a mean $\log_{10}$ CFU of 3.21 (95% CI of 2.75 to 3.69) per gram of feces compared to 4.17 (95% CI of 3.70 to 4.65) for the control group, 4.72 (95% CI of 4.26 to 5.20) for the menthol group, and 4.21 (95% CI of 3.74 to 4.69) for the combined zinc and menthol group; once again, this occurred despite the randomization process which should have yielded no differences among the treatment groups on Day 0. It should be noted that of the five samples which exhibited no growth on m-*Enterococcus* agar with erythromycin, four samples belonged to the zinc treatment group collected from Day 0.

There was a significant increase in $\log_{10}$ CFU per gram of feces growth on erythromycin supplemented agar between Day 0 and Day 21 for the zinc group; with a mean $\log_{10}$ CFU of 4.48 (95% CI of 4.02 to 4.96) per gram of feces at Day 21 compared to 3.21 (95% CI of 2.75 to 3.69) at Day 0. However, it was not different from the other treatments, which had a mean $\log_{10}$ CFU of 4.36 (95% CI of 3.90 to 4.84), 4.53 (95% CI of 4.06 to 5.00), and 4.59 (95% CI of 4.12 to 5.06) for the control, menthol, and combined zinc and menthol groups, respectively.

Similarly, the difference in $\log_{10}$ CFU per gram of feces growth between plain and erythromycin-supplemented m-*Enterococcus* agar was significantly different for the zinc group compared to the menthol and the combined zinc and menthol treatment groups, with a mean difference of 2.40 (95% CI of 2.00 to 2.81) for the zinc group, compared to 1.37 (95% CI of 1.28 to 2.08) and 1.45 (95% CI of 1.05 to 1.86) for the menthol and combined zinc and menthol groups respectively. Correspondingly, the zinc group showed a significant decrease from Day 0 to Day 21, from a mean difference of 2.40 (95% CI of 2.00 to 2.81) to 1.26 (95% CI of 0.87 to 1.67) (Figure 2E), suggesting a ten-fold increase in erythromycin resistance. Due to the significant difference in the zinc group on Day 0 compared to the other groups, a *post hoc* pairwise comparison using Bonferroni correction was performed, and the zinc group still showed a significant ($P<0.05$) decrease in difference from Day 0 to Day 21, indicating an increase in resistance.

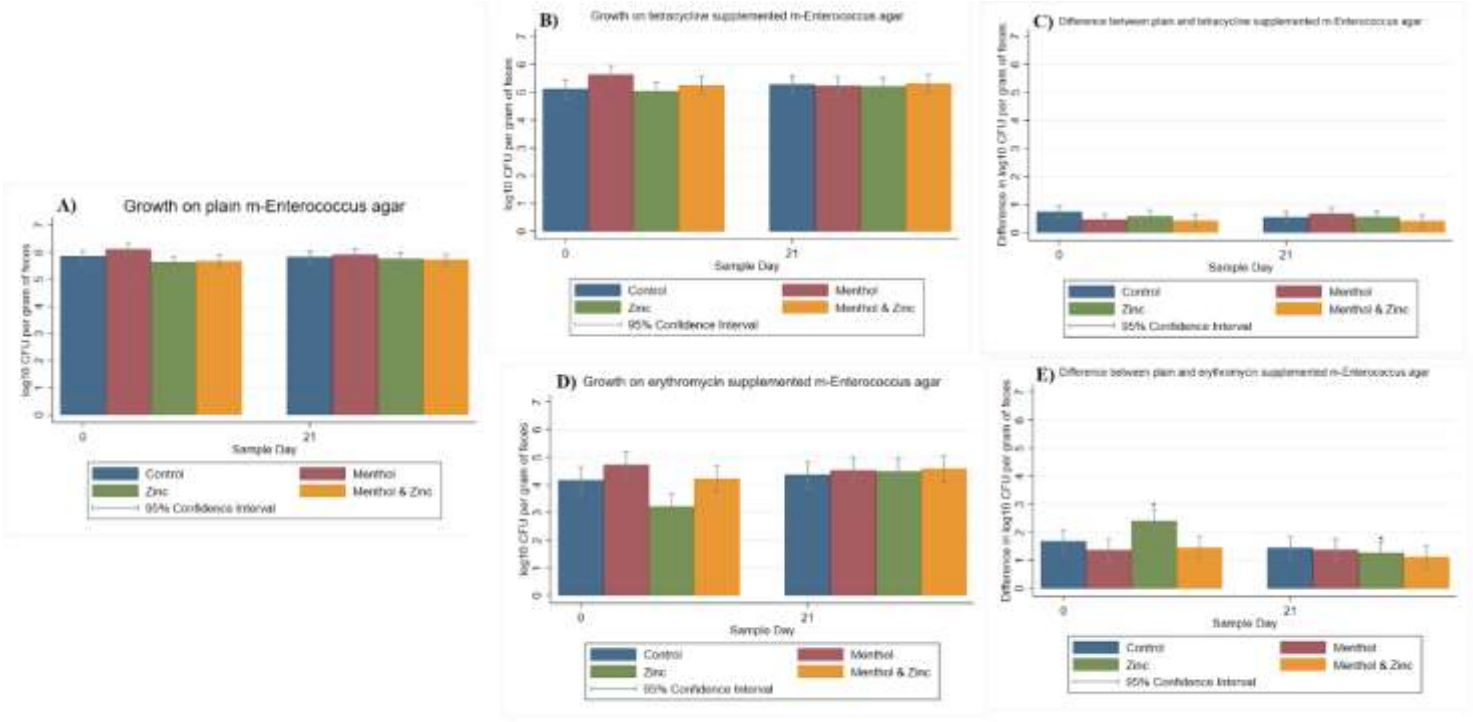


Figure 2 A) log_{10} CFU per gram of feces on plain m-*Enterococcus* agar B) log_{10} CFU per gram of feces on tetracycline supplemented m-*Enterococcus* agar C) Difference in log_{10} CFU between plain and tetracycline supplemented m-*Enterococcus* agar D) log_{10} CFU per gram of feces on erythromycin supplemented m-*Enterococcus* agar E) Difference in log_{10} CFU between plain and erythromycin supplemented m-*Enterococcus* agar

* significantly different using a *post hoc* Bonferonni pairwise comparison

Descriptive statistics of phenotypic resistance of isolates

For phenotypic resistance of *E. coli* isolates (Figure 3), all isolates were susceptible to ciprofloxacin. Nearly half of all isolates were resistant to tetracycline. Over 20% of isolates exhibited resistance to sulfisoxazole and streptomycin, while very few isolates (less than 1%) were resistant to amoxicillin/clavulanic acid, azithromycin, cefoxitin, ceftiofur, ceftriaxone, gentamicin, nalidixic acid, or trimethoprim / sulfamethoxazole. It should be noted that a bimodal distribution of MIC values appeared for isolates susceptible and resistant to ceftiofur and ceftriaxone, respectively, with the majority being susceptible and with a very low MIC. This distribution was also present when contrasting the MICs of susceptible and resistant gentamicin and nalidixic acid.

Similarly, the resistance of *E. coli* isolates to each antibiotic class (Figure 4) by sample day and treatment showed that aminoglycoside resistance tended to increase from Day 0 to Day 21 across all treatment groups, from 16.88% to 28.75%. However, resistance to the tetracycline class tended to increase more for the menthol, zinc, and combination treatment groups from Day 0 to 21. The menthol group increased from 42.5% isolates resistant to 52.5%, the zinc group increased from 40% to 60% isolates resistant, and the combined zinc and menthol group increased from 45% to 52.5% isolates resistant to tetracycline class antibiotics. The increase in antibiotic resistance to aminoglycosides and tetracyclines was later statistically tested for significance using a multi-level mixed logistic regression.

	# Resistant (of 320 tested)	% Resistant	95% Confidence Interval		0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Amoxicillin/ Clavulanic Acid	3	0.94	0.19	2.72							4.69	18.75	60.94	14.38	0.31	0.31	0.63		
Ampicillin	42	13.3	9.63	17.3							5.31	42.5	37.5	1.56	0.00	13.13			
Azithromycin	2	0.63	0.08	2.24				0.00	0.00	0.94	0.94	8.13	78.75	10.31	0.31	0.63			
Cefoxitin	3	0.94	0.19	2.72						0.00	0.31	7.81	61.56	28.13	1.25	0.31	0.63		
Ceftiofur	3	0.94	0.19	2.72				2.81	21.25	73.75	0.94	0.31	0.00	0.63	0.31				
Ceftriaxone	3	0.94	0.19	2.72					98.13	0.94	0.00	0.00	0.31	0.00	0.31	0.00	0.31		
Chloramphenicol	48	15	11.3	19.4								2.5	46.25	35.63	0.63	0.63	14.37		
Ciprofloxacin	0	0	0	1.14*	95.94	2.5	0.63	0.31	0.63	0.00	0.00	0.00	0.00						
Gentamicin	1	0.31	0.01	1.73					0.94	75.31	23.44	0.00	0.00	0.00	0.31				
Nalidixic Acid	3	0.94	0.19	2.72						0.31	4.69	76.25	17.81	0.00	0.00	0.31	0.63		
Streptomycin	73	22.84	18.3	27.8								0.00	16.56	50.94	4.06	5.63	11.56	11.25	
Sulfisoxazole	67	20.94	16.6	25.8											73.13	5	0.63	0.31	20.94
Tetracycline	148	46.25	40.7	51.9									44.38	9.38	2.5	4.06	39.69		
Trimethoprim/ sulfamethoxazole	2	0.63	0.08	2.24				87.81	7.5	3.75	0.31	0.00	0.63						
*97.5% One-sided CI																			

Figure 3 Percentage of *E. coli* isolates that were resistant and their distribution across minimum inhibitory concentrations (MIC) for each antibiotic. Black vertical lines indicate the human CLSI (or, NARMS) interpretive breakpoint, grey boxes indicate areas above and below highest and lowest limit of assay antibiotic concentrations, respectively. Isolates which exceeded growth at the highest antibiotic concentration were placed in the next MIC column.

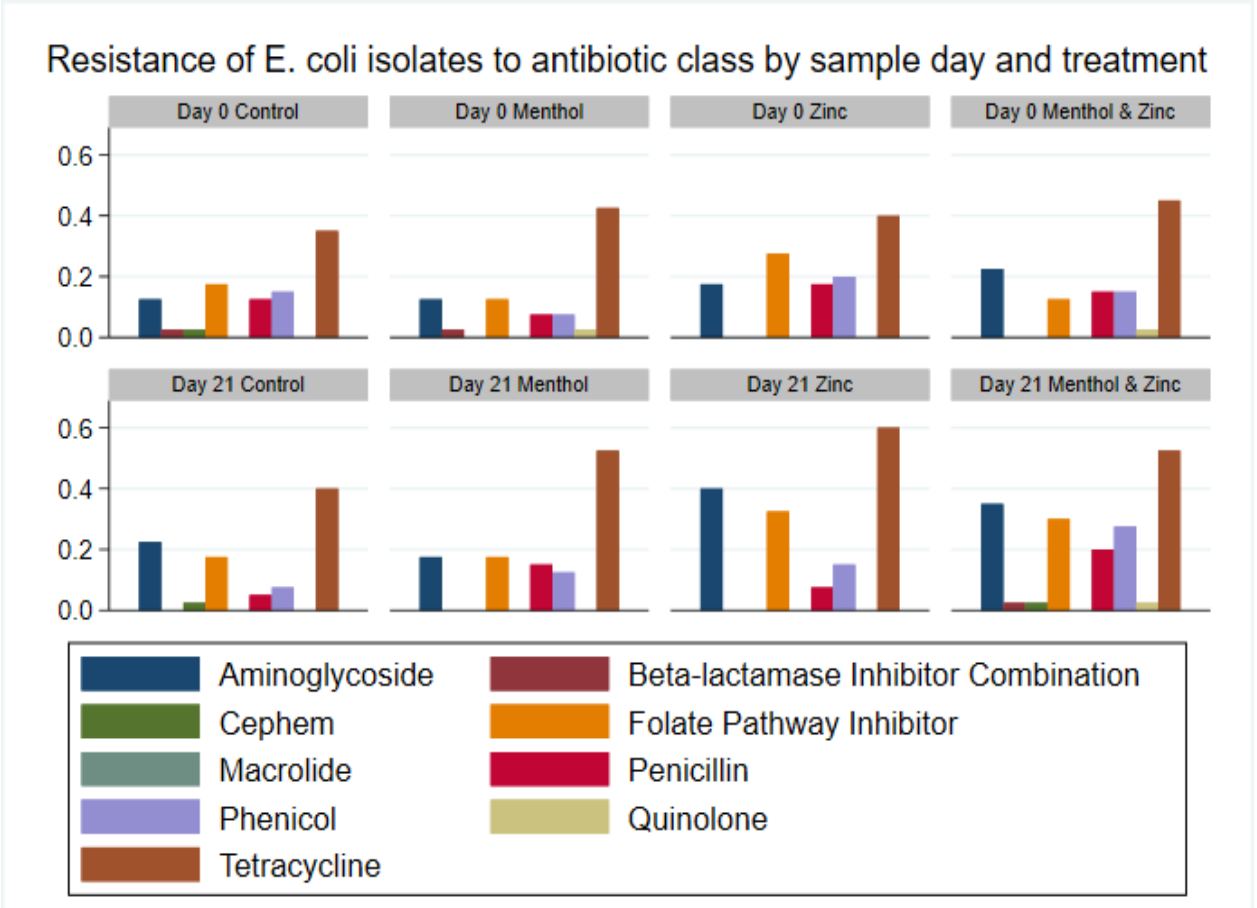


Figure 4 Resistance (proportion) of *E. coli* isolates to each antibiotic class by sample day and treatment

Additionally, the resistance to the number of antibiotic classes by sample day and treatment (Figure 5) showed a trend towards increased multidrug-resistance in the zinc group from a total of 27.5% isolates multidrug-resistant on Day 0 to 32.5% Day 21. Correspondingly, the zinc group had an increase in the percentage of isolates resistant to 3 antibiotic classes, from 7.5% at Day 0 to 18% at Day 21, and an increase in isolates resistant to 5 antibiotic classes, from 5% at Day 0 to 7.5% at Day 21. The menthol group did not show a trend for overall multi-drug resistance, slightly increasing from 15.5% of isolates classified as multidrug-resistant on Day 0 to 16.5% on Day 21. However, there was an increase in the percentage of isolates resistant to 5 classes of antibiotic among the menthol group, from 2.5% of isolates resistant on Day 0, to 13% on Day 21. The combined zinc and menthol group also showed an increase in percentage of multidrug-resistant isolates, from 19% on Day 0 to 32.5% on Day 21.

Correspondingly, the combined zinc and menthol group had an increase in the percent of isolates resistant to 5 classes of antibiotics, from 5% at Day 0 to 18% at Day 21. Additionally, the zinc and menthol group did not have any isolates resistant to 6 antibiotic classes on Day 0, but on Day 21 2.5% of isolates were resistant. Most of this gain in proportion came at the expense of the proportion of isolates that were pan-susceptible to all antibiotic classes on Day 0. In contrast, the control group seemingly decreased in its number of isolates resistant to multiple classes of antibiotics, with 2.5% of isolates resistant to 7 classes of antibiotics, and none resistant to 7 classes on Day 21. These increases in multi-drug resistance were later statistically tested for significance using a multi-level mixed logistic regression.

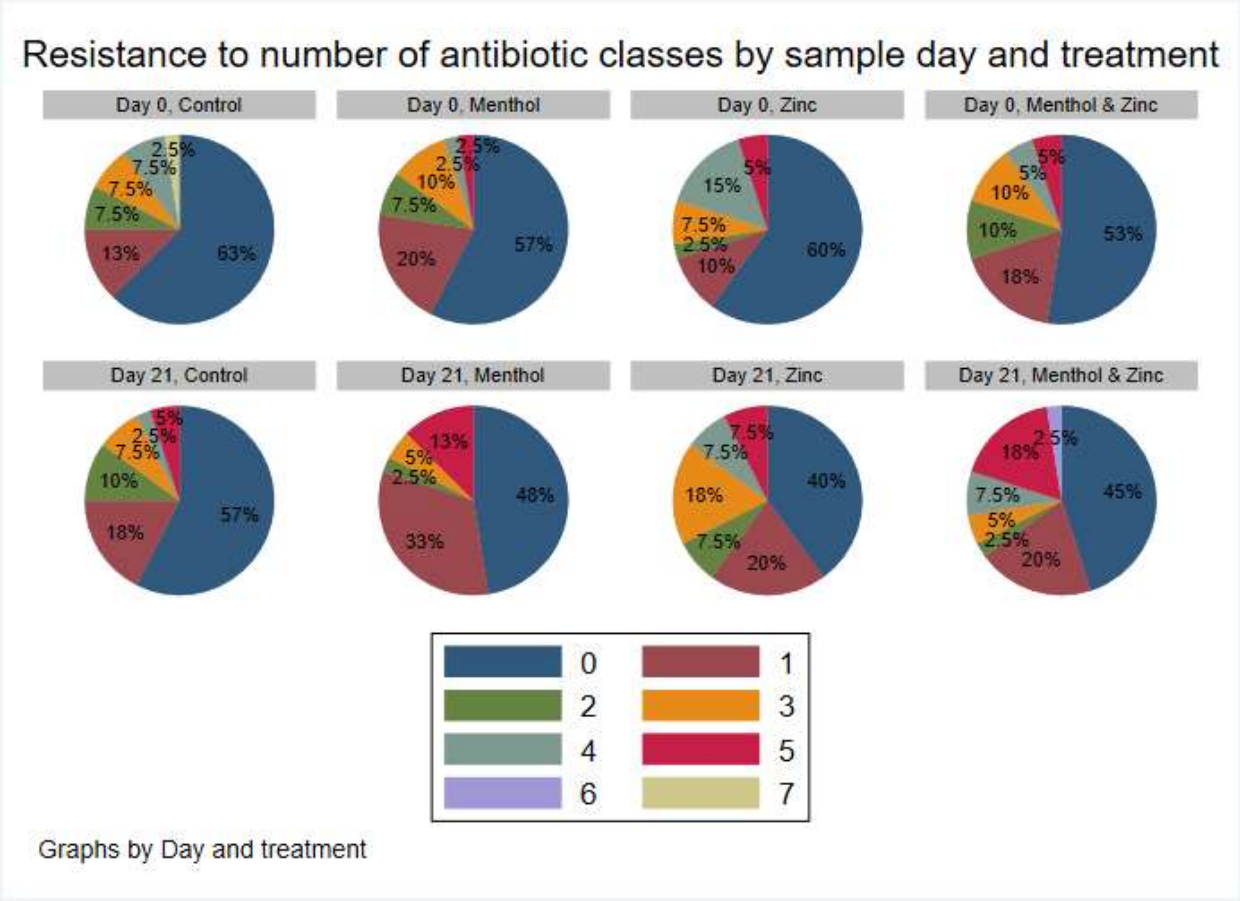


Figure 5 Resistance (%) of *E. coli* isolates to number of antibiotic classes by sample day and treatment

For phenotypic resistance of *Enterococcus* spp. (Figure 6), all isolates were susceptible to gentamicin, tigecycline, and vancomycin. Nearly all isolates (91.25%) were resistant to lincomycin, while approximately a third were resistant to quinupristin/dalfopristin and tetracycline. Not surprisingly, resistance to erythromycin and tylosin (both macrolides) was nearly equal, at 17.81% and 18.44%, respectively. Less than 1% of isolates were resistant to chloramphenicol, kanamycin, linezolid, penicillin, or streptomycin. It should be noted that a bimodal distribution appeared in regards to the MICs of both tetracycline and lincomycin, corresponding to their categorization as either susceptible or resistant.

Additionally, resistance of *Enterococcus* spp. isolates to antibiotic class and sample day (Figure 7), showed that tetracycline resistance tended to decrease from Day 0 to Day 21 in the menthol/zinc combination treatment group; that is, 37.5% of isolates were resistant on Day 0 compared to 17.5% of isolates resistant on Day 21. Tetracycline resistance tended to increase during the same period in the control group, from 20% of isolates on Day 0 to 35% on Day 21. Similarly, the percentage of macrolide resistant isolates tended to increase in the control group from 15% on Day 0 to 22.5% on Day 21. There was also an increased percentage of macrolide resistant isolates in both the menthol group and the zinc group, an increase from 2.5% on Day 0 to 15% on Day 21 in the menthol group, and from 12.5% to 40% on Day 21 in the zinc group. The combined zinc and menthol group tended to have a decreased percentage of macrolide resistant isolates, from 30% on Day 0 to 17.5% on Day 21. The trends in antibiotic resistance to

tetracyclines and macrolides were later statistically tested for significance using a multi-level mixed logistic regression.

The percentage of isolates resistant to the number of antibiotic classes by sample day and treatment (Figure 8) showed all *Enterococcus* spp. isolates were resistant to at least one class of antibiotic, with no pan-susceptible isolates. The menthol group showed an increase in the percentage of multidrug-resistant isolates (i.e., resistant to 3 or more classes of antibiotics) from 30% on Day 0 to 52% on Day 21. Correspondingly, the menthol group also showed an increase in the percentage of isolates resistant to 4 classes of antibiotics from 5% on Day 0 to 15% on Day 21. Additionally, the menthol group also had 2% of isolates resistant to 6 antibiotic classes on Day 21, compared to 0 isolates resistant to 6 classes on Day 0. The zinc group did not exhibit an increase in overall percentage of multidrug-resistant isolates, with 62% of isolates resistant to 3 or more classes on Day 0, and 60% resistant on Day 21. However, on Day 0 all isolates in the zinc group were resistant to at least 2 classes of antibiotic, but on Day 21 there were 10% of isolates resistant to only 1 antibiotic class. The percentage of multidrug-resistant isolates in the combined zinc and menthol group decreased from 63% on Day 0 to 47.5% on Day 21. However, the combined zinc menthol group also had 2.5% of isolates resistant to 6 antibiotic classes on Day 21, compared to none on Day 0. These increases and decreases in multi-drug resistance were later statistically tested for significance using a multi-level mixed logistic regression.

	# Resistant (of 320 tested)	% Resistant	95% Confidence Interval		0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Chloramphenicol	2	0.63	0.076	2.239								0.31	13.75	80	5.31	0.63						
Ciprofloxacin	18	5.63	3.367	8.744				0.00	0.31	17.81	43.44	32.81	4.69	0.94								
Daptamycin	28	8.75	5.893	12.4					1.25	0.31	2.5	20.63	66.56	8.75	0.00							
Erythromycin	57	17.81	13.78	22.45					50.31	7.81	2.5	8.13	13.44	3.75	14.06							
Gentamicin	0	0	0	1.1461														100	0.00	0.00	0.00	
Kanamycin	3	0.94	0.194	2.715														91.88	6.56	0.63	0.31	0.63
Lincomycin	292	91.25	87.6	94.11							8.44	0.31	0.00	5.31	85.94							
Linezolid	1	0.31	0.008	1.729						0.31	6.88	68.13	24.38	0.31								
Nitrofurantoin	52	16.25	12.38	20.76								0.00	0.00	2.19	2.18	16.25	62.5	16.25				
Penicillin	1	0.31	0.008	1.729					2.5	7.19	12.19	15.63	54.38	7.81	0.31							
Streptomycin	3	0.94	0.194	2.715																99.06	0.63	0.31
Quinupristin/ Dalfopristin	106	33.13	27.99	38.58						10	1.56	55.31	32.81	0.31	0.00	0.00						
Tetracycline	106	33.13	27.99	38.58							64.06	1.25	0.00	1.56	2.5	3.75	26.88					
Tigecycline	0	0	0	1.1461	0.00	11.25	61.88	26.88	0.00	0.00												
Tylosin	59	18.44	14.34	23.13					0.00	0.00	1.25	16.88	20.31	37.5	5.63	0.94	17.5					
Vancomycin	0	0	0	1.1461					0.94	57.19	34.38	5	1.88	0.63	0.00	0.00						
*97.5% One-sided CI																						

Figure 6 Percentage of *Enterococcus* spp. isolates that were resistant and their distribution across minimum inhibitory concentrations (MIC) for each antibiotic. Black vertical lines indicate the human CLSI (or, NARMS) interpretive breakpoint, grey boxes indicate areas above and below highest and lowest limit of assay antibiotic concentrations, respectively. Isolates which exceeded growth at the highest antibiotic concentration were placed in the next MIC column.

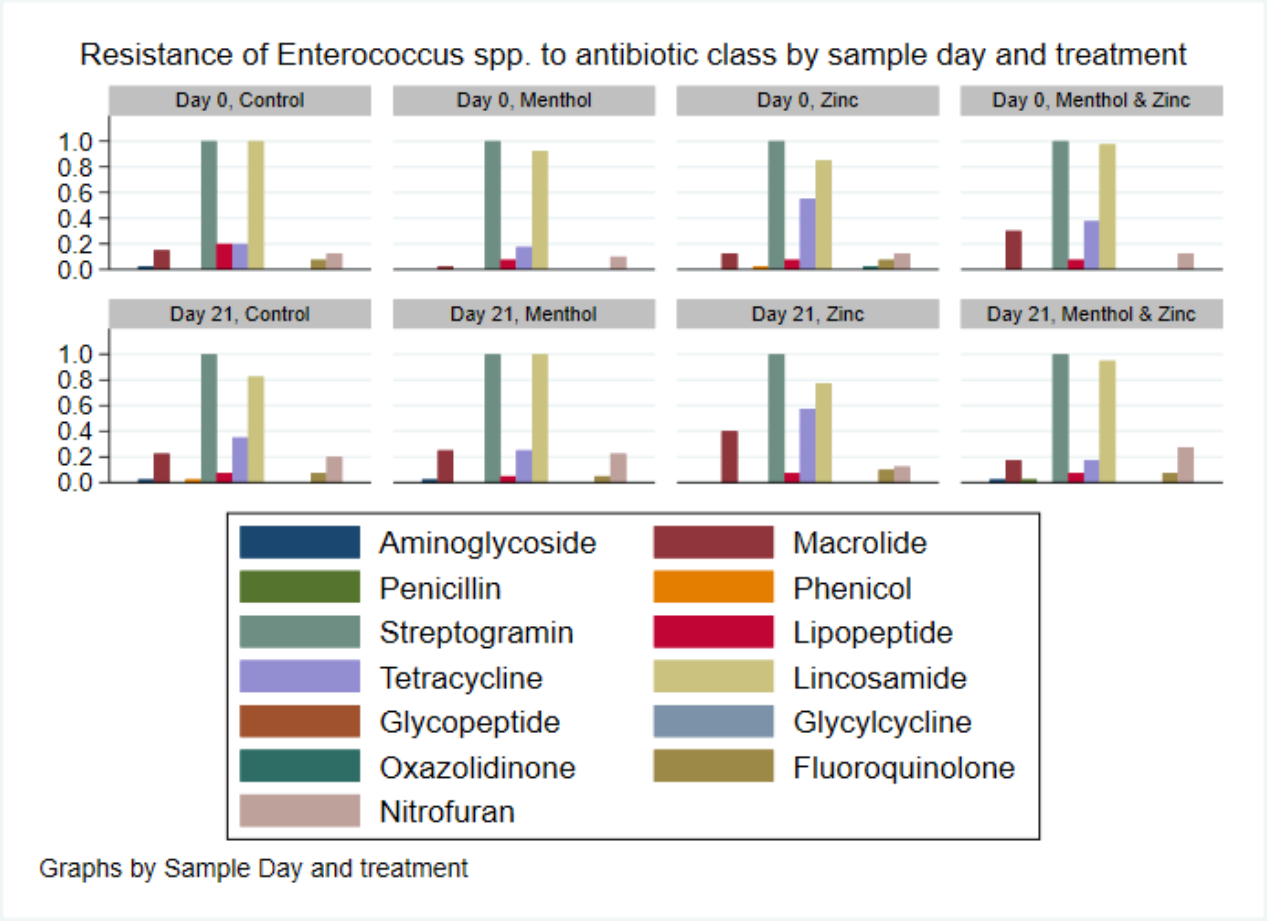


Figure 7 Resistance (proportion) of *Enterococcus* spp. isolates to each antibiotic class by sample day and treatment

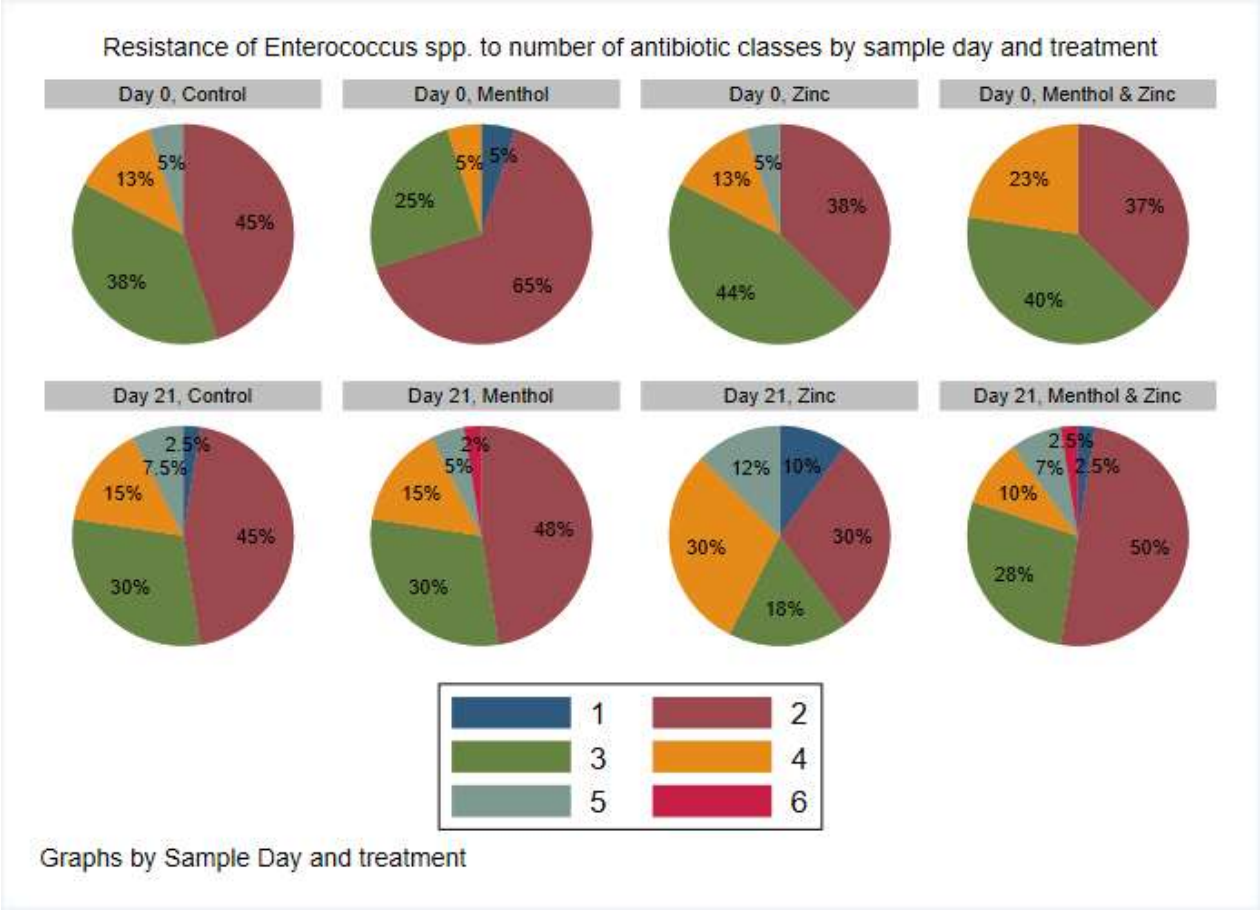


Figure 8 Resistance (%) of *Enterococcus* spp. isolates to number of antibiotic classes by sample day and treatment

Multi-level mixed effects logistic regression modeling of binary multi-drug resistance

Multi-level mixed effect logistic regression modeling was performed on the binary outcome of multidrug-resistant (i.e., resistance to ≥ 3 antibiotic classes) *E. coli* isolates (Figure 9). There were no significant differences among treatment groups, the marginal predicted prevalence of MDR isolates ranging from 0.15 to 0.275 on Day 0 and 0.15 to 0.325 on Day 21. The control group exhibited a decrease in the predicted prevalence of MDR isolates from Day 0 to Day 21, from 0.175 (95% CI of 0.06 to 0.29) to 0.15 (95% CI of 0.04 to 0.26); however, this decrease was not significant (P>0.05).

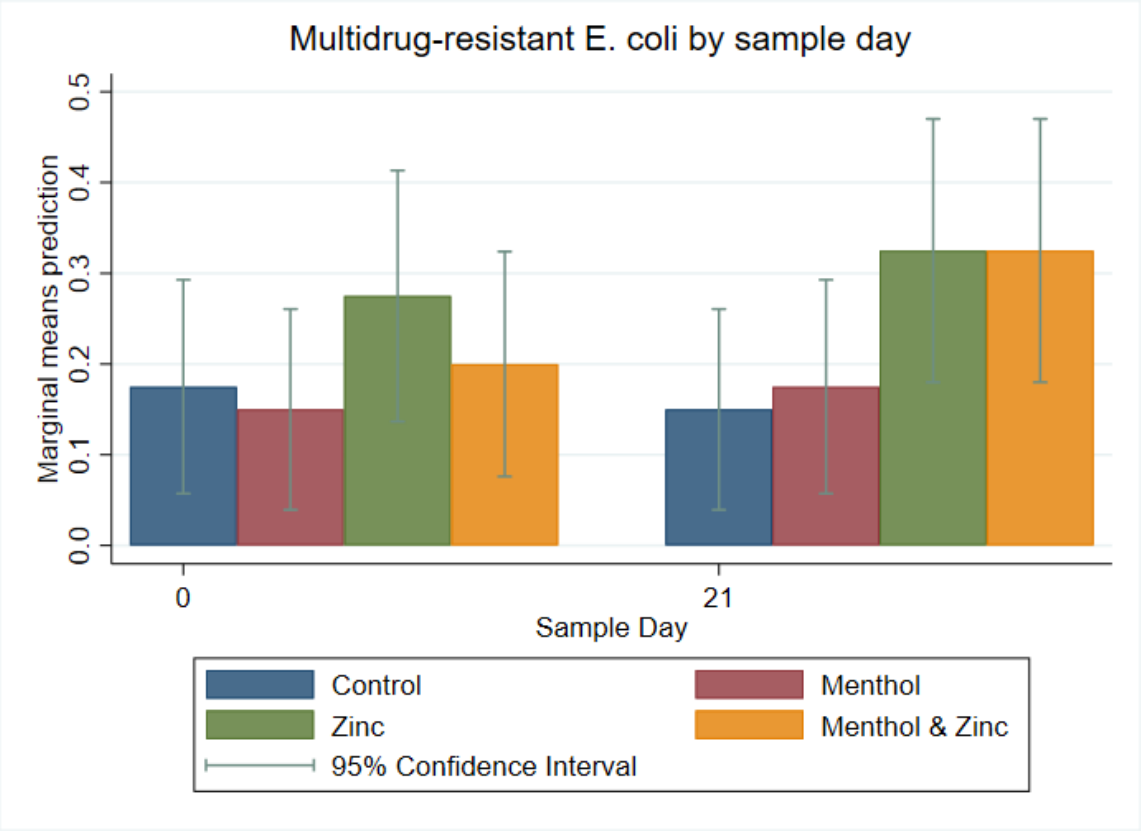


Figure 9 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of multidrug-resistant (resistant to ≥3 classes of antibiotics) *E. coli*

The menthol group increased from 0.15 (95% CI of 0.04 to 0.26) to 0.175 (95% CI of 0.06 to 0.29) from Day 0 to Day 21, the zinc group increased from 0.275 (95% CI of 0.14 to 0.41) to 0.325 (95% CI of 0.18 to 0.47) and the combined zinc and menthol group increased from 0.20 (95% CI of 0.08 to 0.32) to 0.325 (95% CI of 0.18 to 0.47); however, none of these increases in the predicted prevalence of MDR isolates was significant ($P>0.05$). Overall, there was an increase in the predicted prevalence of MDR isolates from 0.20 (95% CI of 0.08 to 0.32) on Day 0 to 0.243 (95% CI of 0.17 to 0.31) on Day 21 in multi-drug resistance for sample day alone; again, this was also not significant ($P > 0.05$).

As previously mentioned, the aminoglycoside class of antibiotics appeared to increase over time for all treatment groups. Multi-level mixed effect logistic regression modeling was performed on the binary outcome of resistance to the aminoglycoside class antibiotics. The predicted prevalence of aminoglycoside resistant isolates increased from 0.175 (95% CI of 0.057 to 0.293) on Day 0 to 0.40 (95% CI of 0.248 to 0.552) on Day 21 for the zinc group; however, this increase was not significant (Figure 10). The combined menthol and zinc group also showed an increase in the predicted prevalence of aminoglycoside resistant isolates, from 0.225 (95% CI of 0.096 to 0.354) on Day 0 to 0.35 (95% CI of 0.202 to 0.498) on Day 21, but these increases were also not significant. Sample day alone showed an increase from 0.168 (95% CI of 0.057 to 0.279) to 0.287 (95% CI of 0.148 to 0.426); however, this increase was not significant.

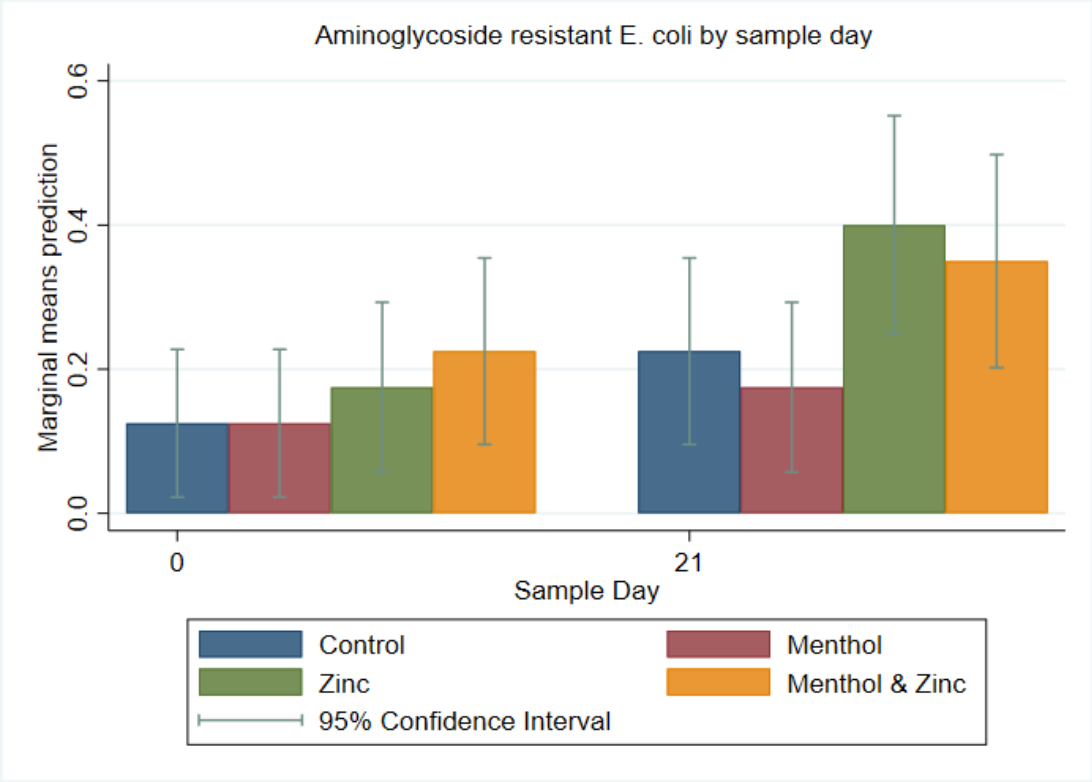


Figure 10 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of aminoglycoside resistant *E. coli*

Multi-level mixed effect logistic regression modeling was also performed on the binary outcome of resistance to tetracycline class antibiotics. The predicted prevalence of tetracycline-resistant *E. coli* tended to increase from Day 0 to Day 21 among all groups, ranging from 0.35 to 0.45 on Day 0, to 0.40 to 0.60 on Day 21. Most notably, the zinc group increased in the predicted prevalence of tetracycline resistant *E. coli*, from 0.40 (95% CI of 0.248 to 0.552) on Day 0 to 0.60 (95% CI of 0.448 to 0.752) on Day 21; however, this increase was not significant. Additionally, sample day alone was not significant in the predicted prevalence of tetracycline resistant *E. coli* (Figure 11).

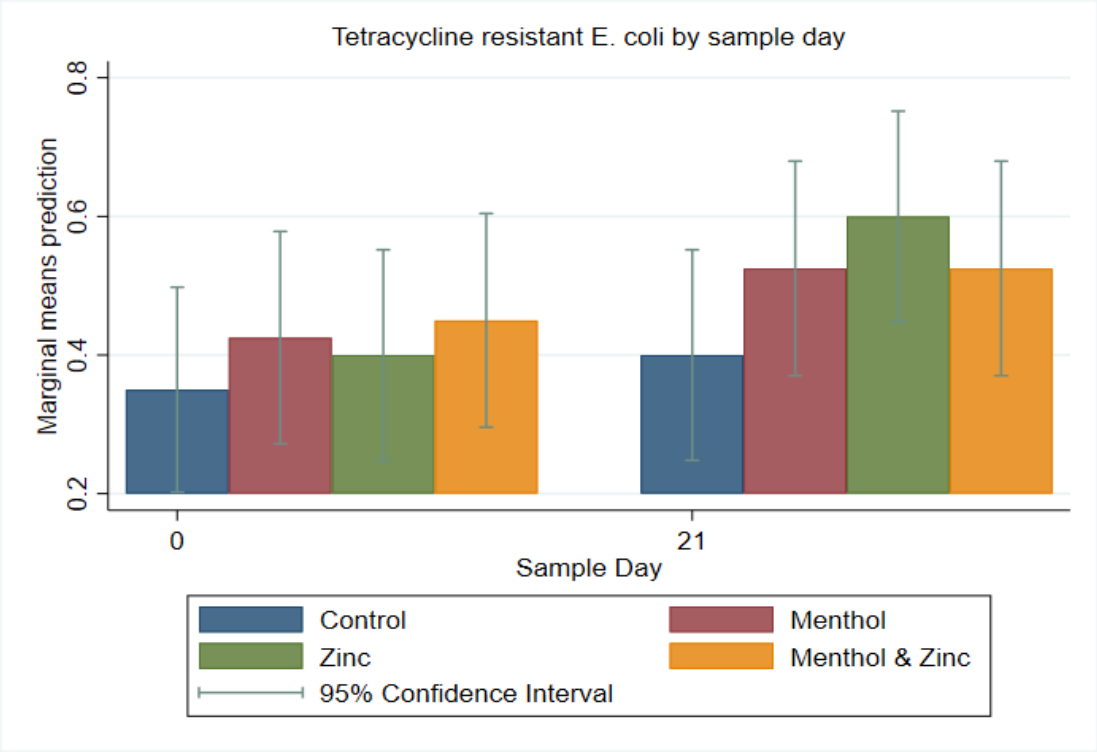


Figure 11 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of tetracycline resistant *E. coli*

The results of a multi-level mixed effect logistic regression model on the binary outcome of multidrug-resistant (i.e., resistance to ≥ 3 antibiotic classes) *Enterococcus* spp. isolates (Figure 12) showed on Day 0 the menthol group had a significantly decreased predicted prevalence of MDR isolates, of 0.30 (95% CI of 0.158 to 0.442) compared to the zinc or zinc/menthol combined group which both had 0.625 (95% CI of 0.475 to 0.775). The predicted prevalence of MDR isolates from the menthol group also tended to increase from Day 0 to Day 21, from 0.30 (95% CI of 0.158 to 0.442) to 0.525 (95% CI of 0.370 to 0.680); however, this was not significant (P>0.05). This increase was also not significantly different from the other treatments on Day 21. Additionally, the predicted prevalence of MDR isolates from the combined zinc and menthol group

decreased from 0.625 (95% CI of 0.475 to 0.775) on Day 0 to 0.475 (95% CI of 0.320 to 0.630) on Day 21; however, this decrease was not significant (P>0.05).

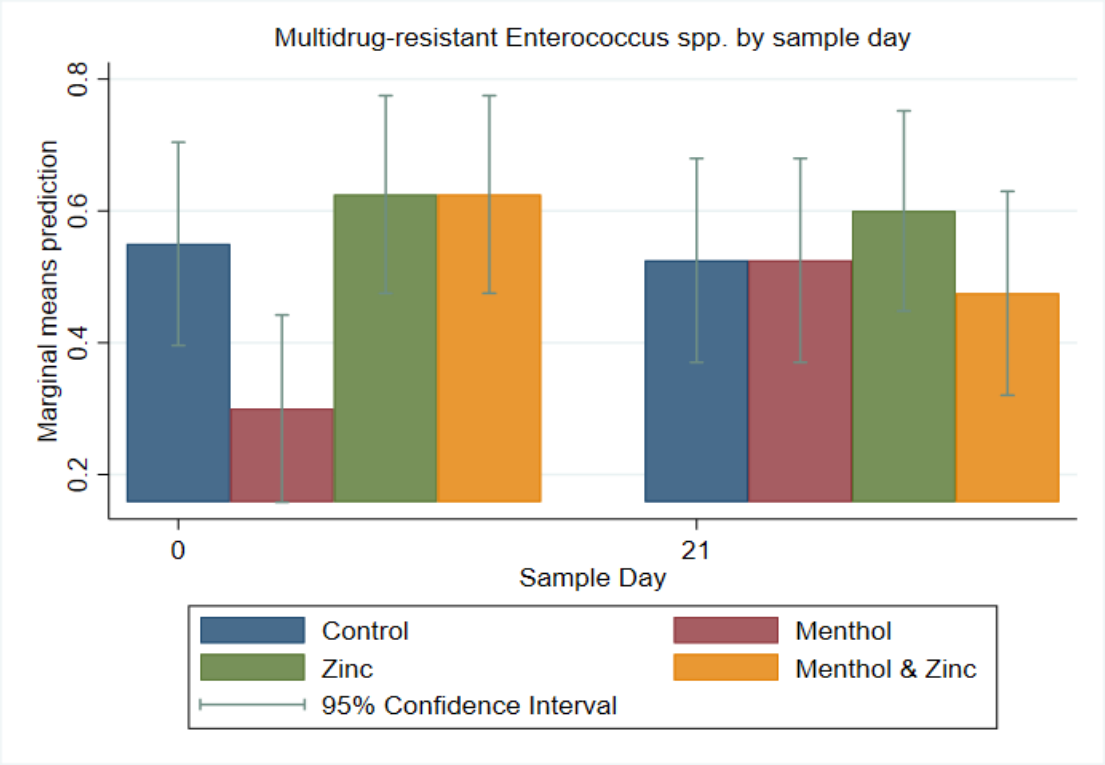


Figure 12 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of multidrug-resistant (resistant to ≥3 classes of antibiotics) *Enterococcus* spp

A multi-level logistic regression on the binary outcome of tetracycline-resistance for *Enterococcus* spp. isolates showed an increase in the predicted prevalence of tetracycline resistant isolates from the control group, from 0.20 (95% CI of 0.076 to 0.324) on Day 0 to 0.35 (95% CI of 0.202 to 0.498) on Day 21; however, this increase was not significant (P>0.05). The combined zinc and menthol group also showed a decrease in the predicted prevalence of tetracycline resistant isolates, from 0.375 (95% CI of 0.225 to 0.525) on Day 0 to 0.175 (95% CI of 0.057 to 0.293) on Day 21, but this decrease was also not significant. The zinc group showed a significantly higher predicted

prevalence of tetracycline resistant isolates of 0.55 (95% CI of 0.396 to 0.704) on Day 0, compared to 0.20 (95% CI of 0.076 to 0.324) in the control group and 0.175 (95% CI of 0.057 to 0.293) in the menthol group. On Day 21, the zinc group showed a significantly higher proportion of tetracycline resistant isolates at 0.575 (95% CI of 0.422 to 0.728) compared to 0.25 (95% CI of 0.116 to 0.384) compared to the menthol group, versus 0.175 (95% CI of 0.057 to 0.293) from the combined zinc and menthol group (Figure 13).

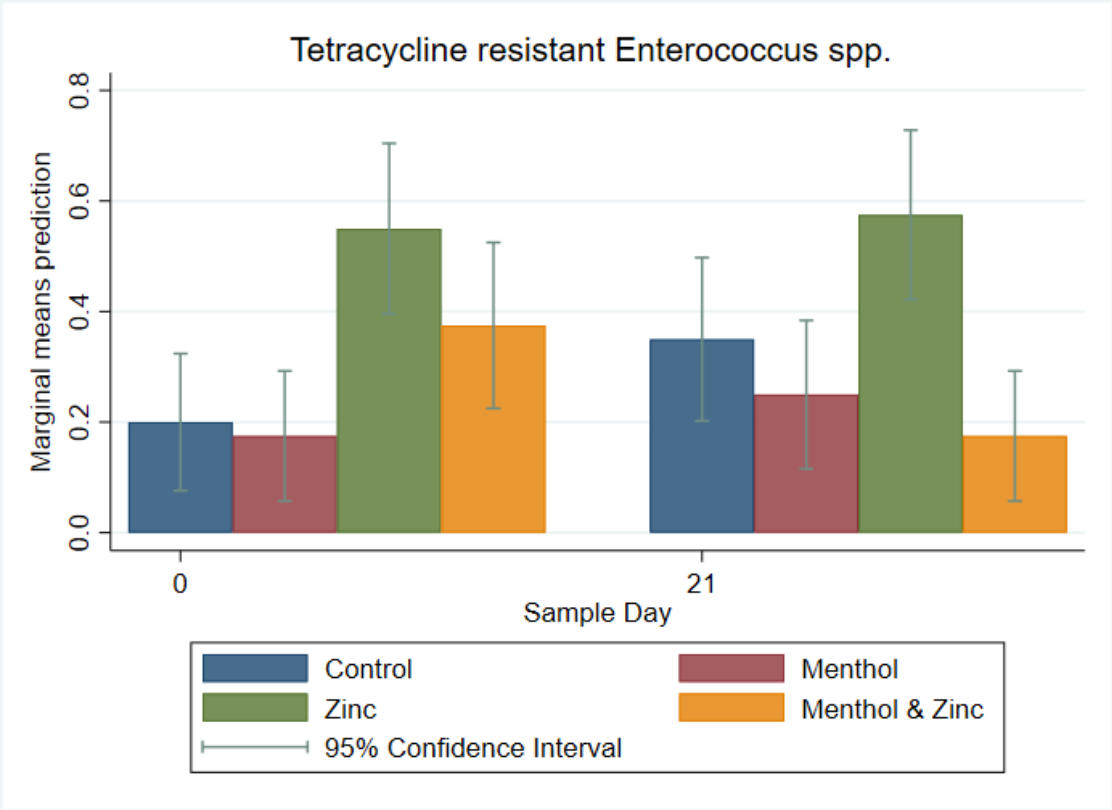


Figure 13 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of tetracycline resistant *Enterococcus* spp

A multi-level logistic regression on the binary outcome of macrolide-resistance for *Enterococcus* spp. isolates showed no significant differences in the predicted

prevalence of macrolide resistant isolates among the treatments on Day 21. Day 0 to Day 21 The menthol treatment and the zinc treatment significantly increased in the predicted prevalence of macrolide resistant enterococci, from 0.025 (95% CI of -0.023 to 0.073) on Day 0 to 0.25 (95% CI of 0.116 to 0.384) on Day 21 in the menthol group and from 0.125 (95% CI of 0.023 to 0.227) on Day 0 to 0.40 (95% CI of 0.248 to 0.552) on Day 21 in the zinc group. The menthol group showed a significantly lower predicted prevalence of macrolide resistant enterococci compared to the combined menthol and zinc group on Day 0. Therefore, a *post hoc* Bonferroni multiple comparison adjustment, which showed the increase in macrolide resistant enterococci in the menthol group was still significant. In contrast, the combination of menthol and zinc treatment group tended

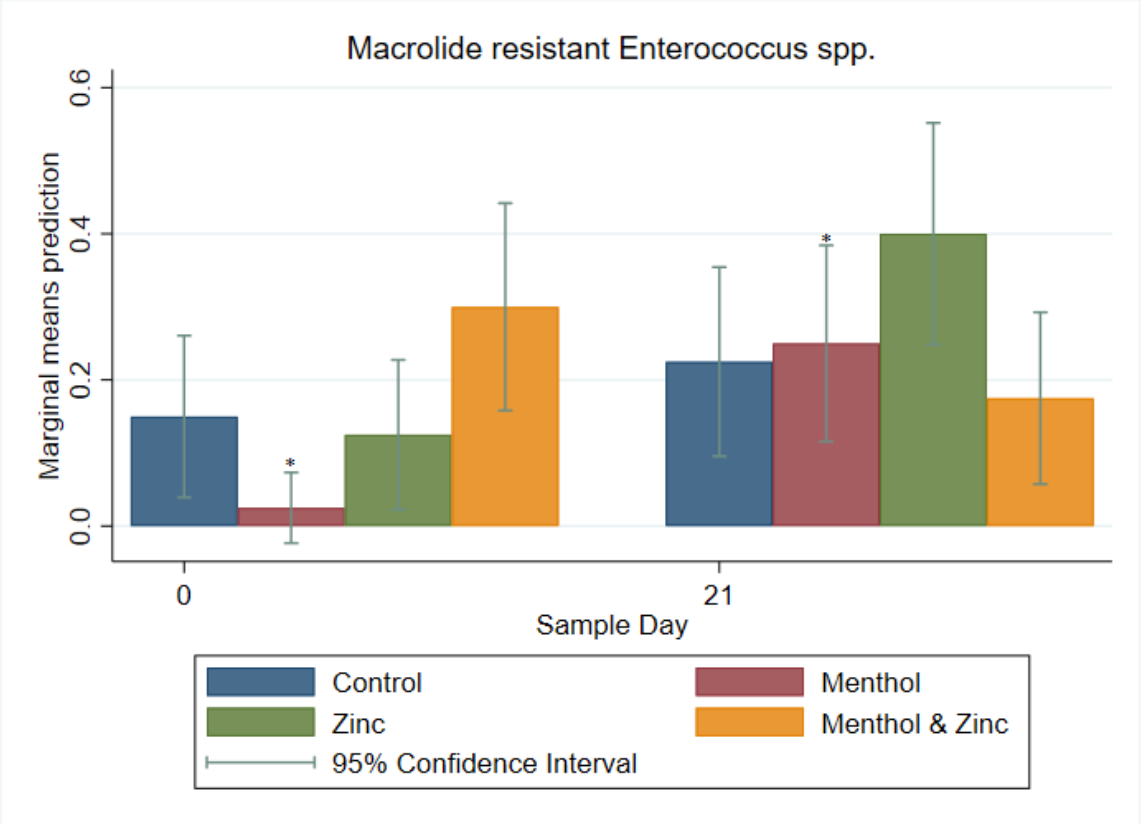


Figure 14 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of macrolide resistant *Enterococcus* spp
* significantly different using a *post hoc* Bonferonni pairwise comparison

to decrease in macrolide resistance from 0.30 (95% CI of 0.158 to 0.442) on Day 0 to 0.175 (95% CI of 0.057 to 0.293) on Day 21; however, this decrease was not significant (Figure 14).

Discussion

This randomized controlled trial demonstrated that there were some trends, but little to no significant effects of zinc or menthol supplementation as alternatives to antibiotic on *E. coli*; in contrast, some significant effects of zinc and menthol supplementation on enterococci were present. The $\log_{10}$ CFU per gram of feces on plain MacConkey agar was significantly affected by sample day. Tetracycline resistance tended to increase for the combination zinc and menthol group on both tetracycline supplemented MacConkey agar and phenotypic resistant *E. coli* isolates. This was similar to results reported by Aperce *et al.* who found that menthol significantly increased the prevalence of tetracycline resistant *E. coli* (Aperce *et al.*, 2016). However, *mentha piperita* (peppermint) essential oil and menthol have been shown to inhibit quorum sensing, which regulates the expression of certain genes, and biofilm of gram-negative organisms including *E. coli* (Husain *et al.*, 2015). Our data showed no noticeable trends of sample day, zinc, menthol, or the combination of zinc and menthol, pertaining to growth on ceftriaxone-supplemented MacConkey. Similarly, there was seemingly no effect on resistance of *E. coli* isolates to other cephem-class antibiotics as determined using isolate-based analyses from plain MacConkey agar. However, while zinc was associated with higher aminoglycoside and tetracycline resistance among *E. coli* isolates, this difference did not prove to be statistically significant ($P>0.05$). This is in contrast to a previous study in pigs, in which heavy metals, particularly mercury, were

associated with a decrease in aminoglycoside, tetracycline, and cephalosporin resistance (Hölzel *et al.*, 2012). Additionally, multi-drug resistance tended to increase, though also not significantly, for the zinc and the combination zinc/menthol group among *E. coli* isolates. Interestingly, two previous studies suggest that high dietary zinc promotes multi-drug resistance in pigs (Ciesinski *et al.*, 2018) (Bednorz *et al.*, 2013). Yet, another more recent study also conducted in swine cautioned against this correlation and stated that that tolerance to zinc was not associated with multidrug-resistance (Ghazisaeedi *et al.*, 2020).

Among enterococci, menthol tended to decrease growth on tetracycline supplemented agar for *Enterococcus* spp.; however, these results were not significant ($P>0.05$) and also were not matched in an observed decrease in tetracycline resistance among isolates grown on plain m-*Enterococcus* agar. Menthol was associated with significantly ($P<0.05$) increased macrolide resistance among *Enterococcus* spp. isolates from Day 0 to Day 21. Additionally, the menthol group tended to increase the prevalence of multi-drug resistance among isolates from Day 0 to Day 21. There were few studies examining the effects of essential oils on antimicrobial resistance at the time of publication, even though essential oils such as tea tree oil have been licensed for medicinal use in Australia since the 1920s (Yap *et al.*, 2014). When a variety of essential oils were screened for bactericidal activity, Shapiro *et al.* found that peppermint oil, which contains menthol, and tea tree oil were the most potent essential oils against obligate anaerobes and facultative anaerobes (Shapiro *et al.*, 1994). While tea tree oil has been licensed for the past 100 years, clinical resistance has yet to be reported (Burt and Reinders, 2003). However, after several generations of methicillin-resistant

Staphylococcus aureus (MRSA) were exposed to tea tree oil, a resistant subpopulation emerged (Cho *et al.*, 2011). It is therefore plausible that after repeated exposure to menthol, in addition to antibiotic exposure, a multidrug-resistant subpopulation of *Enterococcus* spp. would emerge.

Conversely, after repeated exposure to oregano essential oil, *Serratia marcescens*, *Morganella morganii*, and *Proteus mirabilis* exhibited a changed antibiotic resistance profile; however. this was not associated with any increased resistance to oregano oil itself (Becerril *et al.*, 2012). Zinc was associated with increased erythromycin and macrolide resistance, with significant increases in measured growth on erythromycin supplemented m-*Enterococcus* agar, and a significantly higher macrolide resistance among isolates analyzed using broth microdilution. These results are similar to a previous study by Hasman et al, which showed supplementation of copper in piglets selected for the *tcr*B gene, which is strongly associated with a gene (*ermB*) encoding increased resistance to macrolides (Hasman and Aarestrup, 2002). It should be noted, however, that the similarity is strictly between heavy metals and co-selection for macrolide resistance among enterococci, as the previous study used copper instead of zinc. Conversely, Jacob *et al.* found that when cattle were fed a combination of zinc and copper, there were minimal effects on any associated increase in antimicrobial resistance, and those authors did not find *tcrB* in the samples or among the enterococcal isolates (Jacob *et al.*, 2010).

Conclusions

Although no significant treatment effects were present for *E. coli*, these trial data suggest that there is potential co-selection pressures occurring in populations of

Enterococcus spp. when using supranutritional zinc and menthol as alternatives to antibiotics. No mechanistic explanations were pursued in this study. One limitation of this study relates to time constraints, since a longer period of supplementation with supranutritional zinc and menthol would have the potential to yield more sustained and significant effects. In all of the previous reported studies, animals were supplemented for at least 28 days. By increasing the amount of time exposed to the alternatives, such as throughout the cattle feeding period, further co-selection expanding resistance would likely occur. Longer and more definitive studies to further explore any associations are necessary, especially with menthol.

CHAPTER IV

EFFECTS OF A DIRECT-FED MICROBIAL AND ENVIRONMENTAL PEN CHANGE ON PHENOTYPIC RESISTANCE IN ENTEROCOCCI

Background

Since 2017 antibiotic use in animals in the U.S. has been restricted to prevention, control and treatment indications in food animals, including cattle. Tylosin falls under these categories with respect to its use for the prevention and control of liver abscesses. However, it also has been associated with increased resistance to macrolides among enterococci (Jacob *et al.*, 2008; Zaheer *et al.*, 2013). This is cause for concern as the macrolide class (including erythromycin and azithromycin) was deemed of highest priority and critically important by the World Health Organization (WHO).

In response, several studies have been performed exploring ways to limit tylosin use, or else to find non-antibiotic alternatives for liver abscess prevention. In 2015, Beukers *et al.* suggested that antibiotic withdrawal prior to slaughter contributed to a reduction in the proportion of macrolide resistant enterococci (Beukers *et al.*, 2015). Additionally, in 2018, Muller *et al.* showed no difference in resistance between intermittent tylosin supplementation and continuous treatment; thereby, suggesting that environmental factors may be more important in carrying over resistance from one lot of cattle to the next than contemporaneous selection during the actual feeding period (Müller *et al.*, 2018). In another direction, *Saccharomyces cerevisiae* fermentation products (SCFP) have been suggested for use to prevent liver abscesses, though there have been no statistically significant difference reported among treatment groups with respect to abscess prevalence or severity (Huebner *et al.*, 2019). *Enterococcus faecium* is

a unique bacterium in that it can be deployed as a probiotic, due to its bile tolerance and bacteriocins which are antagonistic towards organisms such *as Listeria monocytogenes* (Izquierdo *et al.*, 2009). Under other circumstances it can also be an opportunistic pathogen. Therefore, we performed a feeder cattle study testing the combined effects of pre-slaughter withdrawal of tylosin, in combination with the use of a *Saccharomyces cerevisiae* and *Enterococcus faecium* probiotic (direct-fed microbial (DFM), with environmental change to pens where antibiotics have never been used. The probiotic was named Tri-Lution and supplied by Agri-King™, consisting of live cultures of *Enterococcus faecium* and *Saccharomyces cerevisiae*. By utilizing fecal samples from this study design, we determined the effects of the above factors on $\log_{10}$ enterococci CFU per gram of feces, plated on plain and erythromycin- and tetracycline-supplemented agar. Additionally, phenotypic resistance of select isolates was determined by microbroth dilution, to explore additional effects on multidrug-resistance and resistance to single antibiotics.

Materials and methods

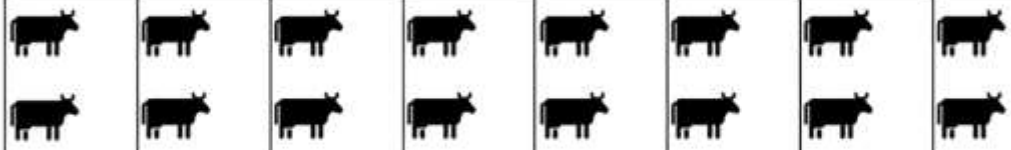
Experimental design

A randomized controlled trial consisting of two replicates in a 2x2x2 factorial design was conducted at the Texas A&M Agri-Life Research experimental feedlot in McGregor, Texas (AACUC AUP #2015-026A; IBC #2017-049 and #2017-021). This facility was unique in that it had 8 pens in use, in addition to 8 new adjacent pens purpose-built new for this study and in which no antibiotics had ever been fed or animals housed that had previously been treated. Antibiotic-free and grass-fed beef cattle were allowed access to the pens to prepare a manure pack for 4 weeks prior to the trial;

importantly, this was meant to ensure a homogenous baseline of fecal bacteria were present in the newly constructed pens prior to the trial. At the onset of the trial, steers were placed in the old pens and randomly assigned to a treatment group, treatments being: 1) tylosin (included at 8g/ton), 2) DFM (907g/ton), 3) both tylosin and DFM, and 4) neither tylosin nor DFM (control). These four treatment groups were performed in serial duplicates so each treatment group had a replicate (Figure 15A). Four weeks prior to slaughter (hereafter referred to as the withdrawal timepoint), tylosin was removed from the respective trial ration while keeping the DFM feeding regimen for the appropriate treatment groups; meanwhile, half the steers in each of the four treatment groups were randomly assigned to an adjacent pen constructed for the purpose of this study (Figure 15B).

The first replicate consisted of 90 steers and the second replicate consisted of 96 steers, all sourced from the same ranch birth cohort. Once every 28 days in the morning, fecal samples were collected *per rectum* using individual and new rectal palpation gloves for 3 months (i.e., Day 0 to Day 84) by the McGregor, Texas feedlot crew. After Day 84, starting at the tylosin withdrawal time point, samples were taken weekly until slaughter. Samples were transported directly to the laboratory on ice immediately following the completion of collection. Samples were stored in the refrigerator until the next day, at which time they were processed. Sample processing consisted of aliquoting fecal samples into two 5 ml tubes; specifically, into one tube without glycerol and one tube with sterile 50% glycerol at a 1:1 ratio of glycerol to feces. Tubes were preserved at -80°C until further use. This sample collection schedule, processing scheme, and storage was repeated for each trial replicate.

A)

Day 0-Day 84							
1 Control	2 Tylosin & Probiotic	3 Probiotic	4 Tylosin	5 Control	6 Tylosin & Probiotic	7 Tylosin	8 Probiotic
							
9	10	11	12	13	14	15	16

B)

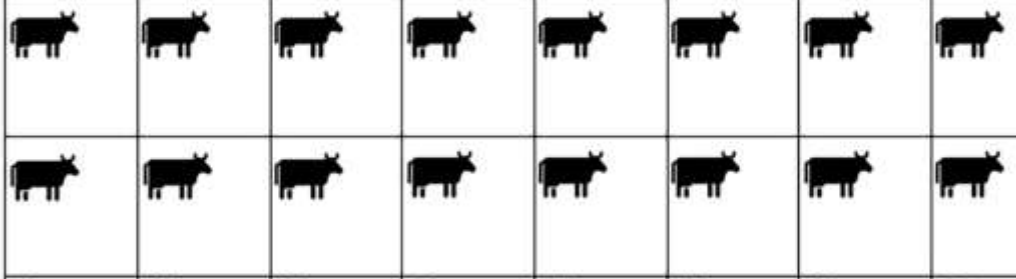
Day 84-Endpoint							
1 Control	2 Tylosin* & Probiotic	3 Probiotic	4 Tylosin*	5 Control	6 Tylosin* & Probiotic	7 Tylosin*	8 Probiotic
							
9 Control	10 Tylosin* & Probiotic	11 Probiotic	12 Tylosin*	13 Control	14 Tylosin* & Probiotic	15 Tylosin*	16 Probiotic

Figure 15 A) Pen layout for first 3 months. Each cartoon figure represents six cattle. B) Pen layout for last 4 weeks.
*Cattle were not fed tylosin from this point forward

Bacterial enumeration, isolation, and speciation

Samples from Day 0 as a baseline, Day 84 as the antibiotic withdrawal time point (i.e., presumed maximum cumulative effect), and slaughter as the final time point, were used for statistical analyses. Samples preserved with glycerol were thawed on ice and mixed thoroughly with phosphate buffered saline (PBS) (Gibco Life Technologies, Thermo Scientific Microbiology, Oakwood Village, OH) using 4.5 milliliters of PBS to 0.5 grams of feces to create a 1:10 dilution. An aliquot of 50 microliters of this dilution was spiral plated to plain m-*Enterococcus* agar (Difco, Becton Dickinson Sparks, MD), along with m-*Enterococcus* agar supplemented with tetracycline and erythromycin at the Clinical and Laboratory Standards Institute (CLSI) human clinical breakpoints of16 mg/L and 8 mg/L, respectively, using an EddyJet 2 Spiral Plater (Neutec Group Inc, Farmingdale, NY). Plates were incubated at 42°C for 48 hours.

Colony counts on each plate were performed using the Flash & Go® System (Neutec Group Inc, Farmingdale, NY). Two colonies presumptive for *Enterococcus faecium* (i.e., dark red to maroon with a cream halo) were selected from each of plain and erythromycin-supplemented plate when possible. When presumptive *Enterococcus faecium* was not available, presumptive *Enterococcus hirae* was selected. Colonies were quadrant streaked for isolation to Tryptic Soy Agar (TSA) with 5% sheep blood agar (Remel™, Lenexa, KS) and incubated at 37°C for 24-48 hours. A single colony from each TSA agar with 5% sheep blood plate was again isolated and streaked fresh to TSA with 5% sheep blood agar and incubated at 37°C for 24-48 hours then saved for further analysis.

Each isolate was subjected to MALDI-TOF for confirmation of genus and species. Using a new sterilized wooden toothpick per isolate, a single isolate of presumptive *Enterococcus faecium* or *Enterococcus hirae*, was spread onto two wells of a reusable 96 well target plate (Bruker Daltonik GmbH., Billerica, MA). Once dry, one microliter of 70% formic acid was added to the first well of each sample spot pair of each *Enterococcus spp.* isolate, in addition to one empty spot to serve as a negative control. One microliter of the bacterial test standard (BTS) solution (Bruker Daltonik GmbH., Billerica, MA) was applied to the first and second wells as a positive control. After drying of all wells, one microliter of HCCA matrix solution (Bruker Daltonik GmbH., Billerica, MA) was added to each well, including all the sample wells, BTS wells, formic acid negative control well, and an additional empty well as a secondary negative control. The target plate was then transferred to the MALDI-TOF Microflex LT/SH for reading, using MBT Compass v1.4 software.

Phenotypic susceptibility testing

To obtain minimum inhibitory concentrations, microbroth dilution using the Sensititre® (TREK, Thermo Scientific Microbiology, Oakwood Village, OH) platform was used. Isolates were freshly plated to TSA with 5% sheep blood agar and incubated at 37°C for 18-24 hours. Afterward, 11 ml of sterilized water was normalized to a 0.5 McFarland standard. Next, 10 µl of the culture suspension was transferred to 11 ml of sterile Mueller-Hinton broth. Subsequently, 50 µl of the broth culture was inoculated to each well of the NARMS gram-positive CMV3AGPF plate for *Enterococcus* spp. using the Sensititre® automated inoculation delivery system (TREK, Thermo Scientific Microbiology, Oakwood Village, OH). The plate consisted of 16 antibiotics from 13

classes, including: chloramphenicol, ciprofloxacin, daptomycin, erythromycin, gentamicin, kanamycin, lincomycin, linezolid, nitrofurantoin, penicillin, quinupristin/dalfopristin, streptomycin, tetracycline, tigecycline, tylosin, and vancomycin (Table 1).

Plates were incubated at 37°C for 24 hours, with *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 serving as quality controls. Plates were read using a Sensititre OptiRead™ instrument (TREK, Thermo Scientific Microbiology, Oakwood Village, OH). The results were interpreted as susceptible, intermediate, or resistant in accordance with CLSI guidelines according to the M100 document (CLSI, 2020), and NARMS breakpoints when CLSI breakpoints were not available, using SWIN software (TREK, Thermo Scientific Microbiology, Oakwood Village, OH).

Statistical analysis

CFU bacterial count data were $\log_{10}$ transformed, and the output was used in 2x2x2 full factorial multilevel mixed effect linear regression models, factors being tylosin, DFM, and sample day with original pen number and replicate treated as random effects. Due to the low percentage of growth on erythromycin-supplemented agar plates, a Cragg hurdle regression model was used for analysis of the $\log_{10}$ CFU per gram of feces for erythromycin resistant enterococci. For statistical analysis of phenotypic resistance using Sensititre™, isolates which were interpreted as intermediate were reclassified as susceptible. Isolates were classified as multidrug-resistant if they were resistant to 3 or more classes of antibiotics and the resulting output was classified into a

binary variable and used as the dependent variable in a 2x2x2 full factorial multilevel mixed effects logistic regression model, factors being tylosin, DFM, and sample day with pen and replicate as random effects. Significant increased or decreased resistance to individual classes of antibiotics was determined using a 2x2x2 full factorial multilevel mixed effect logistic regression model, factors being tylosin, DFM, and sample day with pen and replicate as random effects.

Results

Descriptive statistics

Of 558 total fecal samples, 270 samples were collected from Replicate 1 and 288 fecal samples were collected from Replicate 2. From replicate 1, 98.51% (n=266) of samples were quantifiable on plain m-*Enterococcus* agar, while 83.7% (n=226) of samples were quantifiable on tetracycline-supplemented m-*Enterococcus*, and 31.11% (n=84) of samples were quantifiable on erythromycin-supplemented agar. In replicate 2, 99.31% (n=286) of samples were quantifiable on plain m-*Enterococcus* agar, while 90.28% (n=260) of samples were quantifiable on tetracycline supplemented m-*Enterococcus*, and 48.96% (n=141) of samples were quantifiable on erythromycin supplemented agar. The CFU per gram of feces was normalized using a log10 transformation. Over 75% of the samples had a CFU of less than 1,000,000 on plain m-*Enterococcus* agar (Figure 16A). After $\log_{10}$ transformation, 11.9% had a $\log_{10}$ CFU between 2 and 4, 62.1% had a $\log_{10}$ CFU between 4 and 6, and 24.9% had a $\log_{10}$ CFU over 6 (Figure 16B). Similarly, for colony counts on m-*Enterococcus* agar supplemented with tetracycline, nearly 90% of samples had a CFU of less than 1,000,000 (Figure 16C. After $\log_{10}$ transformation, 29.6% had a $\log_{10}$ CFU between 2 and 4, 44.9% had a $\log_{10}$

CFU between 4 and 6, and 12.1% had a $\log_{10}$ CFU over 6 (Figure 16D). For growth on m-*Enterococcus* agar supplemented with erythromycin, over 98% of samples had a CFU less than 1,000,000 and nearly 90% had a CFU less than 10,000 (Figure 16E). After $\log_{10}$ transformation, 29.2% of samples had a CFU between 2 and 4, 9.86% had a CFU between 4 and 6, and 1.26% had a CFU greater than 6 (Figure 16F).

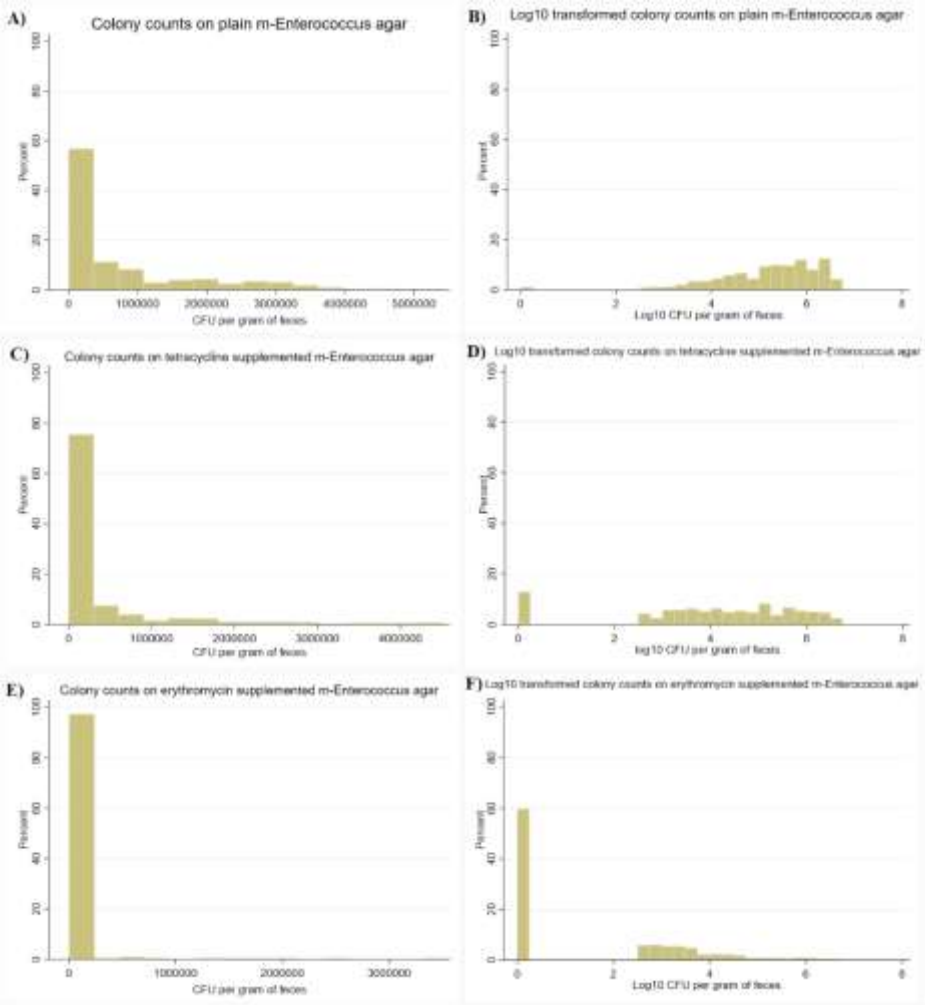


Figure 16 Histogram comparison of CFU per gram before and after log10 transformation A) Distribution of colony counts on plain m-*Enterococcus* agar B) Distribution of colony counts on plain m-*Enterococcus* agar after $\log_{10}$ transformation for normalization C) Distribution of colony counts on tetracycline supplemented m-*Enterococcus* agar D) Distribution of colony counts on tetracycline supplemented m-*Enterococcus* agar after $\log_{10}$ transformation for normalization E) Distribution of colony counts on erythromycin supplemented m-*Enterococcus* agar F) Distribution of colony counts on erythromycin supplemented m-*Enterococcus* agar after $\log_{10}$ transformation for normalization

Multi-level mixed effects linear regression modeling of quantification

For the quantification of total enterococci, a multi-level linear regression was performed on the $\log_{10}$ CFU per gram of feces colony counts, in a 2x2x2 full factorial design, factors being tylosin, DFM and sample day, with pen and replicate as random effects. For Day 0, the lowest and highest CFU per gram of feces collected ranged from $\log_{10}$ 5.68 to 5.82, with a mean of 5.76 from 186 samples. Overall, from Day 0 to Day 84 there was a decrease in the mean $\log_{10}$ CFU to 4.79 per gram of feces, with a range from 4.56 to 5.21 from 184 samples. The two samples that did not grow on plain m-*Enterococcus* agar on Day 84 were both from the tylosin group. The range of mean $\log_{10}$ CFU per gram of feces on Day 119 was 4.83 to 5.18, with a mean of 5.04 from 182 samples. Of the 4 samples that did not grow on plain m-*Enterococcus* agar on Day 119, one was from the control group, one was from the DFM group, and the remaining pair were from the combined tylosin and DFM group.

Period effects significantly impacted the $\log_{10}$ CFU per gram of feces from Day 0 to Day 84 among the DFM, tylosin, and combined DFM/tylosin groups (Figure 17A). The DFM group had a significantly ($P<0.05$) decreased mean $\log_{10}$ CFU per gram of feces on Day 84, from 5.82 (95% CI of 5.54 to 6.11) on Day 0 to 4.83 (95% CI of 4.55 to 5.12) on Day 84. From Day 84 to Day 119 the $\log_{10}$ CFU per gram of feces for the DFM group increased from 4.84 (95% CI of 4.55 to 5.12) to 5.18 (95% CI of 4.90 to 5.48); however, the Day 119 $\log_{10}$ CFU per gram of feces was not significantly different from Day 0 or Day 84 ($P>0.05$). The tylosin group also had a significant ($P<0.05$) decrease in the $\log_{10}$ CFU per gram of feces from Day 0 to Day 84, with a mean of 5.82 (95% CI of 5.54 to 6.11) on Day 0 and 4.57 (95% CI of 4.29 to 4.86) on Day 84.

Similarly, the tylosin group increased from Day 84 to Day 119 at 5.18 (95% CI of 4.90 to 5.47); likewise, this later value was not significantly different from Day 0 $\log_{10}$ CFU per gram of feces or Day 84 $\log_{10}$ CFU per gram of feces. Lastly, the combined tylosin DFM group had a significantly ($P<0.05$) decreased mean $\log_{10}$ CFU per gram of feces on Day 84, from 5.68 (95% CI of 5.40 to 5.97) on Day 0 to 4.56 (95% CI of 4.28 to 4.85) on Day 84. There was also an increase from Day 84 to Day 119 in the $\log_{10}$ CFU per gram of feces of the combined DFM tylosin groupto 4.83 (95% CI of 4.55 to 5.12); once again, this was not significantly different from either Day 0 or Day 84. The control group decreased in mean $\log_{10}$ CFU per gram of feces from 5.71 (95% CI of 5.42 to 6.00) on Day 0 to 5.21 (95% CI of 4.92 to 5.50) on Day 84, but this decrease was not significant ($P>0.05$). This decrease continued from Day 84 to Day 119 to 4.96 (95% CI of 4.67 to 5.25). The decrease from a mean $\log_{10}$ CFU of 5.71 (95% CI of 5.42 to 6.00) per gram of feces on Day 0 to 4.96 (95% CI of 4.67 to 5.25) on Day 119 was significant ($P<0.05$).

As previously mentioned, by subtracting the $\log_{10}$ growth on tetracycline-supplemented agar from corresponding growth on plain agar, the resulting difference can be transformed into a proportion (i.e., the size of the difference is inversely related to resistance such that a decrease in the difference between plain and tetracycline-supplemented agar should be interpreted as an increase in resistance). The difference between plain and tetracycline supplemented agar illustrated a decrease in the proportion of resistance for the DFM group, with the difference shifting from a mean of 1.29 (95% CI of 0.68 to 1.90) on Day 0 to a mean of 1.95 (95% CI of 1.34 to 2.56) on Day 84

(Figure 17B). While this indicates a decrease in resistance, it was not statistically significant ($P>0.05$).

The tylosin group and the combined tylosin/DFM group also demonstrated an increase in the difference in growth between plain and tetracycline-supplemented agar, from a mean of 1.23 (95% CI of 0.63 to 1.83) on Day 0, to 1.54 (95% CI of 0.94 to 2.15) on Day 84 for the tylosin group, and from a mean of 1.37 (95% CI of 0.77 to 1.98) to 1.41 (95% CI of 0.81 to 2.00) for the combined tylosin/DFM group. These changes in growth differences on Day 84 also were not significantly different from Day 0. For Day 119, all treatment groups decreased in the mean difference from Day 84, indicating an increase in resistance from Day 84 to Day 119; once again, these differences were not significantly different from Day 0 or Day 84 ($P>0.05$). Concerning growth on erythromycin-supplemented agar, a Cragg's hurdle model was used to account for the high number of zero counts (Figure 17C). The DFM group tended to have slightly lower counts on erythromycin supplemented agar, with a mean $\log_{10}$ CFU of 1.38 (95% CI of 0.58 to 2.20) per gram of feces on Day 0 compared to 1.55 (95% CI of 0.74 to 2.36) for the control group, 1.78 (95% CI of 0.98 to 2.59) for the tylosin group, and 1.70 (95% CI of 0.90 to 2.51) for the combined tylosin/DFM group, though none of the treatment groups was significantly different from any of the other treatment groups. Similarly, on Day 84, the mean $\log_{10}$ CFU per gram of feces of the DFM group was 1.05 (95% CI of 0.25 to 1.87), compared to 1.75 for the control group (95% CI of 0.95 to 2.57), 2.13 (95% CI of 1.33 to 2.95) for the tylosin group, and 1.39 (95% CI of 0.60 to 2.19) for the combined tylosin/DFM group.On Day 84, the $\log_{10}$ CFU per gram of feces for the DFM group was not significantly different from the other treatment groups, or from the

baseline $\log_{10}$ CFU per gram of feces at Day 0. For Day 119, the DFM remained lower than the other treatment groups with a mean of 1.14 (95% CI of 0.36 to 1.92) compared to 1.21 (95% CI of 0.43 to 2.00) for the control group, 1.62 (95% CI of 0.84 to 2.40) for the tylosin group, and 1.22 (95% CI of 0.45 to 2.00) for the combined tylosin/DFM group, but these differences were not significant for the treatment groups. The tylosin group did tend to slightly increase from Day 0 to Day 84, with a mean $\log_{10}$ CFU of 1.78 (95% CI of 0.98 to 2.59) per gram of feces on Day 0 to 2.13 (95% CI of 1.33 to 2.95) on Day 84; then, after tylosin was withdrawn and half the cattle were moved to a new pen there was a decrease in mean $\log_{10}$ CFU to 1.61 (95% CI of 0.84 to 2.40) on Day 119. None of the treatment-specific temporal changes were not statistically significant ($P>0.05$).

Concerning the difference between plain and erythromycin-supplemented agar, in the first replicate there was a significantly decreased difference ($P<0.05$), and therefore increased resistance, in the tylosin group with a mean difference of 2.63 (95% CI of 1.87 to 3.40) on Day 84 compared to Day 0 which had a mean difference of 5.11 (95% CI of 4.37 to 5.87) (Figure 17D). The tylosin group on Day 84 was also significantly different from the DFM group on Day 84 which had a mean of 4.26 (95% CI of 3.50 to 5.03). On Day 112, 4 weeks after half the cattle were moved to new pens, and 28 days after the withdrawal of tylosin from the feed, the tylosin-fed group still had a significantly lower mean difference of 2.95 (95% CI of 2.26 to 3.65) when compared to Day 0; however, thisdifference was not significantly different from Day 84, and likewise was not significantly different ($P>0.05$) compared to any of the other treatment groups on Day 112.

In contrast, the combined tylosin/DFM group was not significantly different from the tylosin group, the DFM group or the control on Day 84. However, with a mean difference of 3.69 (95% CI of 2.96 to 4.42) on Day 84 the DFM/tylosin group was significantly lower than the baseline mean difference of 5.48 (95% CI of 4.74 to 6.24) on Day 0. By Day 112, it had increased to a mean difference of 4.04 (95% CI of 3.36 to 4.74), and was not significantly different from Day 0 or Day 84 (Figure 17D).

When both replicates were combined, treating both pen and replicate as random effects, there was still a tendency for a decreased difference, and therefore increased resistance, in the tylosin group on Day 84, with a mean difference of 2.81 (95% CI of 1.95 to 3.68) compared to 4.08 (95% CI of 3.21 to 4.93) on Day 0. However, this decreased difference was not statistically significant (Figure 17E). Additionally, when both replicates were combined, the tylosin group was not significantly different from the DFM group on Day 84, with a mean difference of 3.75 (95% CI of 2.89 to 4.62) for the DFM group and 2.81 (95% CI of 1.95 to 3.68) for the tylosin group. Additionally, when replicates were combined, the combined tylosin/DFM group mean difference of 3.31 (95% CI of 2.46 to 4.17) on Day 84 was not significantly different from the mean difference of 4.07 (95% CI of 3.21 to 4.93) on Day 0.

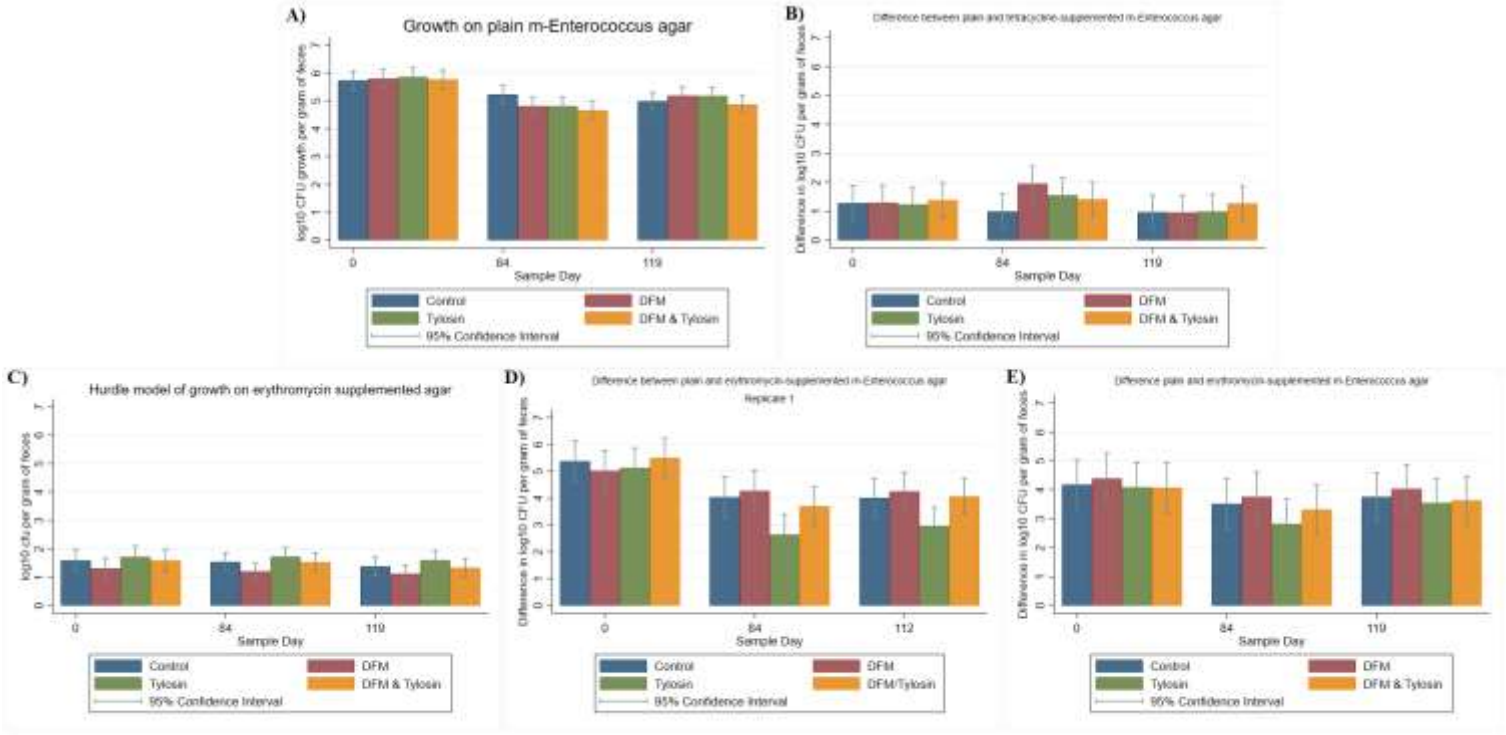


Figure 17 A) log_{10} CFU per gram of feces on plain m-*Enterococcus* agar, B) Difference in log_{10} CFU between plain and tetracycline supplemented m-*Enterococcus* agar, C) Cragg hurdle model of log_{10} CFU per gram of feces on erythromycin supplemented m-*Enterococcus* agar, D) Difference in log_{10} CFU between plain and erythromycin supplemented m-*Enterococcus* agar from Replicate 1, and E) Difference in log_{10} CFU between plain and erythromycin supplemented m-*Enterococcus* agar from both replicates

Descriptive statistics of phenotypic resistance

For phenotypic resistance of *Enterococcus* spp. isolates, all 693 of either *E. faecium* or *E. hirae* isolated from plain m-*Enterococcus* agar, were susceptible to gentamicin, linezolid, tigecycline, and vancomycin (Figure 18). A majority of isolates, 73.3%, were resistant to tetracycline. Additionally, over 50% of the tetracycline-resistant isolates were right-censored when considering their MIC and continued to grow at the highest concentration of 32 mg/L. Over half of the isolates, a total of 59.6%, were resistant to lincomycin. Only 11.54% of isolates were resistant to erythromycin. Less than 25% of the isolates were resistant to daptamycin or nitrofurantoin. Less than 10% of isolates were resistant to chloramphenicol, ciprofloxacin, kanamycin, penicillin, streptomycin, quinupristin/dalfopristin, or tylosin. Of the 8.66% of isolates resistant to tylosin, 7.65% grew at the highest concentration of 32 mg/L, and were therefore right-censored.

Multidrug-resistant isolates (i.e., ≥3 antibiotic classes) showed an increased percentage in the control group, from 8% on Day 0 to 22.6% on Day 84, and on to 31.8% on Day 119 (Figure 19). Similarly, the tylosin group increased in the overall percent of multidrug-resistant isolates from 19% on Day 0 to 49% on Day 84 and stayed similar at 48% on Day 119. Additionally, on Day 119 1.6% of isolates in the tylosin group were resistant to 6 antibiotic classes, compared to 0 on both Day 0 and Day 84. The combined tylosin and DFM group also increased in the percent of MDR isolates from 12% on Day 0 to 41.6% on Day 84. The combined tylosin/DFM group also showed

2.2% of isolates from Day 84 were resistant to 7 antibiotic classes, compared to none that were resistant to 7 classes on both Day 0 and Day 119. Interestingly, the percent of MDR isolates in the DFM group remained the same at 18% for both Day 0 and Day 84, but then increased from 18% on Day 84 to 52% by Day 119. The increase in multi-drug resistance was later tested for statistical significance using multi-level mixed logistic regression.

Resistance of *Enterococcus* spp. isolates to each antibiotic class by sample day and treatment showed a trend for increasing resistance to macrolides in the tylosin group, from 9.45% of isolates on Day 0 to 26.0% on Day 84. This trend also occurred in the combination tylosin/DFM treatment, from 4.68% on Day 0 to 35.5% on Day 84 (Figure 20). Macrolide resistance decreased in both the tylosin-fed groups (following withdrawal) from Day 84 to Day 119; that is, from 26.0% to 20.31% in the tylosin group, and from 35.5% to 10.5% in the combined tylosin/DFM treatment group. Additionally, there was decreased resistance to tetracycline among isolates in the DFM group, from 84.61% of isolates on Day 0 to 69.96% on Day 84, and further to 65.38% on Day 119. The combination tylosin/DFM group also showed decreased resistance to tetracycline from 75% on Day 0 to 60% on Day 84. However, the tylosin group increased from 75.67% on Day 0 to 80% on Day 84%, but then decreased to 65.62% on Day 119. The increase in macrolide resistance, and the pattern of both increased and decreased resistance to tetracycline was later tested for statistical significance using multi-level mixed logistic regression

	# Resistant (of 693 tested)	% Resistant	95% Confidence Interval		0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Chloramphenicol	2	0.29	0.035	1.039								1.73	65.22	32.32	0.435	0.145	0.145					
Ciprofloxacin	22	3.17	2	4.767				0.58	7.94	47.04	30.45	10.82	3.03	0.145								
Daptamycin	124	17.89	15.11	20.95					0.87	0.43	2.45	13.85	64.5	16.88	0.575	0.435						
Erythromycin	80	11.54	9.261	14.16					48.92	24.1	2.16	5.19	8.18	4.04	7.5							
Gentamicin	0	0	0	0.53*														100	0.00	0.00	0.00	
Kanamycin	3	0.435	0.089	1.26														83.26	14.43	1.88	0.435	
Lincomycin	413	59.6	55.84	63.27							35.96	2.45	2.02	11.43	48.2							
Linezolid	0	0	0	0.53*						0.87	3.75	76.48	18.9	0.00								
Nitrofurantoin	141	20.35	17.41	23.54								1.3	0.29	0.435	18.9	28.72	30.01	20.35				
Penicillin	20	2.89	1.772	4.422					7.79	4.18	16.88	40.12	25.69	2.45	2.89							
Streptomycin	1	0.145	0.004	0.801																99.86	0.145	0.00
Quinupristin/ Dalfopristin	57	8.23	6.289	10.53						39.25	5.05	47.47	7.94	0.145	0.145	0.00						
Tetracycline	508	73.3	69.84	76.57							22.37	1.88	0.87	1.59	3.17	18.04	52.09					
Tigecycline	0	0	0	0.53*	0.58	17.75	26.7	53.1	1.88	0.00												
Tylosin	60	8.66	6.672	11					0.435	0.72	8.51	61.33	13.28	5.77	1.3	1.01	7.65					
Vancomycin	0	0	0	0.53*					0.87	52.38	44.73	0.72	1.3	0.00	0.00	0.00						
*97.5% One-sided CI																						

Figure 18 Percentage of *Enterococcus* spp. isolates that were resistant and their distribution across minimum inhibitory concentrations (MIC) for each antibiotic. Black vertical lines indicate the human CLSI (or, NARMS) interpretive breakpoint, grey boxes indicate areas above and below highest and lowest limit of assay antibiotic concentrations, respectively. Isolates which exceeded growth at the highest antibiotic concentration were placed in the next MIC column.

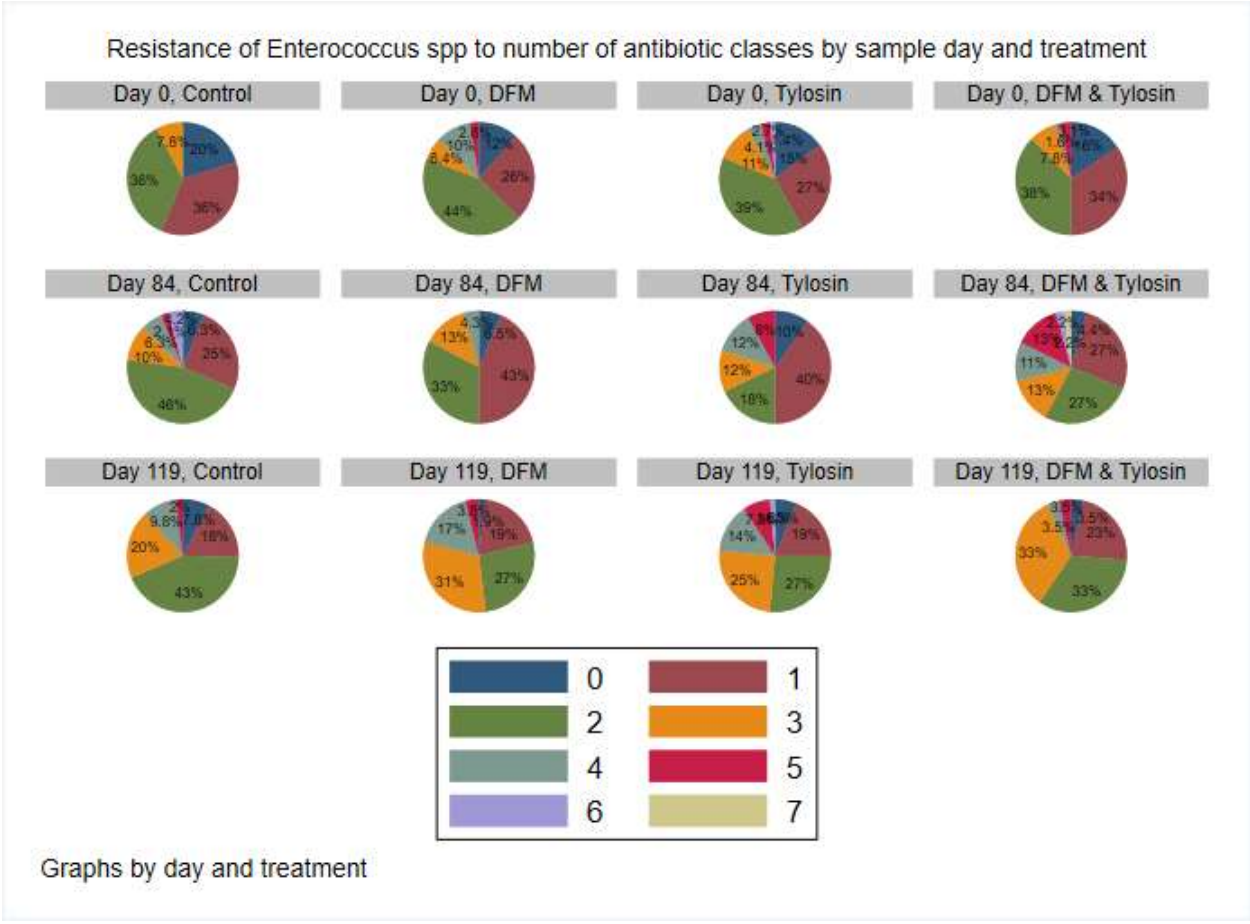


Figure 19 Resistance (%) of *Enterococcus* spp. isolates to number of antibiotic classes by sample day and treatment

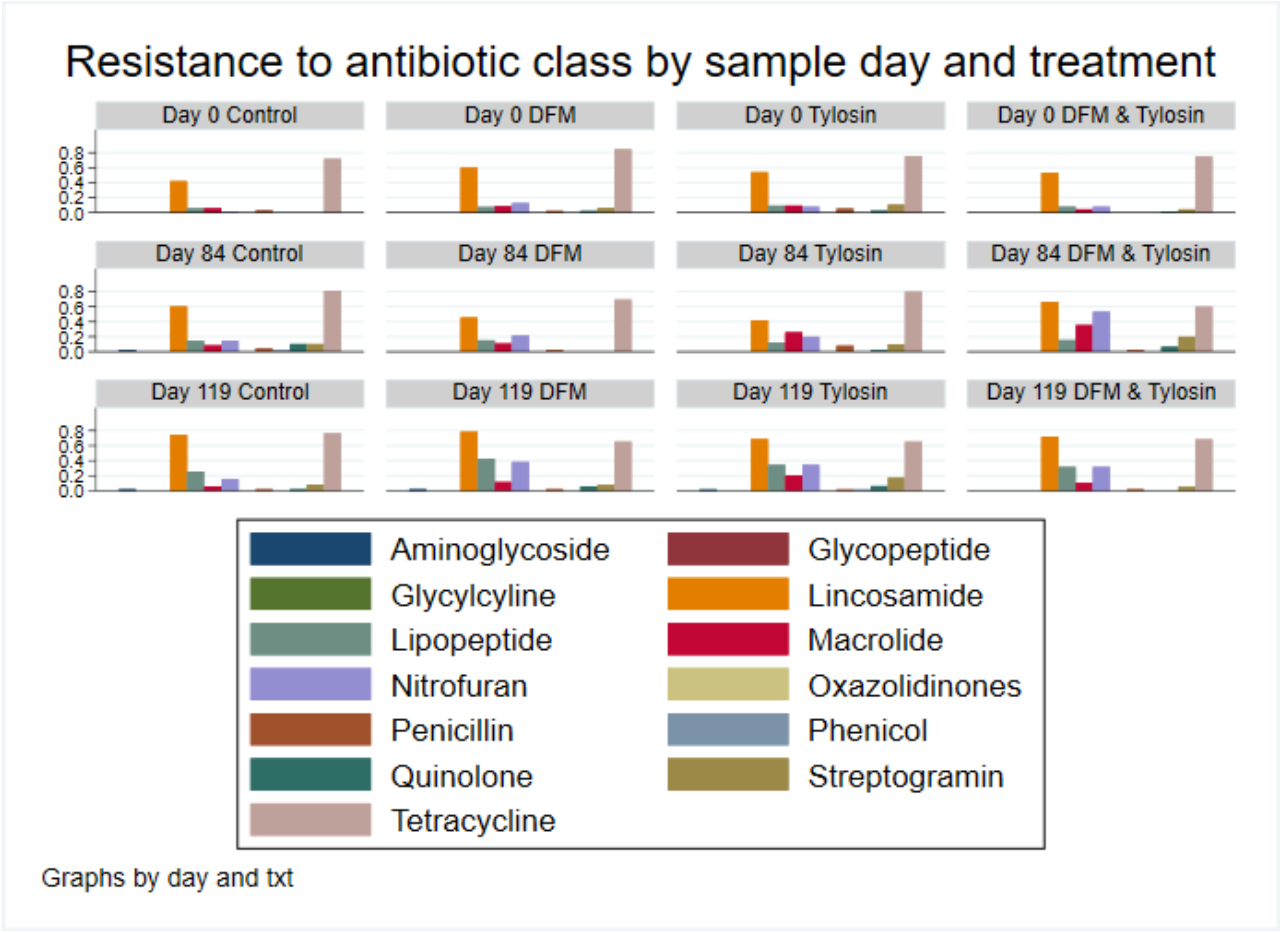


Figure 20 Resistance (proportion) of *Enterococcus* spp. isolates to each antibiotic class by sample day and treatment

Multi-level mixed effects logistic regression modeling of multi-drug resistance

A multi-level mixed effect logistic regression performed on multidrug-resistant enterococci with binary endpoints showed no significant differences among the treatment groups across all sampling periods. However period main effects were very important. The predicted prevalence of MDR isolates ranged from 0.08 to 0.21 on Day 0, 0.15 to 0.33 on Day 84, and 0.32 to 0.54 on Day 119. There was a significant increase in multi-drug resistance among enterococcal isolates from the DFM group from 0.15 (95% CI of 0.04 to 0.27) on Day 84 to 0.54 (95% CI of 0.37 to 0.71) on Day 119. There was also a significant increase in the predicted prevalence of multidrug-resistance from 0.11 (95% CI of 0.02 to 0.20) on Day 0 to 0.42 (95% CI of 0.26 to 0.58) on Day 119 among isolates from the combination tylosin/DFM treatment group (Figure 21). The percentage of multidrug-resistant isolates also tended to increase in the tylosin group, from 0.21 (95% CI of 0.08 to 0.34) on Day 0 to 0.33 (95% CI of 0.16 to 0.51) on Day 84 to 0.48 (95% CI of 0.33 to 0.63) on Day 119; however, these increases were not significant ($P>0.05$)

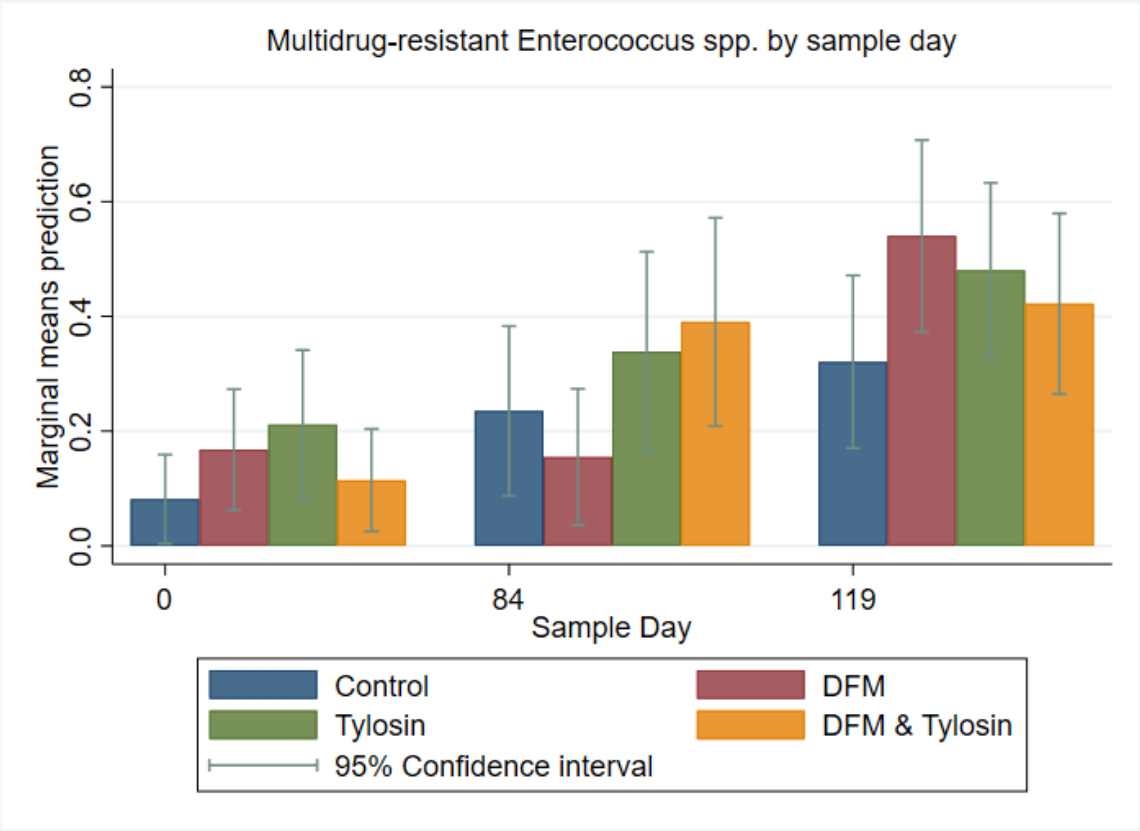


Figure 21 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of DFM, tylosin, and sample day on the binary outcome of multidrug-resistant *Enterococcus* spp.

A multi-level mixed effects logistic regression on tetracycline-resistant enterococci with binary endpoints showed a trend for decreased resistance to tetracycline from Day 0 to Day 84 for the DFM and combination tylosin/DFM groups (Figure 22). The predicted prevalence for the DFM group decreased from 0.83 (95% CI of 0.73 to 0.94) on Day 0 to 0.69 (95% CI of 0.53 to 0.86) on Day 84. Similarly, the combined tylosin/DFM group decreased from 0.75 (95% CI of 0.62 to 0.89) to 0.64 (95% CI of 0.47 to 0.82). However, these decreases were not significant (P>0.05). Additionally, there was a trend for increased resistance to tetracycline from 0.65 (95% CI of 0.49 to

0.83) on Day 0 to 0.77 (95% CI of 0.61 to 0.93) on Day 84 in the control group, but this too was not significant (P>0.05). Lastly, there was decreased resistance to tetracycline in the tylosin group from Day 84 to Day 119 (following product withdrawal), from 0.77 (95% CI of 0.63 to 0.92) to 0.64 (95% CI of 0.50 to 0.79); however, these differences also were not significant.

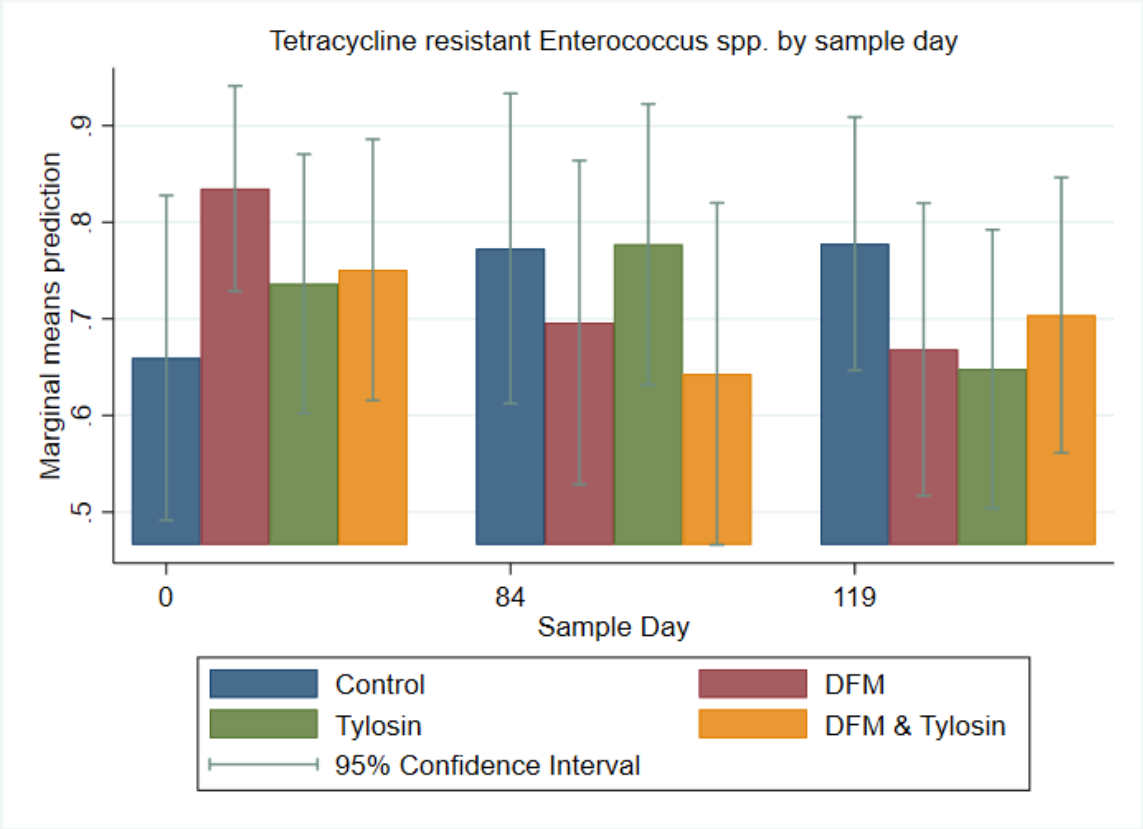


Figure 22 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of DFM, tylosin, and sample day on the binary outcome of tetracycline resistant *Enterococcus* spp.

A multi-level mixed effects logistic regression on erythromycin-resistance among enterococci isolates with binary endpoints showed a trend of significantly higher resistance to macrolides in the combination DFM/tylosin group on Day 84 at 0.35 (95%

CI of 0.201 to 0.513), compared to 0.04 (95% CI of -0.007 to 0.095) on Day 0 (Figure 23). Additionally, after the withdrawal of tylosin, resistance to macrolides significantly decreased in the combination group, from 0.35 (95% CI of 0.201 to 0.513) on Day 84 to 0.11 (95% CI of 0.023 to 0.197) on Day 119. The tylosin group showed a similar

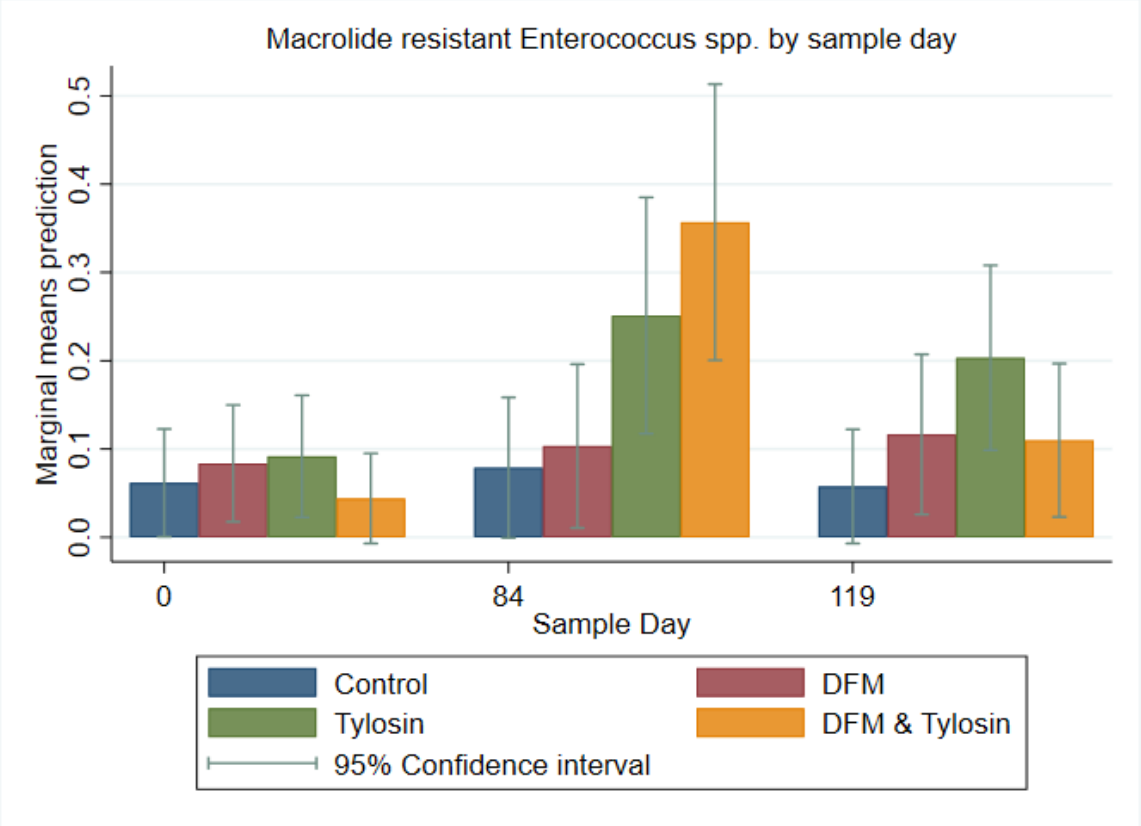


Figure 23 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of DFM, tylosin, and sample day on the binary outcome of macrolide resistant *Enterococcus* spp.

increase from 0.09 (95% CI of 0.023 to 0.161) on Day 0 to 0.25 (95% CI of 0.117 to 0.385) on Day 84, however this increase was not significant.

Discussion

Overall, there was a significant difference ($P<0.05$) in quantification of enterococci between plain and erythromycin supplemented agar in the first replicate for the tylosin group. This difference was not significant ($P>0.05$) 4 weeks after the withdrawal of tylosin at the end of the trial, and was not present in any of the other groups, namely the combined tylosin/DFM group. This indicates the DFM may have an attentuating effect on macrolide resistance when fed in conjunction with tylosin. Additionally, the isolates from tylosin/DFM group also had significantly more phenotypic resistance to macrolides on Day 84 when compared to Day 0, and less resistance was found at slaughter compared to Day 84. This trend of increased resistance after a period of time being fed tylosin, then a decrease following its withdrawal, agrees with previous results from Beukers *et al.*, in that a withdrawal of tylosin prior to slaughter contributes to a decrease in macrolide-resistant enterococci (Beukers *et al.*, 2015).

Additionally, a systematic review by Cazer *et al.* on the effects of tylosin on antimicrobial resistance in beef cattle found that tylosin increased the proportion of macrolide resistant enterococci in the gastrointestinal tract (Cazer *et al.*, 2020). When fed in conjunction with the ionophore monensin, enterococci have also been shown to have increased resistance to macrolides (Jacob *et al.*, 2008). However, it has been suggested that more than simple antibiotic use is at play, as a study by Jackson *et al.* showed that while macrolide resistant *Enterococcus* spp. were higher on a farm which

used tylosin, they were still present on a farm which did not use tylosin (Jackson *et al.*, 2004). This implies that the environment must play an important role in sustaining resistance and its magnitude likely reflects past use. As suggested previously by Muller *et al.*, in a study in which cattle which were not fed tylosin did not have significantly less macrolide resistant enterococci than cattle which were consistently fed tylosin. (Müller *et al.*, 2018), but both groups (and a negative control group) showed a significant increase in erythromycin resistance from arrival until late in the feeding period.

Others have asserted that tylosin minimally affects resistance in beef cattle, and suggest that resistance may be seasonal; however, it should be noted that in the month in which the tylosin-treated cattle exhibited a higher prevalence of macrolide-resistant enterococci, the corresponding pen also had a higher prevalence of macrolide resistant *Enterococcus* spp. (Schmidt *et al.*, 2020). It was of interest in our study that the DFM was associated with decreased tetracycline resistance from Day 0 to Day 84, which occurred in both quantification and phenotypic resistance among isolates, though it was not significant. Additionally, the DFM group had significantly higher multi-drug resistance among isolates at slaughter when compared to Day 0 and Day 84, though this was not significantly different from the other treatments on any of the sample days.

A study by Amachawadi *et al.* pointed out the potential problems with using commercial probiotics, including the isolation of multidrug-resistant *E. faecium* from the product (Amachawadi *et al.*, 2018). Even though the probiotic used in this study was not multidrug-resistant, it could still have significant impacts on antimicrobial resistance, be subjected to selection pressures, or conjugate plasmids with resistant bacteria, as

Enterococcus faecium is known to readily share plasmids with *Staphylococcus aureus* (Clewell *et al.*, 1985) as well as members of its own genus and species. Therefore, the lack of significant differences in macrolide resistance between the combined tylosin/DFM group compared to the tylosin group could imply the DFM may mitigate this resistance among both $\log_{10}$ CFU per gram of feces quantification and enterococcal isolates. Adding a macrolide-susceptible probiotic, withdrawing tylosin before slaughter, and moving cattle to new pens may be a viable future alternative to combat erythromycin resistance in beef cattle fed tylosin.

Conclusions

In conclusion, tylosin and its subsequent withdrawal seem to have a quantitative effect on macrolide-resistant enterococci, and macrolide resistance among isolates, which agrees with previous studies. While the results in the first replicate tended to favor use of a probiotic to mitigate erythromycin resistance in enterococci, the results in the second replicate were inconclusive. This may have been due to contamination of the probiotic, or improper storage conditions leading to its lack of efficacy. Starting levels

(Day 0) of macrolide resistance may differ between trial replicates, and if cattle spend extended periods of time in any feedlot environment the levels of resistance will rise uniformly across all groups reducing the potential for differences to emerge between treatment groups. Thus, trial Replicate 1 may have differed from trial Replicate 2. Meanwhile, other factors, such as seasonality or cattle age may be at play. Therefore, further studies to determine the use of a probiotic and evaluating pre-existing conditions and product longevity and durability must be performed.

CHAPTER V

EFFECTS OF A DIRECT-FED MICROBIAL AND ENVIRONMENTAL CHANGE ON GENOTYPIC RESISTANCE FACTORS IN *ENTEROCOCCUS FAECIUM*[1]

Background

In an updated version of the Antimicrobial Resistance Threats Report, the CDC noted that *E. faecium* is the most common cause of central line-associated bloodstream infections (CDC, 2019). While *E. faecium* is less likely to possess virulence factors than *E. faecalis*, it is more likely to be multidrug-resistant (Arias and Murray, 2012; Huycke *et al.*, 1998). However, when multidrug-resistant *E. faecium* were phylogenetically traced, it was found that the hospital-adapted lineage appeared around 80 years ago, parallel with the beginning of the use of antibiotics, but at that point the bacterial population consisted primarily of animal strains, not those presently associated with human commensals (Lebreton *et al.*, 2013).

Additionally, when considering the possibility and impact of shared mobile genetic elements there is a risk of plasmid transference between enterococci of different origins. For instance, Rosvoll *et al.* found the plasmid pRE25 was widely shared between geographically diverse enterococcal isolates of animal and human clinical origin (Rosvoll *et al.*, 2010). When accounting for resistance to erythromycin, this could pose a problem due to the fact that *erm* family genes which confer resistance to

[1] Part of the data and figures reported in this chapter are reprinted with permission from "Macrolide-susceptible probiotic *Enterococcus faecium* ST296 exhibits faecal-environmental-oral microbial community cycling among beef cattle in feedlots" by Murray, S., Holbert, A., Norman, K., Lawhon, S., Sawyer, J. and Scott, H. (2020), Letters in Applied Microbiology, 70: 274-281 Copyright [2020] by John Wiley & Sons, Inc

erythromycin also are active against other macrolides, lincosamides and streptogramin B (MLS_B). Additionally, a study by Chen *et al.* demonstrated the possibility for co-selection of resistance to tetracycline when feeding tylosin (a macrolide) to cattle (Chen *et al.*, 2008).

Therefore, it is necessary to sequence and phylogenetically trace the *E. faecium* isolated from the second trial in order to determine possible fecal-environmental-oral cycling patterns that may be temporally present, and investigate if the supplemented probiotic could survive these same patterns. This was achieved by sequencing *Enterococcus faecium* isolates from an experimental study at a beef feedlot, and sequencing *Enterococcus faecium* isolated from the supplemented probiotic product. Additionally, through whole genome sequencing we determined associations present among multi-locus sequence type, resistance genes, sample day, and treatment.

Materials and methods

Isolate origin

Enterococcus isolates originated from a study at an experimental feed yard in McGregor, Texas, conducted in a 2x2x2 factorial design, in which cattle were fed tylosin, and/or a macrolide susceptible probiotic, or neither, and subjected to environmental pen change after 84 days on feed. In summary, the cattle were randomized to one of the four fed treatments (control, tylosin, DFM, tylosin/DFM), then placed into one of eight pens, with each treatment being allocated to two pens at the beginning of the study. Fecal samples were collected on Day 0, then every 4 weeks for

the next 12 weeks. After the 12th week, (hereafter, known as Day 84) half the cattle in each pen were put into a new adjacent pen in which antibiotics had never been used, and weekly samples were collected until slaughter. This was then repeated in a second replicate, using animals from the same birthing cohort.

Fecal samples from day 0, day 84 and at slaughter, were spiral plated to m-*Enterococcus* agar, and m-*Enterococcus* agar supplemented with erythromycin. These were incubated at 48°C for 48 hours, then each presumptive *E. faecium* colony was streaked to TSA agar with 5% sheep blood and subjected to MALDI-TOF for confirmation of genus and species. Additionally, 12 *E. faecium* colonies were isolated from the probiotic product, and their genus and species were also confirmed by MALDI-TOF.

DNA extraction and quantification

Enterococcus faecium DNA from isolates derived from each of Day 0, Day 84 and slaughter was isolated in the QIAcube HT automated system using a QIAamp 96 DNA QIAcube HT Kit (Qiagen, Valencia, CA). An *Enterococcus faecium* colony was suspended into 5 ml of Trypic Soy Broth (TSB) and subjected to shaking incubation at 37°C for 18-24 hours. From the broth culture, 2 ml was transferred to a microcollection tube and centrifuged at 10,000 rpm for 10 minutes at room temperature. The supernatant was removed and the pellet was resuspended with 180 µl of Buffer P1 and 20 µl of freshly prepared lysozyme solution (5mg/ml), then transferred to LifeLINE TM (Dot Scientific, Burton, MI) microcollection tubes and incubated at 37°C and 900 rpm for 1 hour on an Eppendorf ThermoMixer TM (Eppendorf, Hauppauge, NY).

One tube of small pathogen lysis beads (Qiagen, Valencia, CA) was added to the suspension and disrupted using the Qiagen TissueLyser system TM (Qiagen, Valencia, CA), in combination with the proprietary Qiagen ATL buffer with DX reagent to prevent foaming of the suspension. The tubes were briefly centrifuged and 40 µl of Proteinase K was added to each tube. The tubes were incubated at 56°C and 900 rpm for 1 hour on the Eppendorf ThermoMixer TM (Eppendorf, Hauppauge, NY), followed by a heat shock at 95°C for 10 minutes. The suspension was then cooled to room temperature and 4 µl of RNAse A was added to each tube. The prepared samples were then loaded into the QiaCube HT to undergo vacuum-based DNA extraction through a modified protocol provided by Qiagen (Valencia, CA). The quality of DNA was be determined by using the 260/280 absorbance ratio on the Omega Fluostar microplate reader (BMG LABTECH, Cary, NC). The quantity of double-stranded DNA was measured using the Qubit TM (Invitrogen, Thermo Scientific Microbiology, Oakwood Village, OH) fluorometric quantification system.

Next Generation Whole Genome Sequencing

To determine multi-locus sequence types (MLST), resistance genes, and phylogeny among the *Enterococcus faecium* isolates, whole genome sequencing (WGS) was performed using the Illumina MiSeq® platform (Illumina, San Diego, CA, USA). Libraries for *Enterococcus faecium* isolates were prepared using the Illumina Nextera DNA Flex Library Preparation Kit following the manufacturer's instructions, and run using the MiSeq V2 500 cycle Reagent kit with paired end reads (Illumina, San Diego, CA, USA). Data were analyzed on the Texas A&M High Performance Research

Computing (HPRC) Ada and Terra clusters. Fastq files obtained from sequencing were trimmed using Trimmomatic version 1.8.0 (Bolger *et al.*, 2014) and assembled using SPAdes version 3.13.0 (Bankevich *et al.*, 2012). MLST types and resistance genes were determined using the Batch Uploader Pipeline through the Center for Genomic Epidemiology (CGE). Phylogeny was determined using the HPRC Ada cluster, analyzed using ParSNP version 1.2-Linux64, Model Test-NG version 0.1.5-linux64, and IQ-Tree 1.6.6-intel-2018 and visualized using ITOL (Letunic and Bork, 2019).

Results

Resistance genes and Multi-Locus Sequence Types

Whole genome sequencing was conducted on 293 *E. faecium* isolates. Fifteen isolates were excluded due to poor assembly and/or poor coverage depth. Of the 278 remaining *E. faecium*, including 12 isolated from the DFM product, the coverage depth ranged from 20.35 to 41.19, with a median of 26.76 and a mean of 30.47. The number of contigsgreater than 200bp of the assembled reads ranged from 43 to 2,078, with a median of 113 and a mean of 170. The N50 of the assembled read lengths ranged from 44,568 to 607,712 with a median of 180,319 and a mean number of base pairs in length of 179,403. The genome size ranged from 2.56 megabases (Mb) to 3.54 Mb with a median of 2.84 Mb and a mean size of 2.92 Mb.

The dominant sequence types found were ST240 and ST296 in Replicate 1, and the dominant sequence type in Replicate 2 was ST240, with no ST296 isolated from fecal samples, and a single ST296 isolated from a pen-level sample. Minority sequence types included ST32, ST94, ST108, ST212, and ST1216. Additionally, a few isolates

were of unknown sequence type. Singular isolates of ST22, ST27, ST100, ST107, ST178, ST540, ST717, ST936, and ST1442 also were found. On Day 0 in the first replicate the majority (55%) of *E. faecium* isolates were ST240. By Day 84, the variety of sequence types increased, including 33% of ST296 (Figure 24). By the slaughter date of the first replicate, 25% of isolates were ST240 while over one third were ST296. In the second replicate, the majority of *E. faecium* isolates on Day 0 was still ST240. On Day 84, the percentage of ST240 isolates decreased, and the variety of sequence types again increased. By the slaughter date of the second replicate, the percentage of ST240 isolates had decreased to 38%, and the variety of sequence types increased.

ST296 was the only sequence type of *E. faecium* isolated from the probiotic, and did not appear on Day 0 in either replicate; that said, it was also not found among any sequenced isolates from the second trial replicate. One ST296 isolate was recovered in the second replicate from a pen-environment sample which had been dried and milled.

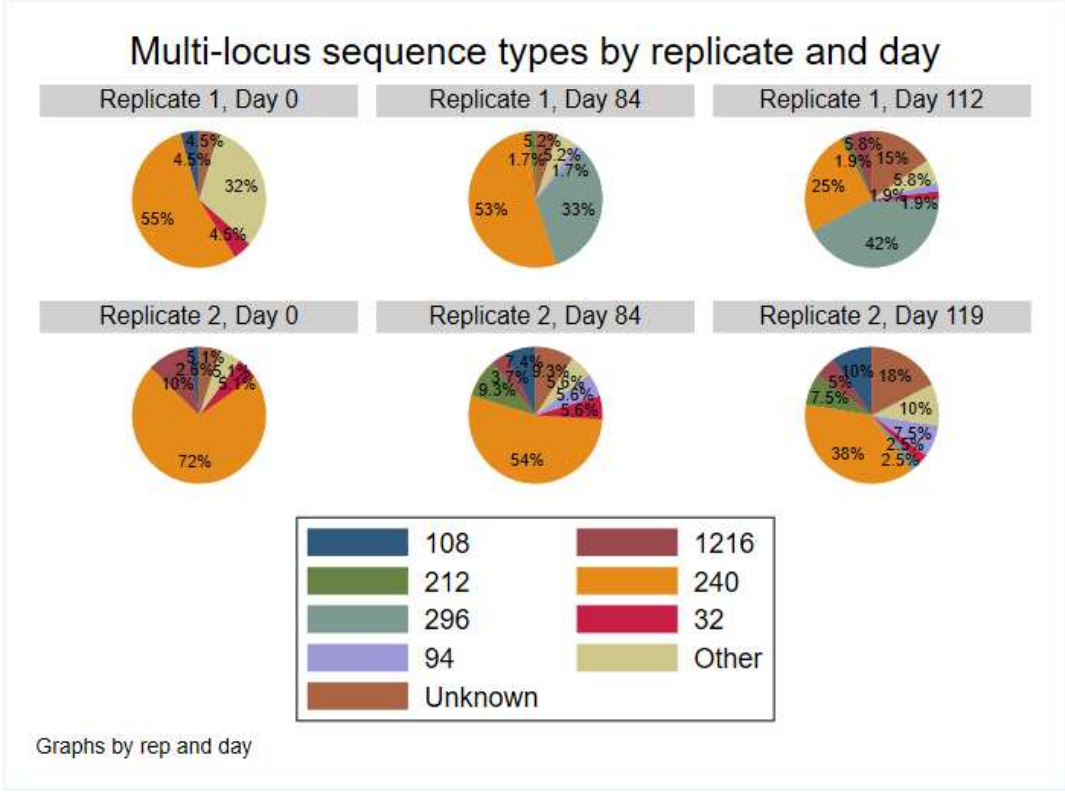


Figure 24 Dominant MLST *Enterococcus faecium* isolates by sample day and replicate

Of the unknown sequence types, nearly all carried the *msrC* gene, which encodes resistance to macrolide-lincosamide-streptogramin (MLS_B) antibiotics. This gene was common among nearly all sequence types, only being absent in ST212, ST27, and a single unknown ST. Additionally, tetracycline resistance genes, including *tet*(M) and *tet*(L) were common among isolates, and present in all ST108 and ST212. Of the minority sequence types, including ST22, ST27, ST100, ST107, ST178, ST540, ST717, ST936, and ST1442, all carried *msrC* (except ST27) with some sequence types also harboring *ermB*, *tet*(L), and *tet*(M).

All ST108 carried *ms*rC and *tet*(M), and all were isolated from the plain m-*Enterococcus* agar. None of the ST212 isolates had *msrC*, though all had *ermB* and *tet*(M), and the majority were isolated from erythromycin-supplemented agar. The majority of ST32 was isolated from plain m-*Enterococcus* agar, all carried *msrC* with some also harboring *tet*(M). Many of the ST94 isolates also carried *ermB* and *tet*(M) in addition to *msrC*, with the majority of isolates coming from erythromycin supplemented agar. Importantly, all ST296 isolates appeared to carry only *msrC* as a resistance gene, and did not harbor *ermB*, or *tet*(M) resistance genes. Conversely, ST240 was found in all the sample days to varying degrees, and in the first replicate decreased in lockstep with the observed increase of ST296. ST240 was highly associated with *ermB*, *tet*(M) and *tet*(L). An abridged list of isolates and ST types, illustrating common resistance genotypes, is presented in Table 3.

Sample Day	Pen	Lab ID	Media Origin	MLST	*ermB*	*msrC*	*tet(M)*	*tet(L)*	*tet(M)*
D0	3	119	ME1	108					
D84	4	395	MEery1	212					
D0	3	119	ME1	108					
D84	1	369	ME2	296					
D0	4	124	ME1	32					
D112	2	726	MEery1	94					
D84	2	374	ME1	240					
D112	6	749	ME1	240					

Table 3 Representative resistance genes for each multi-locus sequence type of *Enterococcus faecium* sequenced
*Full tables of all sequenced isolates for all ST types can be found in the appendix

Phylogeny

Of the *E. faecium* isolates sequenced, the majority of isolates were ST240 and ST296 (Figure 25). Of the minority sequence types, ST1216 appeared most frequently, with a total of 11 isolates. The ST1216 isolates were collected from the end of trial Replicate 1 and across all sample days from trial Replicate 2; of interest, the majority were from tylosin treatment groups. Similarly, ST108 had 10 isolates, all within the same phylogenetic node (a grouping at which branches of a phylogenetic tree terminate, indicating a shared evolutionary history) and these were isolated across all sample days and treatments. Multi-locus sequence type 212 appeared in similar frequency, with a total of 10 isolates, though only from day 84 and the slaughter date, and none were from the DFM treatment group. The minority sequence types of ST32 and ST94 had a total of 8 isolates each, both spread across all treatment groups, though ST32 was isolated across all sample days and ST94 was only isolated from day 84 and the slaughter date. Interestingly, while all ST32 isolates appeared in the same phylogenetic node, ST92 and ST957 also appeared in the same group as ST32, with branches between ST32 isolates.

The majority of ST240 *E. faecium* was isolated from the tylosin and combination tylosin and DFM groups. However, there was some crossover, with a few ST240 coming from the DFM-only or control groups (Figure 25). There was a total of 133 ST240 isolates that were closely phylogenetically related; that is, all clustering together in the same node. One isolate of unknown sequence type also appeared in this node. Of the ST240 isolates, they all carried at least one acquired (i.e., non-chromosomal, as determined by CGE) antimicrobial resistance gene in our sequencing analysis; importantly, ST240 was highly associated with the presence of *ermB* and/or *tet*(M). Additionally, most of these ST240 were from Day 0 or Day 84, with few isolated from the slaughter date.

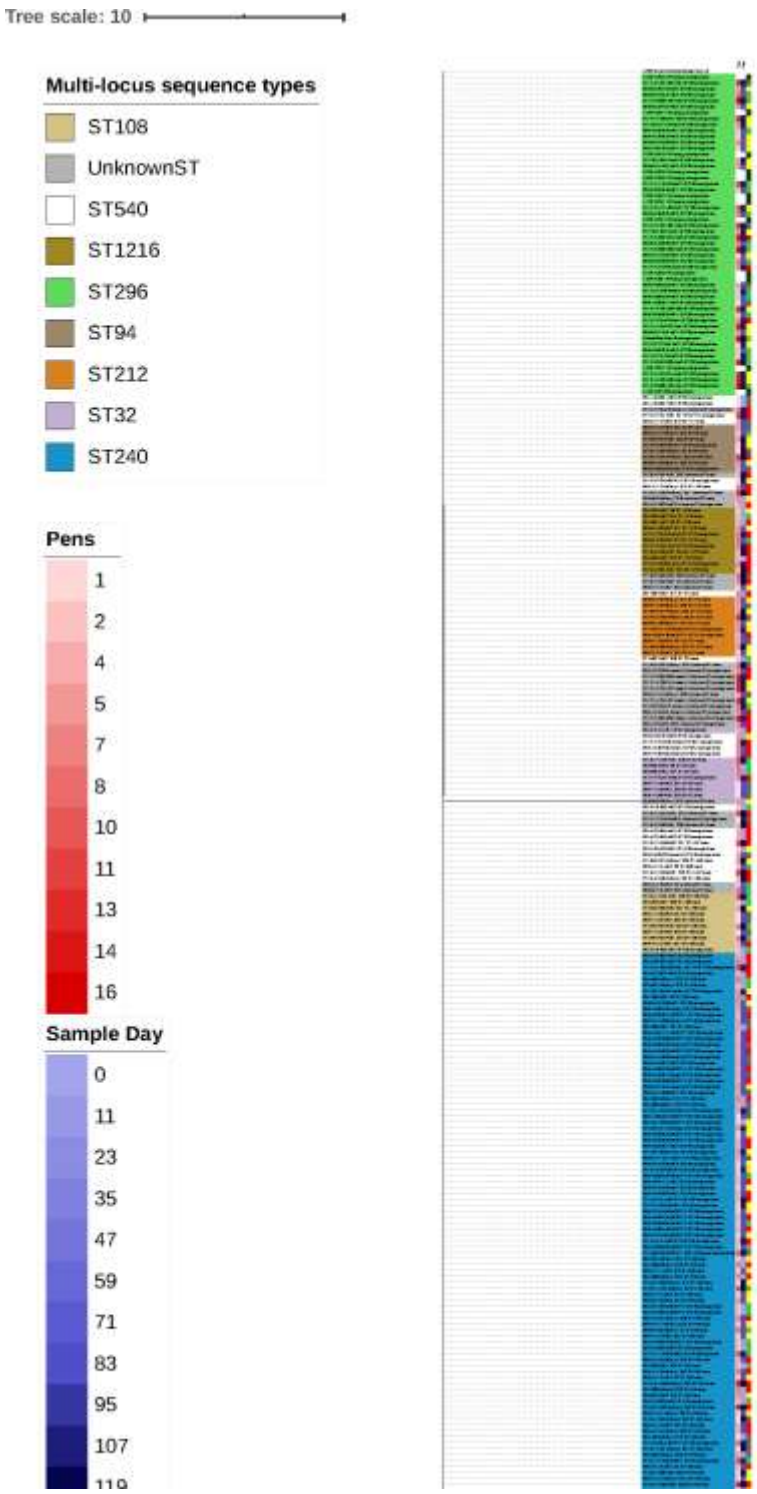


Figure 25 Phylogeny of all *E. faecium* isolates via whole-genome sequencing.

In contrast, most ST296 came from the DFM or combination DFM/tylosin treatment groups; however, these were only isolated during the first replicate, though with very few isolates coming from the control or the tylosin groups. It should be noted as a limitation of the study (though a practical feature of most agricultural operations) that the pen fences were permeable, allowing for some interaction between animals and permitting fecal material and dust to transfer between treatment groups. Additionally, there was a very limited amount of animal crossover of fences, as animals occasionally moved from their own treatment group to another of their own volition.

All *E. faecium* ST296 recovered had *msrC*, which encodes for decreased susceptibility to streptogramins. A total of 36 ST296 isolates were recovered, and only after supplementation with the DFM began. Interestingly, nearly all the ST296 isolates came from the first replicate of the study, with only one isolate coming from the second replicate, from an environmental sample. There were three ST296 from the tylosin-fed group, but it should also be noted that they were isolated from cattle housed in pens with the DFM treatment being fed to cattle in adjacent pens on each side of the tylosin-fed pen. There was a total of 4 ST296 isolates recovered from the control group (i.e., fed neither tylosin nor DFM); specifically, two from Replicate 1 Day 84, and two from the end of Replicate 1. Of the ST296 isolated from the control group on Day 84, one isolate was in a different clade than the sequenced DFM, while the other was in the same clade but from a different node (see Figure 26). Of the ST296 from the control group isolated at the end of the trial, both were isolated from animals in the same pen, but each was in a different clade; one isolate belonging to the same clade as the DFM in a different node,

with the other isolate belonging to a different clade than any of the ST296 from the control groups.

Additionally, two animals had ST296 repeatedly isolated from different sample days. Interestingly, animals from pens which were fed both the DFM and tylosin shed ST296 isolates, along with ST240 isolates. By Day 84 of trial Replicate 1, there were 19 ST296 and 31 ST240 isolates; however, by the end of this trial replicate (Day 112) there were an additional 20 ST296 *E. faecium* isolated, and only 13 ST240. When looking at the SNPs, the ST296 isolates broke into two distinct groups with at least one DFM isolate in each group (Figure 27).

Of note, the single ST296 isolated from the second replicate was recovered from a pen-environment sample taken at the end of the trial, that had been dried and milled to simulate environmental conditions associated with turning manure to dust. This environmental isolate was in the second group, but still bore a very close resemblance to a DFM isolate. This suggests that the probiotic strain can survive environmental conditions in conjunction with resistant enterococci, and therefore may be useful to combat the cyclic resistance model in which enterococci present in cattle (and the prevalence of macrolide resistant enterococci) are affected by the environment (Figure 28).

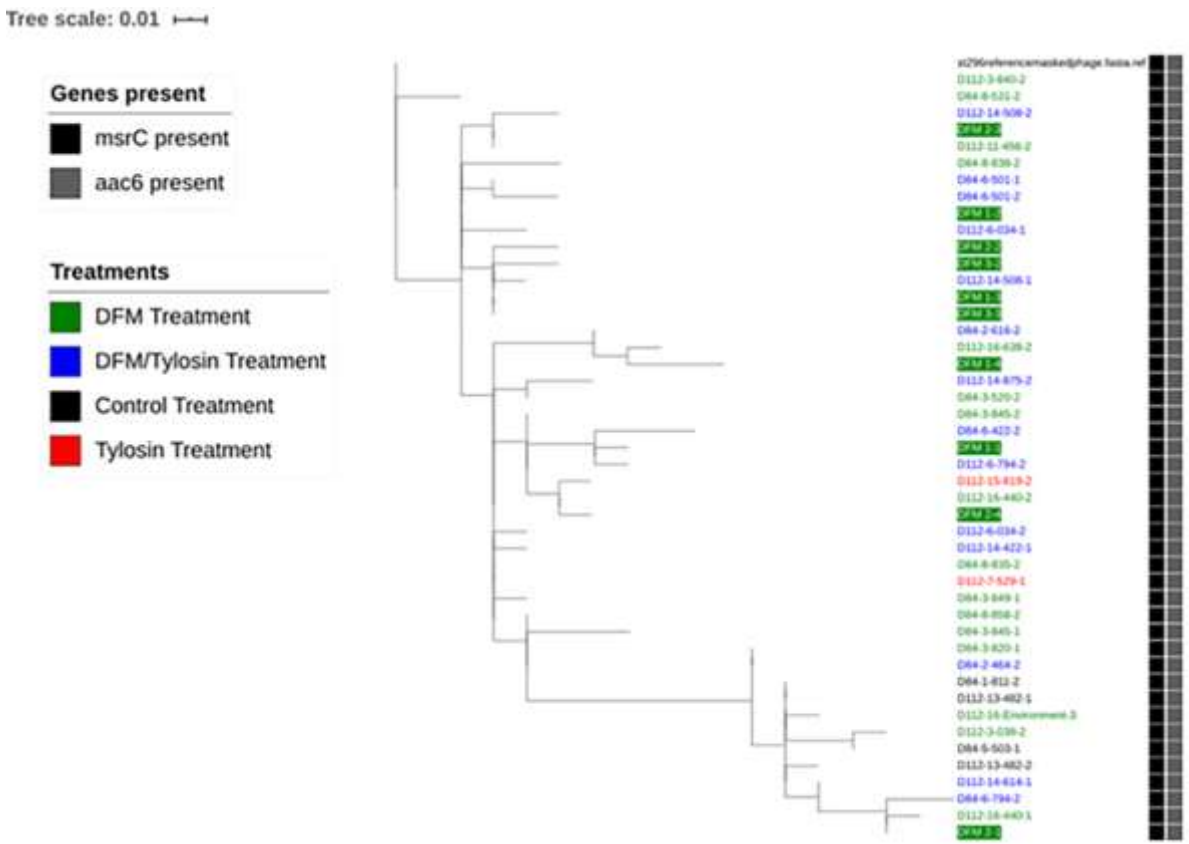


Figure 26 Phylogeny of only ST296 *E. faecium* isolates. Text color indicates treatment group, green=DFM, blue=DFM/tylosin, black=control, red=tylosin. Green highlighted isolates are of probiotic origin.

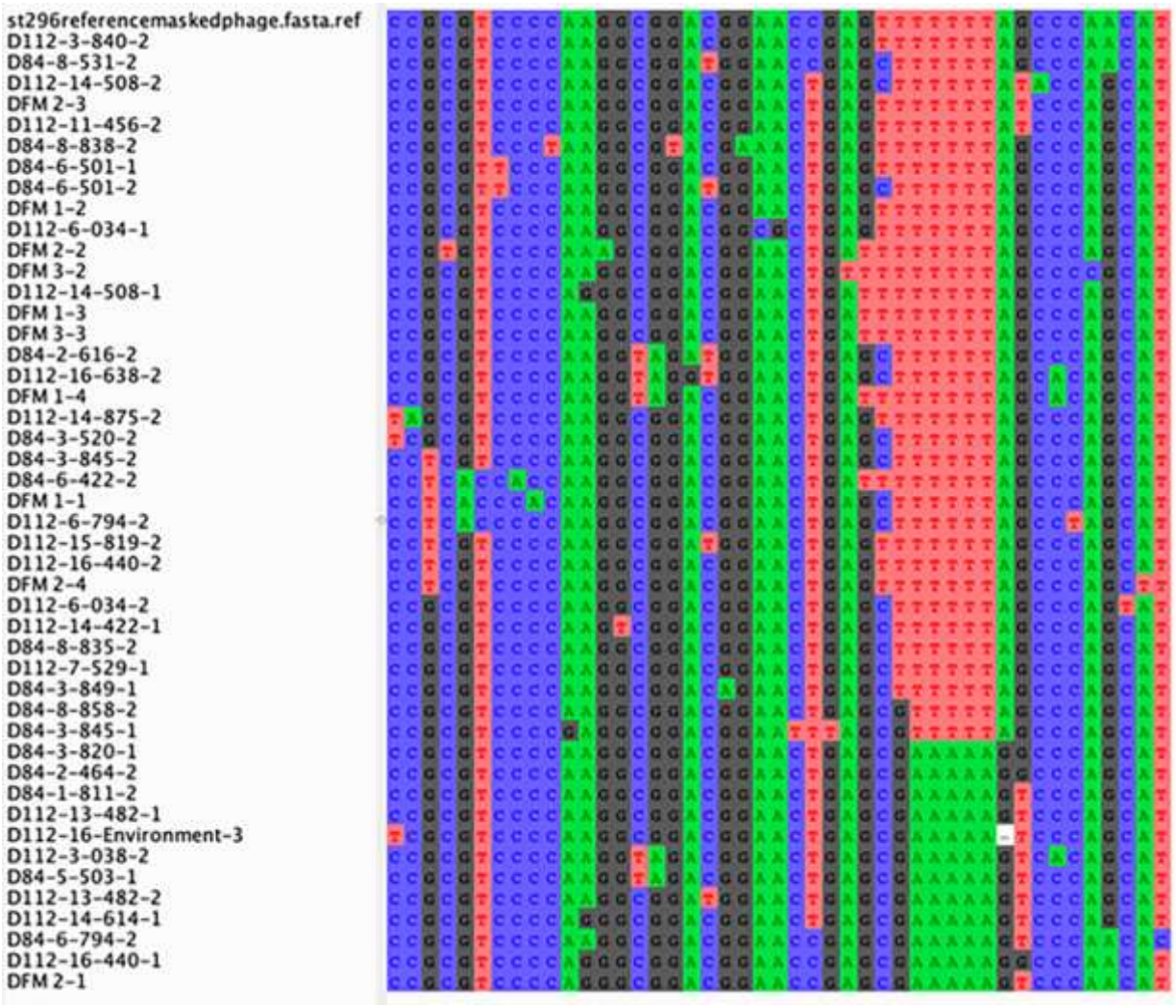


Figure 27 SNPs of *E. faecium* ST296 isolates, sorted by phylogeny, showing two distinct groups of ST296 with isolates of DFM origin in each group

Discussion

The presence of *ermB* and *tet*(M), genes associated with transferable macrolide and tetracycline resistance, respectively, were associated with ST240, which was found in both replicates and on all days of the trial. Additionally, other minor sequence types were also associated with these resistance genes, such as ST212 and ST94. The gene *msrC* was found in nearly all isolates, except for ST212, ST27, and a single isolate of an unknown ST. The ST296 *E. faecium* strain was only found in isolates after Day 0, and only in Replicate 1 fecal samples, though it was present in apen environmental sample from Replicate 2. Interestingly, the importance of the presence of *msrC* in *E. faecium* is widely debated. It was originally thought to be present in all *E. faecium* and responsible for decreased susceptibility to macrolides since it is an MLS_B class gene (Portillo *et al.*, 2000; Singh *et al.*, 2001). However, some argue that it is not evenly distributed amongst all *E. faecium* (Werner *et al.*, 2001). Others contend it is present in all *E. faecium* and thus could be used to help identify species (Zaheer *et al.*, 2020). In this study, it was present in nearly all sequence types, notably ST296, of which the only other resistant gene present was *aac(6')* which is thought to be chromosomal (Salipante and Hall, 2003). The lack of ST296 in Replicate 2 seems likely to be due to improper storage of the DFM, and therefore a lack of viable enterococci were in the supplement fed to cattle.

The results from Replicate 1 indicate that the DFM strain can not only survive, propagate and recycle within and among beef cattle hosts, it can also survive extended periods in environmental conditions that contribute to the cyclic resistance model likely present in beef cattle feed yards. In the current model, robust and resistant bacteria

survive the intensive pen environment and are fed back into the host, thereby increasing the proportion of resistance in the host when compared to extensive pastoral conditions, and regardless of antibiotic selection pressures. We hypothesize that by supplementing with a pan-susceptible DFM which can also propagate following this cycle, the proportion of susceptible enterococci will increase in the environment, which will also feed back into the host, and in the longer term reduce the proportion of resistant enterococci present (Figure 28). Adding a probiotic consisting of macrolide-susceptible *E. faecium* ST296 could have beneficial effects for reduction of resistance, as witnessed by the expansion of the probiotic strain in the cattle and their environment.

Interestingly, the DFM multi-locus sequence type (MLST) is commonly isolated from produce, not cattle fecal samples, which also is consistent with a strain robust to environmental degradation processes (Burgos *et al.*, 2014). However, the company that manufactured this product markets it for use in cattle. Phylogenetic work by Lebreton *et al.* and Raven *et al.* suggest that whole genome sequencing (WGS) is more accurate than traditional MLST typing, and have identified a pylogenetic structure consisting of clade A (hospital-associated) and clade B (community-associated) (Lebreton *et al.*, 2013; Raven *et al.*, 2016) strains in humans. Moreover, Willems *et al.* suggested that Bayesian Analysis of Population Structure (BAPS) divides *E. faecium* isolates into 13 groups (Willems *et al.*, 2012). When ST296 is analyzed, it is found to belong to clade B, and BAPS group 1–2, indicating ST296 has a community associated, nonpathogenic lineage (Freitas *et al.*, 2017).

Conclusions

These results suggest that while supplementation with a pan-susceptible *E. faecium*-based probiotic may not necessarily eliminate sequence types associated with antibiotic resistance gene carriage, the feeding practice may decrease the overall proportion and environmental burden of antimicrobial resistant-sequence types over a cumulative and extended period of time. While this hypothesis of fecal-oral-environmental microbial community cycling may combat antimicrobial resistance at a genotypic level, it remains to be seen if the probiotic sequence type, ST296, would eventually pick up resistance genes after long-term, years-long exposure to a macrolide such as tylosin, under selection pressure favoring acquisition of resistance genes such as *ermB*. Additionally, while we hypothesize ST296 would cycle back into the animal from the environment, there is no definitive proof of that occurrence, since no ST296 isolates were found in fecal samples in the second replicate, and ST296 is more commonly isolated from produce. However, the fact that this sequence type was found in animals housed in pens adjacent to the treated groups suggest that environmental spread is not implausible. These factors, and others, must be further explored before a definitive recommendation for widespread use at a commercial scale can be made.

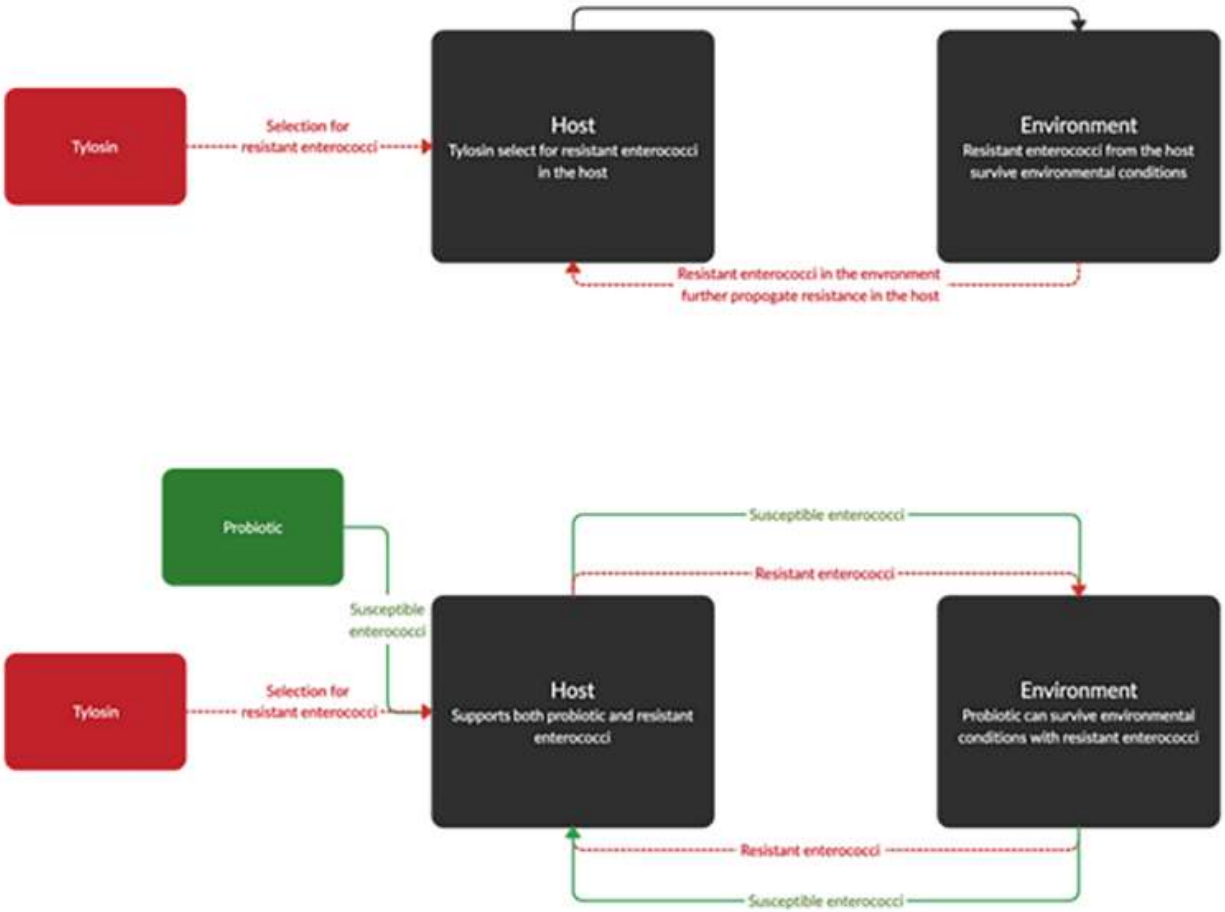


Figure 28 Proposed microbial community cycling of a pansusceptible *Enterococcus faecium* probiotic strain fed in a commercial feedlot setting.

CHAPTER VI

EFFECTS OF TYLOSIN, A DIRECT-FED MICROBIAL, AND ENVIRONMENTAL CHANGE ON MICROBIOME DIVERSITY

Background

It is known that antibiotics can have devastating effects on the microbiome in humans; in particular, long-term antibiotic usage is associated with *Clostridioides difficile* (previously known as *Clostridium difficile*)colitis (Buffie and Pamer, 2013). Additionally, fecal microbiome transplants have been regarded as a valid treatment for this type of microbiome disruption (Bakken *et al.*, 2011), also known as dysbiosis. However, there is currently little data available concerning the effects of probiotics (or, direct-fed microbials) on the fecal microbiome in animals such as beef cattle. A low-dose antibiotic such as tylosin, which is used for the prevention of liver abscesses, appears to have little effect on the microbiome; meanwhile, Weinroth *et al.* found that environment and location had a stronger impact on the microbiome of cattle (Weinroth *et al.*, 2019). Similarly, there was little effect on the microbiome in cattle fed tylosin and the beta-adrenergic agonist ractopamine when compared to a control group receiving no antibiotic or ractopamine. (Thomas *et al.*, 2017).

These studies suggest that the cattle enteric microbiome is robust and less subject to change due to external stimuli and supplementation; however, further studies are needed to examine whether probiotics (i.e., direct-fed microbials) – alone, and in combination with tylosin, have any antagonistic or synergistic effects on the

microbiome, while also considering temporal effects associated with animal aging and dietary changes. Using 16S rRNA metagenomic sequencing we ascertained whether tylosin and/or a supplemented *E. faecium* and *Saccharomyces cerviceae*- based probiotic had any effect on the cattle microbiome; further, we determined if environmental change to new pens is correlated with microbiome changes, and whether any temporal/period effects are present.

Materials and methods

Sample origin and pooling

To determine changing bacterial diversity and ecology, 16S rRNA metagenomics sequencing was performed on fecal samples stored at -80°C without glycerol from a previous study at McGregor, Texas. These fecal samples were collected using aseptic technique, and transported to the ME^2 laboratory at Texas A&M. In brief, this study tested the effects of tylosin, a direct fed microbial (DFM) and a pen-environmental change in fed beef cattle. The cattle were divided into 4 treatment groups, consisting of a control, DFM, tylosin, and combination DFM/tylosin, and the treatments were duplicated for a total of 8 pens. After 84 days, the cattle in each pen were split, and half were placed into new pens in which antibiotics had never been used, and weekly samples were collected until slaughter. Fecal samples from Day 0, Day 84, and slaughter were pooled by their pen placement at slaughter, splitting individual cattle fecal samples from each terminal pen into two pools, and thus resulting in 2-3 animals per pool. This pool of animals was kept the same for each sample day, resulting in 32 pools per day, and 96 pools for each replicate.

DNA extraction and 16S rRNA Next Generation Sequencing

DNA was extracted from the fecal pools using the Qiagen Power Soil Pro kit and the Qiagen QiaCube platform (both: Qiagen, Valencia, CA). Libraries from each pool were prepared using the Illumina 16S Metagenomics workflow on the Illumina MiSeq® platform with Illumina Nextera XT Indexes and run using the MiSeq V3 600 cycle Reagent kit with paired-end reads (Illumina, San Diego, CA, USA). Data were analyzed at the taxonomic Order level using Qiime2 (Bolyen *et al.*, 2019).

Alpha diversity, i.e., the evenness of distribution of taxa, was measured using the Shannon index, and statistically analyzed using multi-level mixed linear regression, in a three-way full factorial, factors being sample day, tylosin, and DFM. The Shannon index quantifies the distribution of taxa in each sample by taking the inverse sum of the natural log of the proportion of individual species (Shannon, 1949). Beta diversity, i.e., the difference in microbial abundance between samples, was measured using the Bray-Curtis dissimilarity index, plotted using the principal coordinates analysis (PCoA) to visualize any trends present among treatments or sample days, and statistically analyzed using the analysis of similarities (ANOSIM) non-parametric test. Bray-Curtis dissimilarity is used to quantify the compositional differences between samples, and is bound between 0 and 1, in which 0 means the samples share all species, and 1 in which they share no species (Sorensen, 1948). The ANOSIM is a ranked non-parameteric statistical test which operates on a ranked dissimilarity matrix such as the Bray-Curtis (Buttigieg and Ramette, 2014).

Results

Taxonomy and alpha diversity

The most abundant order in the majority of the samples was *Clostridiales*, as indicated by the green colored bar (Figure 29). The second most abundant family found was *Lactobacillales*, indicated by the grey bar. The probiotic product mainly consisted of *Bacilli*, indicated by the pink bar, without any further classification of taxonomy being possible. The first replicate of the probiotic tended to have less variety in taxonomy than the second replicate. There were no discernable patterns or differences in taxonomy by treatment, pen, sample day; however, replicate seemed to have a string qualitative effect. (see Figure 29).

A mixed multi-level linear regression of the Shannon index of alpha diversity, using tylosin, DFM, and sample day as categorical variables in a 2x2x2 full factorial model showed no significant differences by treatment group or sample day. However, the random effect of replicate represented a variance of 0.17 with a 95% CI of 0.05 to 0.57 (Figure 30). The alpha-diversity score of the combined tylosin/DFM group tended to decrease from a mean of 6.42 on Day 0 to 5.97 on Day 84, then increased to 6.47 at Day 119; however, this change was not statistically significant ($P>0.05$). Similarly, the alpha-diversity of the DFM group decreased from 6.81 on Day 0 to 6.48 on Day 84, then increased to 6.82 by Day 119; similar to the combined group, this also was not significant.

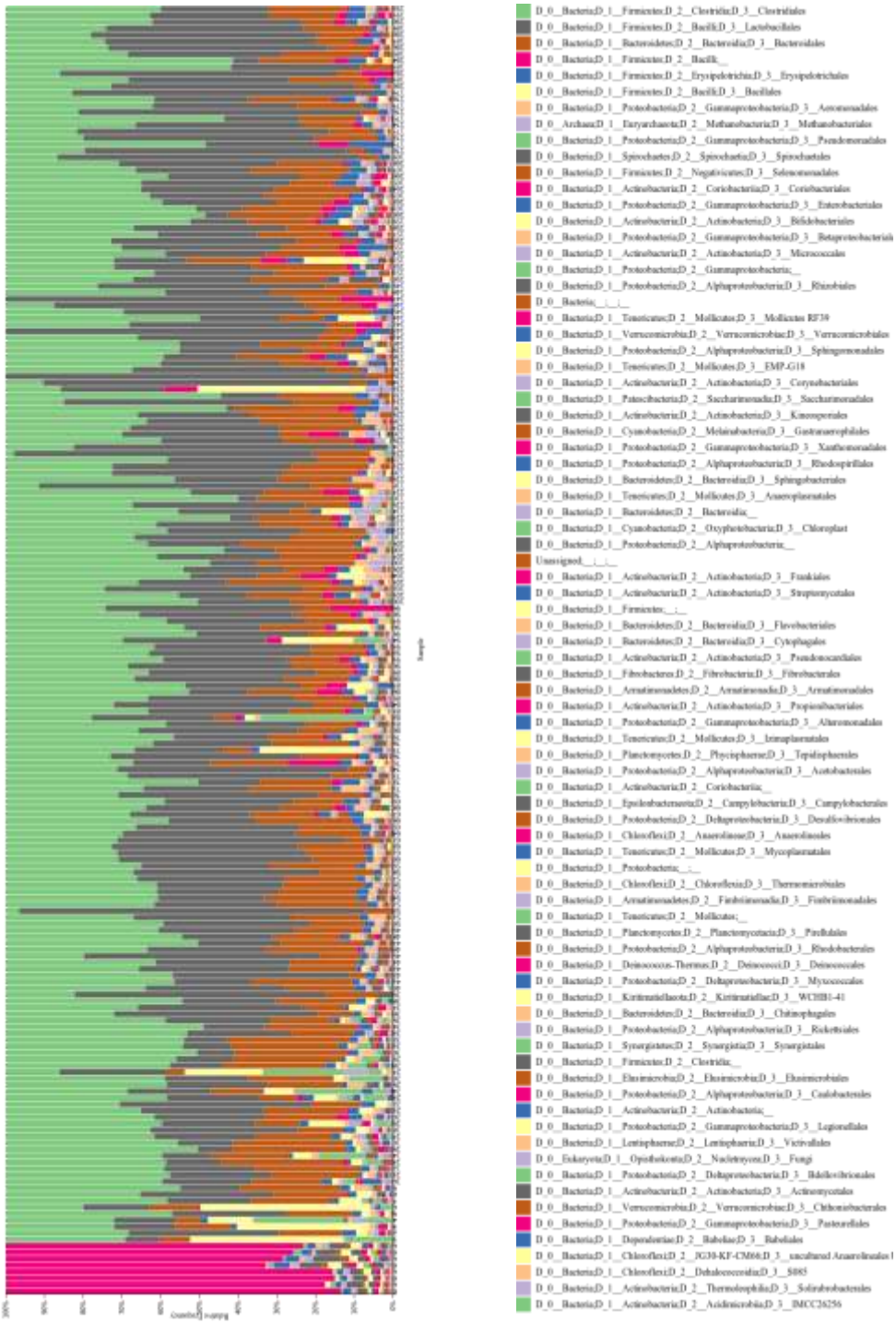


Figure 29 Taxonomic bar plot of each pool and corresponding legend. Abundant orders include *Clostridiales* (green), *Lactobacillales* (grey), *Bacteroidales* (brown), *Erysipelotrichales* (blue), and *Bacilli* (pink).

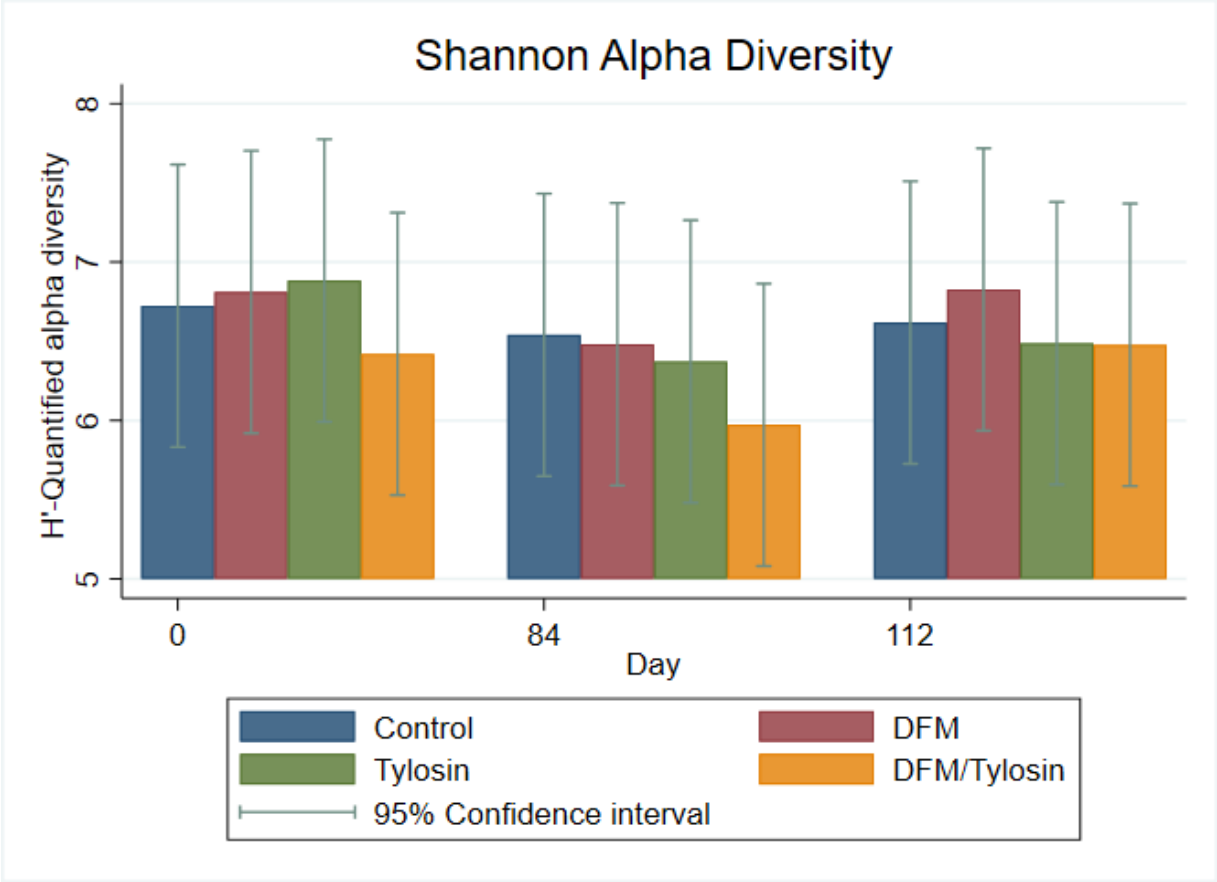


Figure 30 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of DFM, tylosin, and sample day of Shannon alpha diversity among pools representing treatment groups and sampling days.

Beta-diversity

The beta diversity of the DFM product appeared to be different for product samples taken at the beginning of the trial versus samples taken at the end of the trial. The principal coordinates analysis (PCoA) showed a grouping by replicate for the DFM product. The PC1, illustrated by Axis 1 in Figure 31, accounted for the majority of the differences in the two samples, totaling 67.37%. The secondary and tertiary coordinates accounted for a large portion of the remaining variation, with 14.57% and 6.66% respectively.

Performance of a pairwise analysis of similarities (ANOSIM) non-parametric test showed that there was less variance in the first replicate probiotic sample compared to the second. The first replicate probiotic sample had a median distance of 2.8, while the second sample had a median distance of 4.45. The results of the pairwise ANOSIM indicated each sample was more similar to itself than to the other sample, suggesting a significant dissimilarity (P=0.034). Therefore, it can be concluded that the DFM sample from the second replicate was significantly different than the DFM sample from the first replicate (Figure 32, Table 4).

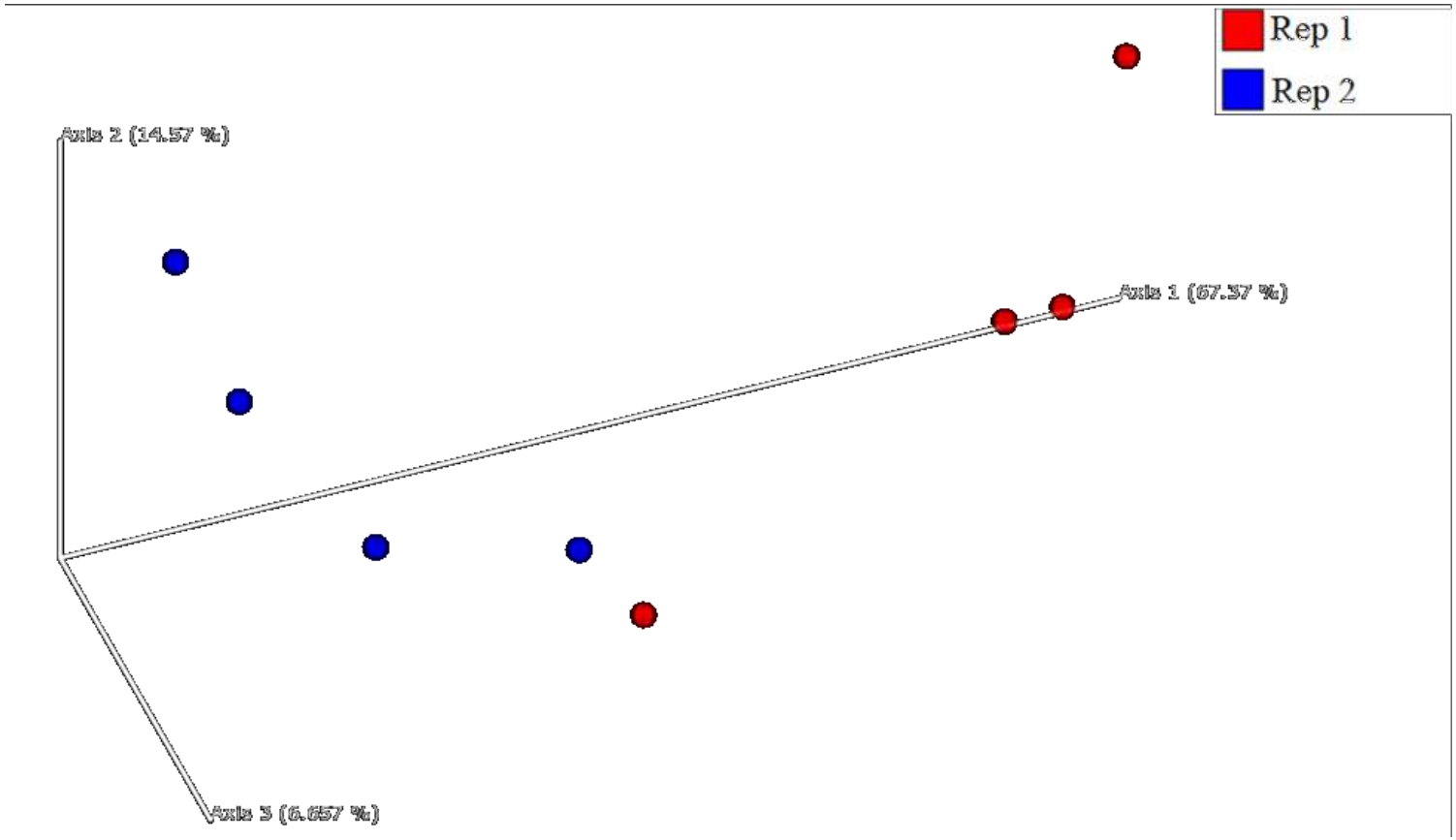


Figure 31 Beta-diversity principal coordinates analysis of the quantified dissimilarity between DFM samples measured by Bray-Curtis index analyzed by trial replicate.

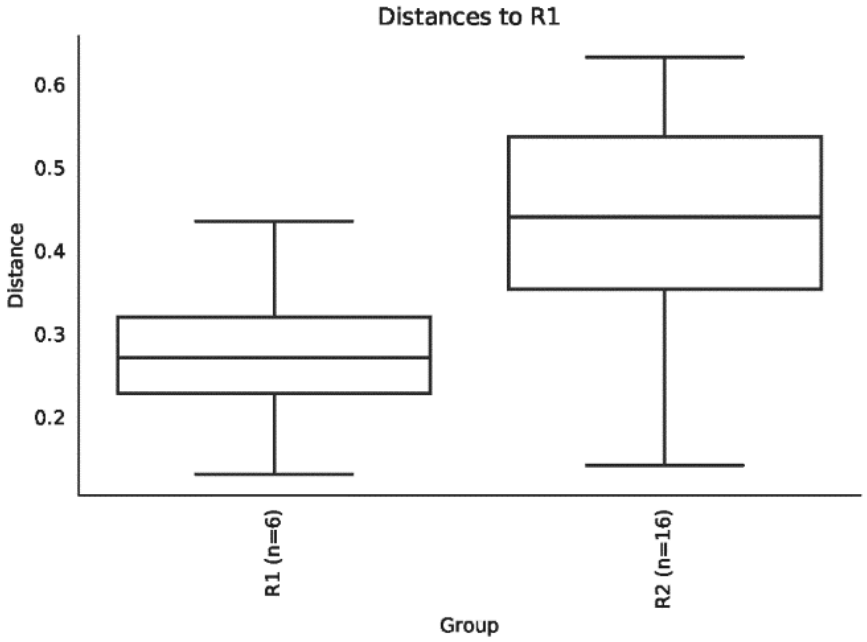


Figure 32 Boxplot of median, range and interquartile range of Bray-Curtis dissimilarity index of DFM sample by trial replicate

anosim-pairwise dfm rep

Group 1	Group 2	Sample size	Permutations	R	p-value	q-value
R1	R2	8	999	0.6875	0.034	0.034

Table 4 Analysis of similarity (ANOSIM) pairwise comparison of DFM samples by trial replicate.

The beta diversity among sample days tended to group together in the PCoA plot (Figure 33). Day 0 samples tended to cluster together regardless of trial replicate, while Day 84 samples were widely spread out, and Day 119 samples again tended to cluster together. However, very little of the diversity was accounted for, with 10.55% for PC1, 9.05% for PC2, and 5.06% for PC3; thereby, totaling less than 25%. The boxplot of the Bray-Curtis dissimilarity index showed a median distance of 0.9 for Day 0, 0.87 for Day 84, and 0.81 for Day 119. Performance of a pairwise ANOSIM showed each sample day was significantly more similar to itself when compared to other sample days, indicating a significant temporal dissimilarity ($P<0.01$). (Figure 34, Table 5)

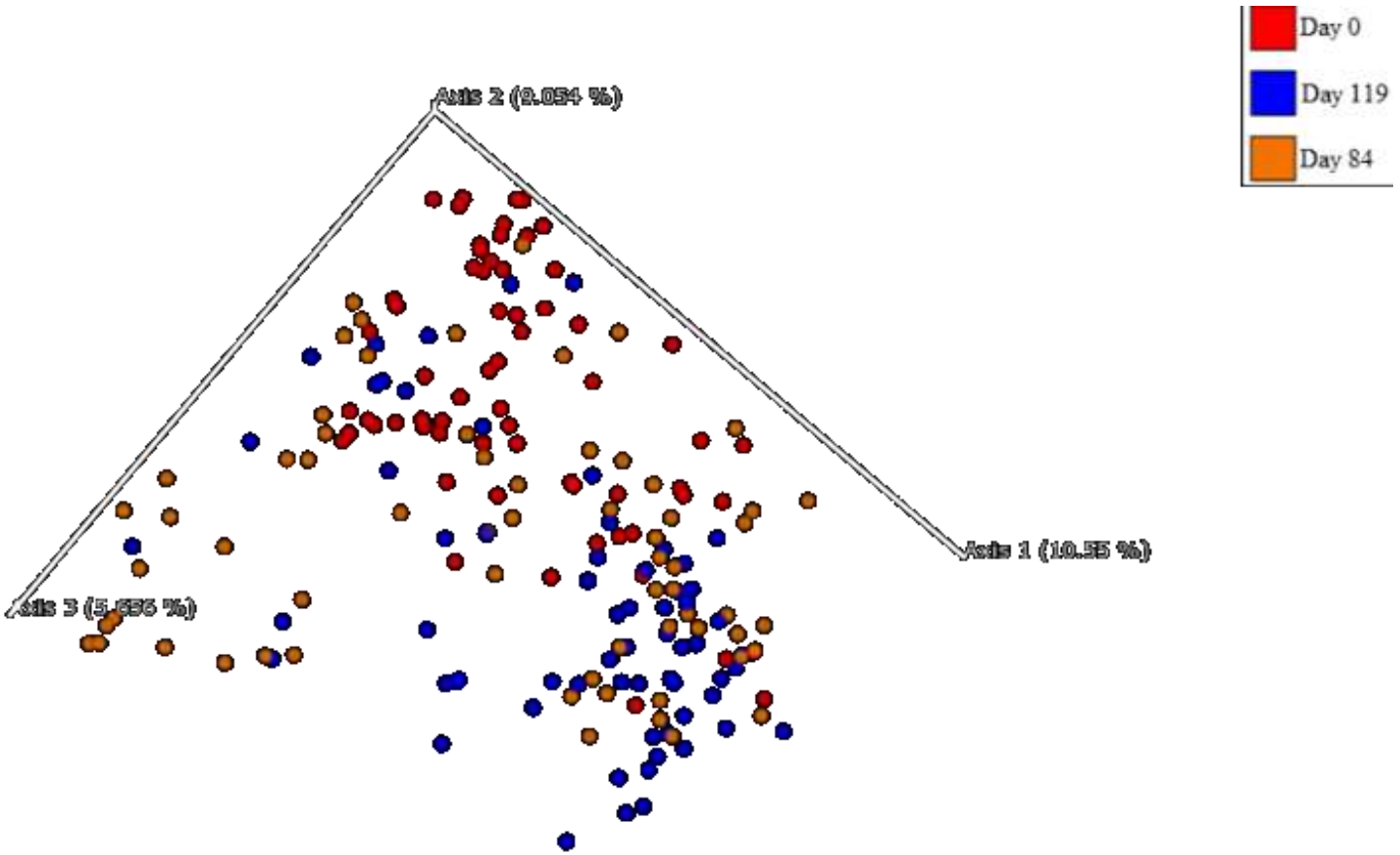


Figure 33 Beta-diversity principal coordinates analysis of the quantified dissimilarity between all samples by sample day measured by Bray-Curtis index.

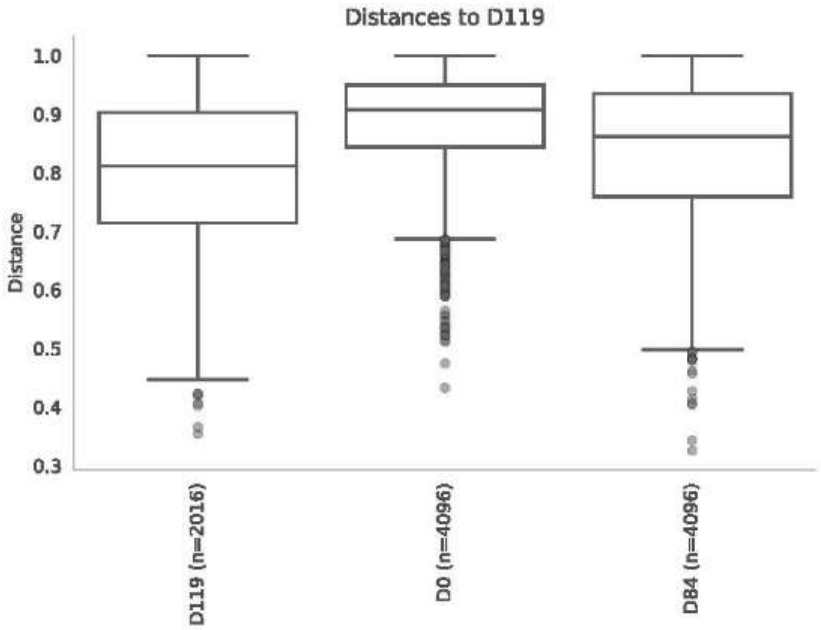


Figure 34 Boxplot of median, range and interquartile range of Bray-Curtis dissimilarity index of all replicate fecal sample by sample day.

anosim-pairwise all reps sample day

Group 1	Group 2	Sample size	Permutations	R	p-value	q-value
D0	D119	128	999	0.22010282486204100	0.001	0.0015
D0	D84	128	999	0.13357537890237500	0.001	0.0015
D84	D119	128	999	0.03156383453853540	0.01	0.01

Table 5 Analysis of similarity (ANOSIM) pairwise comparison of all replicate fecal samples by sample day.

In the principal coordinates analysis for treatment alone, each treatment group appeared scattered across each coordinate (Figure 35). Similar to PCoA by sample day, the coordinates for treatment group alone accounted for very little diversity, with PC1 equal to 10.55%, PC2 equal to 9.05%, and 5.06% for PC3, thus accounting for less than 25% of the total diversity. However, while the Boxplot of the Bray-Curtis dissimilarity showed all the groups had a distance near 0.9, the combined tylosin/DFM group had a slightly larger boxplot indicating much more variance (Figure 36). After performance of a pairwise ANOSIM, this increase was not determined to be significant, indicating the tylosin/DFM treatment group was not significantly different than any of the other treatment groups ($P>0.05$, $Q=0.923$) (Table 6).

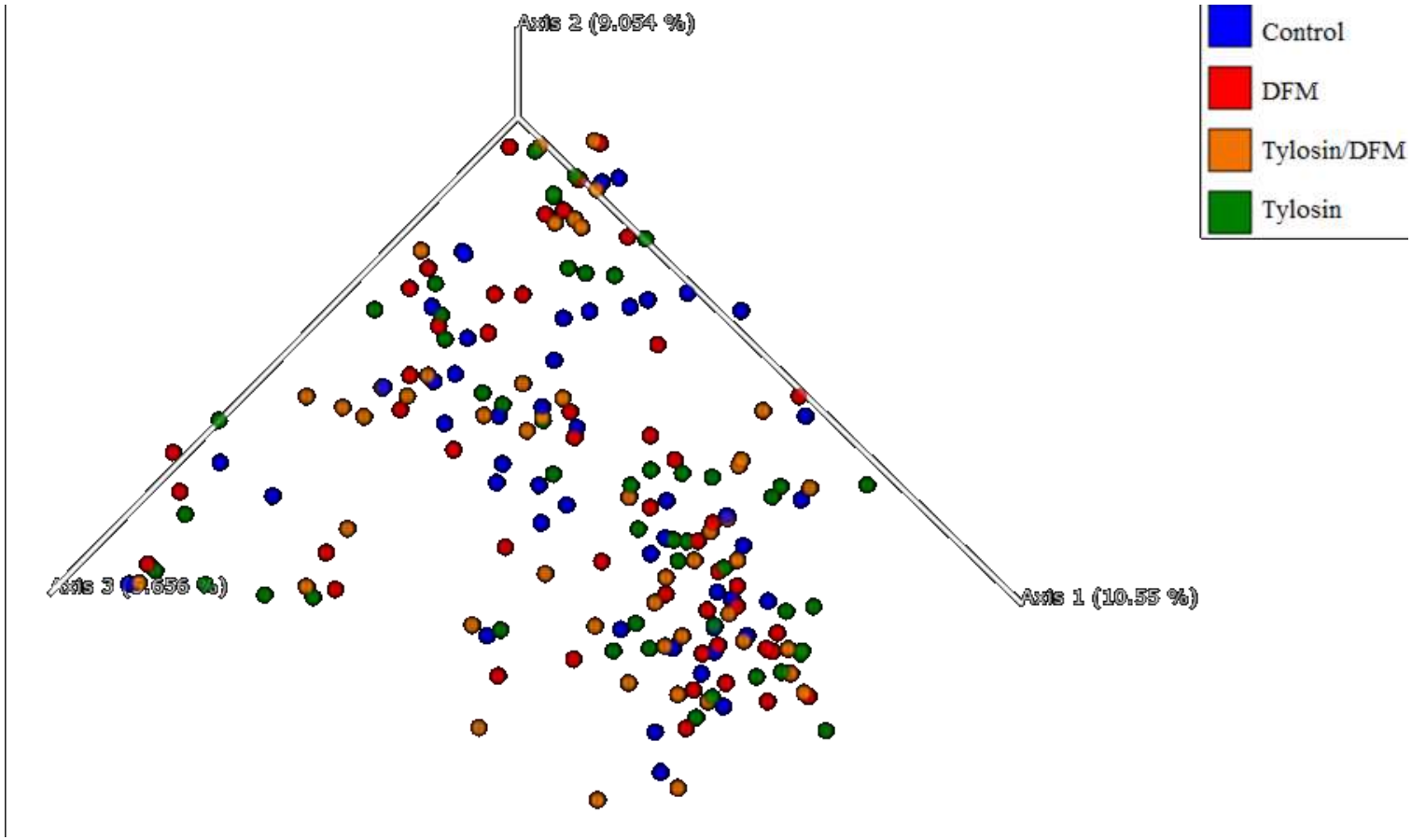


Figure 35 Beta-diversity principal coordinates analysis of the quantified dissimilarity between all samples by treatment measured by Bray-Curtis index.

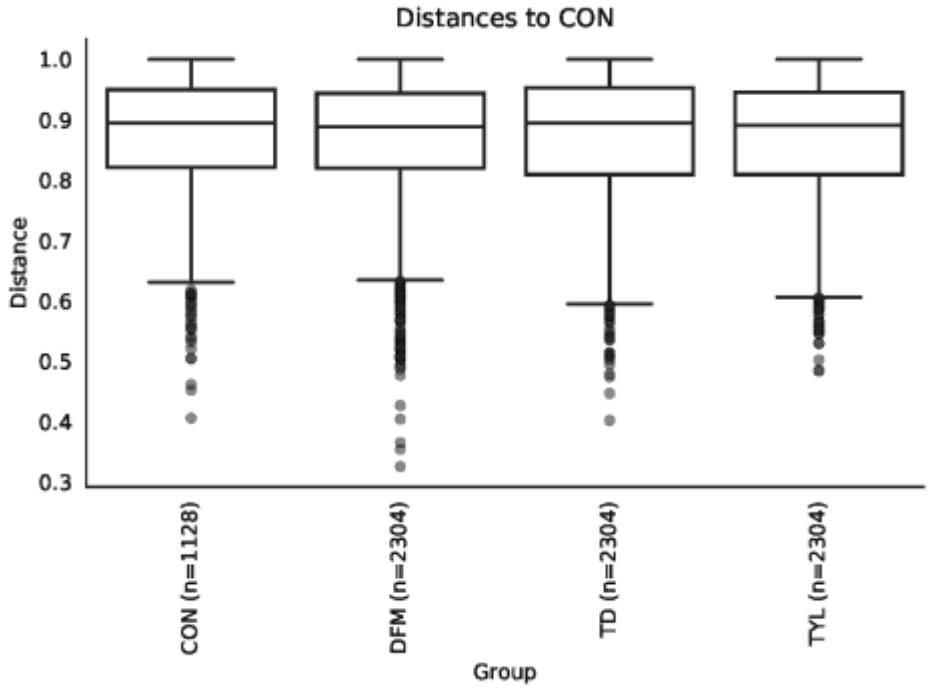


Figure 36 Boxplot of median, range and interquartile range of Bray-Curtis dissimilarity index of all replicate fecal samples by treatment group.

anosim-pairwise all reps txt

Group 1	Group 2	Sample size	Permutations	R	p-value	q-value
CON	DFM	96	999	-0.009958782752166950	0.828	0.923
CON	TD	96	999	-0.007173578789893570	0.702	0.923
CON	TYL	96	999	-0.011727215080772300	0.891	0.923
DFM	TD	96	999	-0.0048903156397753700	0.582	0.923
DFM	TYL	96	999	-0.012550251797675400	0.887	0.923
TD	TYL	96	999	-0.013375404784771400	0.923	0.923

Table 6 Analysis of similarity (ANOSIM) pairwise comparison of all replicate fecal samples by treatment.

Restricting the analysis to Day 84, at the peak of expected effects of tylosin treatment, showed very few patterns across treatment group in the principal coordinates analysis, with treatments scattered across all coordinates (Figure 37). Similar to the PCoA for all samples, very little of the diversity was accounted for; 16.36% for PC1, 10.06% for PC2, and 6.15% for PC3, a sum of 32.56% of the total diversity. The tylosin group appeared to have a lower median distance of 0.875 and a wider confidence interval, indicating more variance but less group similarity when compared to the control group (Figure 38). This was not reflected in the pairwise ANOSIM output; in fact, there were no significant differences among treatment groups when restricting the analysis to Day 84 ($P>0.05$, $Q=0.936$) (Table 7).

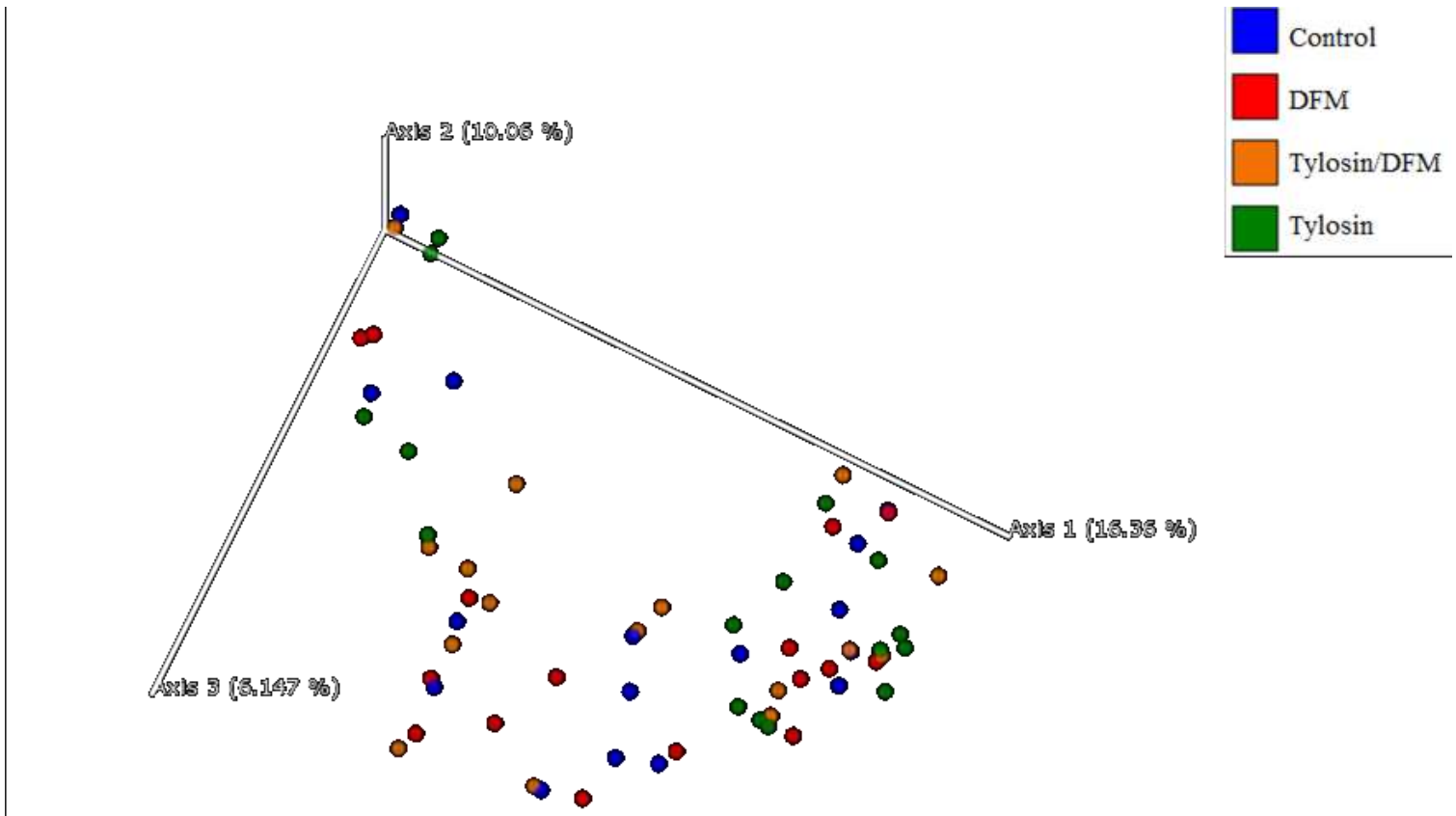


Figure 37 Beta-diversity principal coordinates analysis of of the quantified dissimilarity between only Day 84 samples by treatment measured by Bray-Curtis index analyzed by trial replicate.

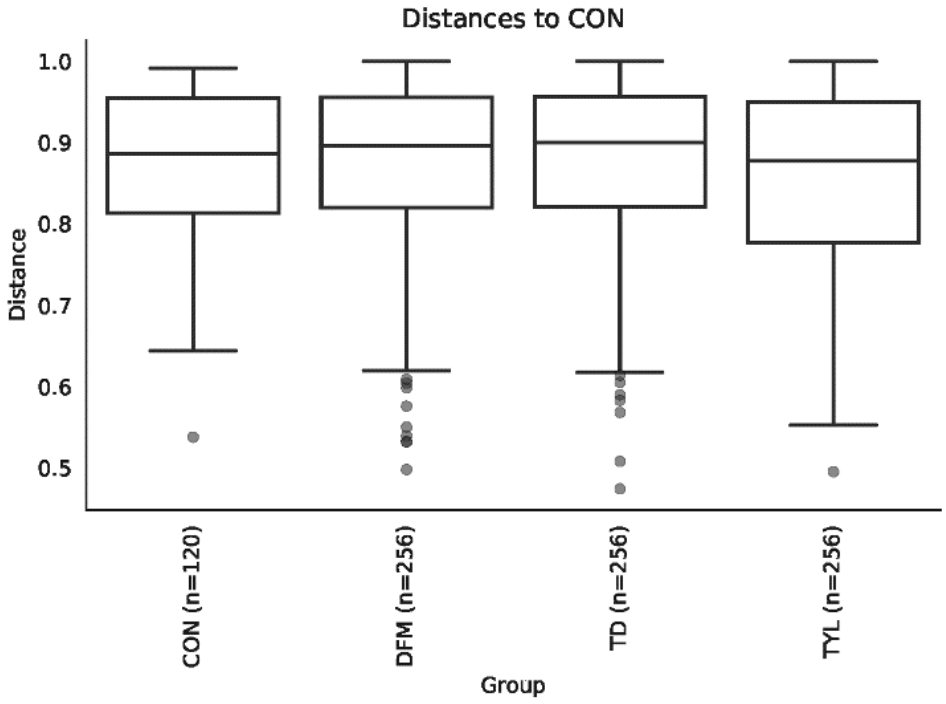


Figure 38 Boxplot of median, range and interquartile range of Bray-Curtis dissimilarity index of Day 84 fecal samples by treatment

anosim-pairwise d84

Group 1	Group 2	Sample size	Permutations	R	p-value	q-value
CON	DFM	32	999	-0.025195312500000000	0.719	0.936
CON	TD	32	999	-0.033203125	0.833	0.936
CON	TYL	32	999	0.005533854166666710	0.344	0.936
DFM	TD	32	999	-0.04641927083333330	0.936	0.936
DFM	TYL	32	999	-0.019205729166666700	0.64	0.936
TD	TYL	32	999	-0.02965494791666660	0.766	0.936

Table 7 Analysis of similarity (ANOSIM) pairwise comparison of Day 84 fecal samples by treatment.

Discussion

There were no major discernable differences in taxa by sample day or treatment visualized in the bar plot of taxa (Figure 29). This was reflected in the Shannon diversity index, showing no statistically significant differences in alpha diversity for any of the treatment groups, albeit with replicate contributing significantly to the variance. This indicates balanced microbes among the treatment groups, without any particular families dominating in the pens or treatment groups (Figure 30).

There was a trend for temporal differences in the PCoA plot, as most sample days tended to cluster together (Figure 33). This was reflected in the ANOSIM results, with each sample day being significantly different than those surrounding it (Figure 34, Table 5). This agrees with previous reports, such as by Jami *et al.*, which suggested that as cattle age, their diversity and within-group similarity increases, indicating a more diverse but more stable bacterial gut population (Jami *et al.*, 2013). Additionally, Shanks *et al.* noted that the transition from a forage-based diet to a grain-based diet was a contributing factor to changes in the gut microbiome, which was perhaps less present in this study (Shanks *et al.*, 2011) though not entirely. Cattle placed on the trial in the first replicate were younger members of the same birth cohort as were later placed in the second trial replicate. In addition to the age differences between the two trial replicates, the access and type of forage in pastures available to the cattle grazing before placement would have been distinctly different between March and July.

Another factor to consider was the relatively low proportion of enterococci present the fecal microbiome (though common to many studies of mammals); therefore, it is

possible that there was a significant effect on enterococci, and increased enterococci in the gut from the DFM product, but this did not have a measurable effect using a total microbiome analysis. On Day 84, at the peak of treatment effects, there were again very few differences in microbial abundances according to the Bray-Curtis dissimilarity index (Figure 38, Table 7). This was also in agreement with previous data concerning the effect of tylosin on the gut microbiome, as Weinroth *et al.* likewise found no significant effects of tylosin on the cattle microbiome (Weinroth *et al.*, 2019).

This may be unique to tylosin as an antibiotic, as Holman *et al.* noted a significant change in the fecal microbiota after administration of oxytetracycline and tulathromycin (Holman *et al.*, 2019). However, oxytetracycline and tulathromycin injections were also administered at 20mg/kg and 2.5mg/kg respectively, which for their average weight of 300 kg would equal 6000 mg (or 6g) of oxytetracycline and 750mg (or 0.75g) of tulathromycin per steer. This is relatively higher than our dose of 8.8 mg/tonne of feed, of which steers only ate an average of 8.6 kilograms per day. Lastly, there were significant differences in the beta diversity of the probiotic from the first replicate and the second. While this was perhaps unexpected, since the probiotic is a live culture and not lyophilized, it is possible that improper storage caused contamination, or else the variability from lot to lot of the probiotic product was not consistent. Winter versus summer temperatures in central Texas could also pose challenges to the consistency of a live-culture product, regardless of adherence to sterile conditions, allowing minor components of the probiotic mixture to dominate at the expense of our preferred source.

Conclusions

This lack of significant differences among treatment group could well be interpreted as a beneficial discovery, demonstrating that no matter what supplementation is given, or lack thereof, there was no negative effect on the fecal microbiome. The robustness of the cattle enteric microbiome, and its imperviousness to outside stimuli, could be worthwhile for further exploration of probiotic effects on resistance without upsetting the balance of microbial diversity and causing dysbiosis. Further research into the extended impacts of a probiotic on the microbiome could be beneficial, in order to determine if probiotic supplementation throughout the entire feeding period would have any discernable effect on microbial ecology and diversity. Additionally, with the significant differences found in the DFM sample from each replicate, this may account for the disagreement in phenotypic and genotypic results from Replicate 1 and Replicate 2.

CHAPTER VII

SUMMARY AND CONCLUSIONS

In our first study, we evaluated the use of supra-nutritional zinc, menthol and their combination, on prevalence and resistance of *Escherichia coli* and *Enterococcus spp*. in feedlot cattle. We examined the ecological abundance of resistance among these bacteria through $\log_{10}$ CFU per gram of feces, and isolate-based antimicrobial susceptibility and minimum inhibitory concentrations through microbroth dilution. While the results of both of these studies do not definitively demonstrate a causal link between antibiotic resistance and non-antibiotic antimicrobial use (i.e., alternatives), or co-selection with non-antibiotic antimicrobials, there are some associations that should be regarded with caution.

The association of menthol feeding with an increase in tetracycline-resistant *E. coli* prevalence found in previous literature has been supported, though the observed association was not significant ($P>0.05$) in our study. Additionally, the link between heavy metals and antibiotic resistance, explored using zinc in this study, was also supported with an observed increased resistance among enterococci to macrolides, both quantitatively with the $\log_{10}$ CFU per gram of feces growth, and phenotypically among isolates. Further research and studies on these samples should be performed in order to explore the genotypic population dynamics underlying the observed phenotypic phenomona. For instance, explorations using quantitative PCR on community DNA looking at the prevalence of *tet* family genes, including *tet*(L), *tet*(M), *tet*(A) and *tet*(B), could be used to determine if there is a significant association with any treatment groups. Similar studies could be done for macrolide resistance genes such as *ermA* and *ermB*. These would further aid in confirming

findings from phenotypic studies and to help explain potential co-selection. Resistance elements are less-well characterized for metals and especially for essential oils though metal resistance genes could be included in such a qPCR experiment.

In our second study, we evaluated the effects of a macrolide-susceptible DFM, tylosin, and environmental pen change on the prevalence and resistance of *Enterococcus spp*, the genotypic patterns and associations of MLST type with sample day, treatment, and resistance genes present in *E. faecium*, and the ecological diversity of the microbiome over time when subjected to the described treatments. The resistance abundance was explored through $\log_{10}$ CFU per gram of feces on plain and antibiotic supplemented agar, and the minimum inhibitory concentrations and resistance of isolates were studied using microbroth dilution among isolates to estimate multidrug and single phenotypic resistance. Genotypic data, including resistance genes, MLST type, and phylogeny, were explored using Next Generation Whole Genome Sequencing via the Illumina Flex Kit and Illumina MiSeq. The metagenomic diversity of the microbiome was studied using 16S metagenomic sequencing, also performed using the Illumina MiSeq.

In the first replicate, tylosin feeding was significantly associated ($P<0.05$) with an increased prevalence of erythromycin resistant enterococci, also illustrated by a significant ($P < 0.05$) decrease in the difference between plain and erythromycin-supplemented agar on Day 84 compared to Day 0, and in comparison to the DFM treatment group. After tylosin was withdrawn and half the cattle were moved to new pens, by the time of slaughter for the first replicate the tylosin group was no longer significantly ($P > 0.05$) different from the other treatment groups in terms of erythromycin-resistant enterococci; however, it was still

significantly increased compared to its own respective Day 0 baseline levels. When both trial replicates were combined, this pattern was still present but the significance was lost ($P > 0.05$). The scale of differences in growth on plain versus erythromycin-supplemented media changed substantially for Day 0 when comparing Replicate 1 to Replicate 2.

This pattern was also observed among the *Enterococcus spp.* isolates, with significantly more phenotypic resistance to macrolides prevalent among cattle groups fed tylosin on day 84 when compared to day 0. Once again, this difference was no longer significant ($P > 0.05$) at slaughter, 4 weeks after the withdrawal of tylosin. This trend of increased resistance after 84 days of feeding tylosin, then decreased resistance following the product withdrawal is supported by previous literature, specifically by Beukers *et al.* (2015), showing a withdrawal of tylosin prior to slaughter may contribute to a decrease in macrolide resistant enterococci.

Of note, this present study illustrates a novel concept, that a macrolide-susceptible probiotic, when fed in combination with tylosin, appears to attenuate the undesired effects of feeding tylosin on enterococci resistance. This effect was demonstrated through the lack of significant differences in prevalence of erythromycin-resistant enterococci in the combined DFM/tylosin group when compared to the control group in Replicate 1. Notably, even at the expected peak of treatment effects at Day 84 there was no significant difference in the prevalence of erythromycin resistant enterococci in the group fed DFM and tylosin compared to Day 0 in Replicate 1.

The probiotic product also showed promise for attenuating or mitigating resistance at the genotypic level, as shown by the increased presence of ST296, the sole sequence type

found in the DFM, and only detected in cattle feces and the enviornment after supplementation with the DFM began. The appearance of ST296 occurred in conjunction with an observed decrease in the prevalence of ST240, a sequence type associated with both of the resistance genes *ermB* and *tet*(M). The ST296 isolates found in cattle feces were also shown to be highly related to the sequenced ST296 isolated from the DFM product.

Unfortunately, ST296 was only found in Replicate 1 fecal samples. However, it was also found in a pen-environment sample from the slaughter date in rRplicate 2, a sample which had been dried and milled to simulate harsh and dry environmental conditions. This latter finding suggests that the DFM strains could cycle into the microbial community, and over time, work to combat resistance by integrating into the fecal-environmental-oral microbial cycle.

The DFM does not appear to have upset or to have changed the microbial ecological diversity, as shown by the results of 16s metagenomic sequencing. There were no significant differences or variances in the alpha- or beta-diversity in regard to treatments, even at the height of treatment on day 84. The single significant effect on on the metagenomics in fecal sample beta diversity was based on sample day (regardless of treatment), indicating temporal effects were the only important factor in changing the microbiome in beef cattle at the finishing stage. Additionally, the significant difference in the beta diversity variance between the DFM samples from the first and second replicate may account for the differences in prevalence and resistance seen between replicates, as the DFM product from the first replicate had very low variance, whereas the variance from the

DFM product in the second replicate was much larger, possibly due to contamination or improper storage.

Therefore, adding a macrolide-susceptible probiotic in combination with tylosin may assist in attenuating or mitigating levels of antibiotic resistance, and therefore reduce potential downstream impacts on human health. This would allow for the continued use of tylosin to reduce the incidence and severity of abscessed livers and dampen their economic impacts. Further research exploring the quantitative effects of this field trial design on resistance genes at a community level could be of use, if only to further confirm this thesis. Quantitative PCR performed on pen-pooled fecal community DNA, using primers for the resistance genes *ermB* and *tet*(M), and compared to the overall bacterial DNA through 16S rRNA gene quantification, would help to explore the overall resistance gene prevalence among all bacteria and not just the trends present in specific subsets of enterococci.

In conclusion, the jury is still out on the appropriateness of zinc and menthol as suitable alternatives to antibiotics in beef cattle, based on the results from this study combined with previous literature. However, the withdrawal of tylosin prior to slaughter, in combination with use of a macrolide-susceptible probiotic and pen environmental change, appears to have a promising effect in decreasing the prevalence of erythromycin resistance among enterococci. Furthermore, as shown in this study, supplementing cattle feed with a macrolide-susceptible probiotic that can survive harsh environmental conditions, and therefore can circulate and propagate both in the host and the environment, and thus cycle back into beef cattle, may help to reduce the prevalence of macrolide resistant enterococci in beef cattle. Additionally, the probiotic – when used alone and in combination with tylosin –

did not negatively affect the cattle microbiome or cause dysbiosis. Therefore, supplementation with a macrolide-susceptible probiotic in combination with tylosin, combined with antibiotic withdrawal prior to slaughter, and moving cattle into pens where antibiotics are not used, is a viable option to decrease the prevalence of antibiotic resistant enterococci without adversely affecting cattle health and productivity.

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APPENDIX

Resistance genes and Multi-Locus Sequence Types (MLST)

Sample Day	Pen	Lab ID	Media Origin	MLST	*erm A*	*erm B*	*msr C*	*optr A*	*spc*	*tet* (L)	*tet* (M)
D0	4	134	ME2	Unknown			■				
D84	7	433	MEery1	Unknown		■	■			■	■
D84	7	433	ME2	Unknown		■	■			■	■
D84	7	433	MEery2	Unknown		■	■			■	■
D112	1	723	ME2	Unknown			■				
D112	7	755	MEery1	Unknown	■		■	■	■		■
D112	8	759	MEery1	Unknown		■	■			■	■
D112	14	794	MEery2	Unknown		■	■			■	■
D112	14	794	MEery1	Unknown		■	■			■	■
D112	14	796	MEery1	Unknown		■	■			■	■
D112	15	804	MEery1	Unknown		■	■			■	■
D112	15	804	MEery2	Unknown		■	■			■	■
D0	6	876	MEery1	Unknown		■					■
D0	6	878	MEery1	Unknown		■	■			■	■
D84	2	1122	ME1	Unknown			■				
D84	3	1128	ME2	Unknown			■			■	■
D84	5	1157	MEery1	Unknown		■	■			■	■
D84	7	1171	MEery1	Unknown			■			■	
D84	8	1191	ME2	Unknown			■			■	■
D119	4	1597	ME1	Unknown			■				
D119	4	1598	ME1	Unknown			■				
D119	7	1616	ME1	Unknown			■				
D119	9	1629	ME2	Unknown			■				
D119	9	1632	MEery1	Unknown		■	■			■	■
D119	12	1647	ME1	Unknown			■				
D119	12	1650	MEery1	Unknown		■	■				■

Table 8 Resistance genes of unknown *Enterococcus faecium* ST types as determined via whole-genome sequencing. Shaded boxes represent strains in which the respective genes were detected. Media origin indicates agar type and isolate number (ME1= first isolate from plain m-*Enterococcus* agar, MEery2 =second isolate from erythromycin supplemented m-*Enterococcus* agar)

Sample Day	Pen	Lab ID	Media Origin	MLST	*aad E*	*erm B*	*lnu A*	*lnu B*	*msr C*	*tet* (L)	*tet* (M)	*vat E*
D0	1	100	ME1	604								
D0	1	100	ME2	604								
D0	2	105	ME1	1036								
D0	3	119	ME2	1258								
D0	4	135	ME2	328								
D0	4	135	ME1	328								
D0	6	156	ME2	92								
D84	5	409	MEery2	540								
D84	7	430	MEery2	957								
D84	7	430	MEery1	957								
D112	2	729	MEery1	178								
D112	12	751	ME1	717								
D112	7	754	MEery2	957								
D0	3	837	ME1	22								
D0	7	894	ME1	27								
D84	3	1131	ME2	936								
D84	5	1147	ME2	717								
D84	7	1172	MEery1	178								
D119	4	1598	MEery1	100								
D119	6	1613	MEery1	540								
D119	12	1646	ME2	107								
D119	12	1648	ME1	1442								

Table 9 Resistance genes of minority *Enterococcus faecium* ST types as determined via whole-genome sequencing. Shaded boxes represent strains in which the respective genes were detected. Media origin indicates agar type and isolate number (ME1= first isolate from plain m-*Enterococcus* agar, MEery2 =second isolate from erythromycin supplemented m-*Enterococcus* agar)

Sample Day	Pen	Lab ID	Media Origin	MLST	*msrC*	*tet*(M)
D0	3	119	ME1	108		
D0	3	840	ME1	108		
D84	1	1100	ME2	108		
D84	1	1102	ME2	108		
D84	1	1102	ME1	108		
D84	4	1137	ME1	108		
D119	6	1609	ME2	108		
D119	8	1623	ME1	108		
D119	8	1623	ME2	108		
D119	11	1641	ME1	108		

Table 10 Resistance genes of *Enterococcus faecium* ST108 types as determined via whole-genome sequencing. Shaded boxes represent strains in which the respective genes were detected. Media origin indicates agar type and isolate number (ME1= first isolate from plain m-*Enterococcus* agar, ME2 =second isolate from m-*Enterococcus* agar).

Sample Day	Pen	Lab ID	Media Origin	MLST	*aadD*	*aph2*	*ermB*	*fexA*	*optrA*	*tet (L)*	*tet (M)*
D84	4	395	MEery1	212							
D112	6	751	MEery2	212							
D84	2	1120	ME1	212							
D84	5	1156	MEery1	212							
D84	5	1156	ME2	212							
D84	6	1162	MEery1	212							
D84	6	1168	MEery1	212							
D119	6	1609	MEery1	212							
D119	6	1614	MEery1	212							
D119	13	1655	MEery1	212							

Table 11 Resistance genes of *Enterococcus faecium* ST212 types as determined via whole-genome sequencing. Shaded boxes represent strains in which the respective genes were detected. Media origin indicates agar type and isolate number (ME1= first isolate from plain m-*Enterococcus* agar, MEery2 =second isolate from erythromycin supplemented m-*Enterococcus* agar).

Sample Day	Pen	Lab ID	Media Origin	MLST	*ermB*	*lnuA*	*msrC*	*tet(L)*	*tet(M)*
D0	4	124	ME1	32					
D112	7	753	MEery2	32					
D0	8	903	ME2	32					
D0	8	903	ME1	32					
D84	1	1099	ME2	32					
D84	1	1109	ME2	32					
D84	1	1109	ME1	32					
D119	11	1642	ME1	32					

Table 12 Resistance genes of *Enterococcus faecium* ST32 types as determined via whole-genome sequencing. Shaded boxes represent strains in which the respective genes were detected. Media origin indicates agar type and isolate number (ME1= first isolate from plain m-*Enterococcus* agar, MEery2 =second isolate from erythromycin supplemented m-*Enterococcus* agar).

Sample Day	Pen	LabID	Media Origin	MLST	*ermB*	*msrC*	*tet(M)*	*vatE*
D84	5	409	MEery1	94				
D112	2	726	MEery1	94				
D84	3	1127	MEery1	94				
D84	5	1153	ME2	94				
D84	6	1164	MEery1	94				
D119	1	1584	MEery1	94				
D119	6	1613	ME1	94				
D119	12	1647	MEery1	94				

Table 13 Resistance genes of *Enterococcus faecium* ST94 types as determined via whole-genome sequencing. Shaded boxes represent strains in which the respective genes were detected. Media origin indicates agar type and isolate number (ME1= first isolate from plain m-*Enterococcus* agar, MEery2 =second isolate from erythromycin supplemented m-*Enterococcus* agar).

Sample Day	Pen	Lab ID	Media Origin	MLST	*msrC*	*tet*(S)
112	7	753	ME1	1216		
112	7	755	ME2	1216		
112	7	756	ME1	1216		
0	2	834	ME1	1216		
0	3	835	ME2	1216		
0	4	851	ME1	1216		
0	4	853	ME1	1216		
84	6	1160	ME2	1216		
84	8	1190	ME2	1216		
119	4	1599	ME1	1216		
119	4	1601	ME1	1216		

Table 14 Resistance genes of *Enterococcus faecium* ST1216 types as determined via whole-genome sequencing. Shaded boxes represent strains in which the respective genes were detected. Media origin indicates agar type and isolate number (ME1= first isolate Media origin indicates agar type and isolate number (ME1= first isolate from plain m-*Enterococcus* agar, ME2 =second isolate from m-*Enterococcus* agar).

Sample Day	Pen	Lab ID	Media Origin	MLST	*aadE*	*ermB*	*lnuB*	*msrC*	*tet (L)*	*tet (M)*
D0	2	107	MEery1	240		■		■	■	■
D0	3	120	ME1	240		■		■	■	■
D0	3	120	ME2	240				■	■	■
D0	3	120	MEery1	240		■		■	■	■
D0	3	120	MEery2	240		■		■	■	■
D0	3	121	MEery1	240		■		■	■	■
D0	4	131	MEery1	240		■		■	■	
D0	4	133	ME1	240			■	■	■	■
D0	4	133	ME2	240			■	■	■	■
D0	4	134	ME1	240				■	■	■
D0	4	135	MEery1	240		■		■	■	■
D0	7	165	ME1	240			■	■	■	■
D84	1	363	MEery2	240		■		■		■
D84	2	374	ME1	240		■		■	■	
D84	2	374	MEery1	240		■		■	■	■
D84	2	374	MEery2	240		■		■	■	■
D84	2	375	ME2	240		■		■	■	■
D84	2	380	MEery2	240		■		■		■
D84	3	386	MEery1	240		■		■		■
D84	3	388	MEery1	240		■		■	■	■
D84	3	388	MEery2	240		■		■	■	■
D84	4	393	ME1	240		■		■		■
D84	4	393	MEery1	240		■		■		■
D84	4	398	MEery1	240		■		■	■	
D84	4	398	MEery2	240		■		■	■	
D84	4	400	MEery1	240		■		■		■
D84	4	401	ME1	240		■		■		■
D84	4	401	MEery1	240		■		■		■
D84	4	402	MEery1	240		■		■		■
D84	4	402	MEery2	240		■		■		■
D84	4	404	ME1	240		■		■	■	■
D84	4	404	MEery1	240		■		■		■

Table 15 Resistance genes of *Enterococcus faecium* ST240 types as determined via whole-genome sequencing. Shaded boxes represent strains in which the respective genes were detected. Media origin indicates agar type and isolate number (ME1= first isolate from plain m-*Enterococcus* agar, MEery2 =second isolate from erythromycin supplemented m-*Enterococcus* agar).

Sample Day	Pen	Lab ID	Media Origin	MLST	*aadE*	*ermB*	*lnuB*	*msrC*	*tet (L)*	*tet (M)*
D84	4	404	MEery2	240						
D84	5	408	MEery1	240						
D84	5	408	MEery2	240						
D84	5	411	MEery1	240						
D84	5	414	ME1	240						
D84	5	414	MEery1	240						
D84	5	414	MEery2	240						
D84	5	416	ME2	240						
D84	5	416	MEery2	240						
D84	6	421	MEery2	240						
D84	6	422	ME1	240						
D112	2	728	ME1	240						
D112	2	728	ME1	240						
D112	2	729	MEery1	240						
D112	2	729	MEery2	240						
D112	4	741	MEery1	240						
D112	4	741	MEery2	240						
D112	5	744	MEery1	240						
D112	6	749	ME1	240						
D112	6	749	ME1	240						
D112	6	749	MEery1	240						
D112	6	751	MEery1	240						
D112	9	763	MEery1	240						
D112	9	765	MEery1	240						
D112	11	779	ME1	240						
D112	11	779	ME1	240						
D112	11	779	ME2	240						
D112	11	779	ME2	240						
D112	12	785	ME1	240						
D0	1	811	MEery1	240						
D0	1	814	MEery1	240						
D0	1	818	MEery1	240						
D0	1	820	MEery1	240						
D0	2	824	MEery1	240						
D0	2	830	MEery1	240						
D0	2	831	MEery2	240						

Table 15 Continued

Sample Day	Pen	Lab ID	Media Origin	MLST	*aadE*	*ermB*	*lnuB*	*msrC*	*tet (L)*	*tet (M)*
D0	2	833	MEery1	240						
D0	3	836	ME1	240						
D0	3	837	MEery1	240						
D0	3	841	MEery1	240						
D0	4	851	MEery2	240						
D0	4	857	ME1	240						
D0	5	860	ME1	240						
D0	5	860	MEery1	240						
D0	5	863	MEery1	240						
D0	5	870	MEery1	240						
D0	6	871	MEery1	240						
D0	6	873	ME2	240						
D0	6	875	ME1	240						
D0	7	888	MEery1	240						
D0	7	888	MEery2	240						
D0	7	889	MEery1	240						
D0	7	890	ME1	240						
D0	7	890	MEery1	240						
D0	8	899	MEery1	240						
D0	8	900	MEery1	240						
D0	8	905	MEery2	240						
D84	2	1111	ME2	240						
D84	2	1115	ME2	240						
D84	2	1117	ME1	240						
D84	2	1117	ME2	240						
D84	2	1117	MEery1	240						
D84	2	1119	ME1	240						
D84	2	1119	MEery1	240						
D84	2	1121	ME1	240						
D84	2	1121	MEery1	240						
D84	2	1121	MEery2	240						
D84	2	1122	MEery1	240						
D84	3	1124	MEery1	240						
D84	3	1133	ME2	240						
D84	4	1137	MEery1	240						
D84	4	1144	ME1	240						

Table 15 Continued

Sample Day	Pen	Lab ID	Media Origin	MLST	*aadE*	*ermB*	*lnuB*	*msrC*	*tet (L)*	*tet (M)*
D84	4	1145	ME1	240						
D84	4	1145	MEery1	240						
D84	6	1159	MEery1	240						
D84	6	1163	ME2	240						
D84	6	1163	MEery1	240						
D84	7	1171	ME1	240						
D84	7	1171	ME1	240						
D84	7	1175	MEery1	240						
D84	7	1176	MEery1	240						
D84	7	1177	MEery1	240						
D84	7	1178	MEery1	240						
D84	7	1181	MEery1	240						
D84	8	1185	MEery1	240						
D84	8	1190	MEery1	240						
D119	1	1581	MEery1	240						
D119	1	1582	ME1	240						
D119	1	1582	MEery1	240						
D119	2	1586	ME2	240						
D119	2	1586	MEery1	240						
D119	3	1591	ME1	240						
D119	3	1591	MEery1	240						
D119	11	1642	MEery1	240						
D119	14	1658	ME1	240						
D119	14	1658	ME2	240						
D119	14	1658	MEery1	240						
D119	15	1663	ME2	240						
D119	15	1663	MEery1	240						
D119	15	1664	MEery1	240						
D119	15	1666	MEery1	240						

Table 15 Continued

Sample Day	Pen	Lab ID	Media Origin	MLST	*msrC*
D84	1	369	ME2	296	
D84	2	374	ME2	296	
D84	2	378	ME2	296	
D84	2	381	ME1	296	
D84	3	386	ME2	296	
D84	3	390	ME1	296	
D84	3	392	ME1	296	
D84	3	405	ME2	296	
D84	3	405	ME1	296	
D84	5	409	ME1	296	
D84	6	420	ME2	296	
D84	6	421	ME2	296	
D84	6	421	ME1	296	
D84	6	423	ME2	296	
D84	6	426	ME2	296	
D84	8	443	ME2	296	
D84	8	448	ME2	296	
D84	8	449	ME2	296	
D84	8	450	ME2	296	
D112	2	727	ME1	296	
D112	3	731	ME2	296	
D112	3	734	ME2	296	
D112	6	748	ME2	296	
D112	6	748	ME1	296	
D112	6	751	ME2	296	
D112	7	754	ME2	296	
D112	7	755	ME1	296	
D112	10	771	ME1	296	

Table 16 Resistance genes of *Enterococcus faecium* ST296 types as determined via whole-genome sequencing. Shaded boxes represent strains in which the respective genes were detected. Media origin indicates agar type and isolate number (ME1= first isolate from plain m-*Enterococcus* agar, MEery2 =second isolate from erythromycin supplemented m-*Enterococcus* agar).

Sample Day	Pen	Lab ID	Media Origin	MLST	*msrC*
D112	11	775	ME2	296	
D112	13	787	ME1	296	
D112	13	787	ME2	296	
D112	14	794	ME1	296	
D112	14	795	ME2	296	
D112	14	795	ME1	296	
D112	14	797	ME1	296	
D112	14	798	ME2	296	
D112	15	802	ME2	296	
D112	16	806	ME1	296	
D112	16	806	ME2	296	
D112	16	809	ME2	296	
D112	16	810	ME2	296	
NA	NA	NA	1_3DFM	296	
NA	NA	NA	3_4DFM	296	
NA	NA	NA	2_3DFM	296	
NA	NA	NA	3_2DFM	296	
NA	NA	NA	3_1DFM	296	
NA	NA	NA	2_2DFM	296	
NA	NA	NA	2_1DFM	296	
NA	NA	NA	1_4DFM	296	
NA	NA	NA	3_3DFM	296	
NA	NA	NA	2_4DFM	296	
NA	NA	NA	1_1DFM	296	
NA	NA	NA	1_2DFM	296	
D119	16	NA	D&MME	296	

Table 16 Continued