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Article

Effects of the antibiotic alternatives zinc and menthol on phenotypic antimicrobial resistance of *E. coli* and *Enterococcus* spp. in beef cattle

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Simple Summary: As antimicrobial resistance increases globally, alternatives to antibiotics are increasingly being investigated as growth promoters, as well as preventive and therapeutic agents, particularly in agriculture. Equally important is the need for investigation into the effect of antibiotic alternatives on antimicrobial resistance and particularly their risk for co-selection, especially among gram-positive and gram-negative indicator organisms. In this study, we explored the prevalence of antibiotic-resistant *E. coli* and *Enterococcus* spp. in cattle fed zinc, menthol, or a combination of the two. We found that zinc supplementation was associated with increased bacterial growth on erythromycin-supplemented agar, with higher levels of macrolide resistance observed among enterococcal isolates.

Abstract: Antimicrobial resistance (AMR) represents a growing crisis in both human and veterinary medicine. We evaluated the use of two categories of antibiotic alternatives – heavy metals and essential oils – in beef cattle, and their effects on gram-negative and gram-positive bacteria. In this randomized 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 exhibited significantly higher macrolide resistance among fecal enterococci isolates.

Keywords: antibiotic resistance; antibiotic alternatives; heavy metals; essential oils

1. Introduction

Antimicrobials have a long history of use in both animals and humans for the prevention, control and treatment of infectious diseases [1-3]. In the 1940s, sulfonamides were found to increase growth in chicks leading to almost seven decades of antimicrobial use for growth promotion (AGP) in food animals. By 1951, a combination vitamin B_{12}/low-dose chlortetracycline product was officially licensed for AGP use in food animals [4-6]. Antibiotics are no longer labeled for growth promotion uses in the United States and most developed countries, prompting research into effective alternatives [7,8]. Some of these alternatives include antimicrobial peptides, probiotics, heavy metals, clay minerals, egg yolk antibodies, essential oils, and recombinant enzymes [9,10]. However, a review by Thacker *et al.* concluded that no currently licensed or approved compounds are equal to antibiotics in efficacy, and most provide inconsistent results [11]. Additionally, there is

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great concern about the possibility of co-selection of antibiotic resistance among antimicrobial alternatives [12].

While research to identify innovative alternatives to antibiotics is necessary, given the current global AMR situation, it is equally important to explore the potential for undesired effects from those alternatives. Heavy metals, including copper and zinc, have been suggested as alternatives to antibiotics and used for growth promotion, as well as disease prevention and control. Zinc oxide (ZnO) fed at supra-nutritional levels has been shown to influence the gut microbiota of weaned piglets in a manner similar to growth-promoting antibiotics; however, differences in average daily gain have not always been shown to be statistically different between treated and untreated groups [13]. It is important to be aware of co-selection potential between tolerance/resistance to heavy metals and antibiotic resistance [14]. Specifically, heavy metal tolerance/resistance and antibiotic resistance genes are often carried on the same mobile genetic elements [12,15,16]. It has been documented that enteric bacterial populations subjected to high levels of copper in feed become more resistant, and copper resistance has been reported in both gram-negative and gram-positive bacteria [17-21]. The link between metal tolerance and antimicrobial resistance, such as for zinc and methicillin in staphylococci (MRSA), or copper and macrolides in enterococci has been reported in Norway [22]. 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 [23,24]. This phenomenon is not restricted to Europe, as swine receiving supra-nutritional zinc in Kansas showed the same co-selection of MRSA in a dose-response relationship [25]. At this point in time, there are no studies reporting on the effects of supranutritional zinc supplemented in the feeder stage of beef cattle production in relation to phenotypic antimicrobial resistance among *E. coli* and *Enterococcus* spp. By studying whether zinc has a similar effect on *E. coli* and enterococci as compared to *Staphylococcus aureus* we can determine if it would be a viable antibiotic alternative in beef cattle.

Another suggested antibiotic alternative is the use of an essential oil such as menthol, origanum, or thymol, among many others. Essential oils have been shown experimentally to be effective against both gram-negative and gram-positive bacteria [26]. 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 [27]. Additionally, menthol has been shown to increase weight gain in poultry [28]. 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 [29]. 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 yields no increase in resistance to many antibiotics among *Escherichia coli* isolates. However, it also was shown to yield increased prevalence of tetracycline-resistant *Escherichia coli* after 30 days in feed [30]. While essential oils have also been suggested as alternatives to tylosin use 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 [31,32]. 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 to antibiotics while assessing potential risks associated with co-selection of antimicrobial resistance.

2. Materials and Methods

2.1 Experimental design

A randomized controlled field trial using a 2x2 factorial design was conducted at Kansas State University (KSU) at the Beef Cattle Research Center and approved by the KSU Institutional Animal

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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 feeding 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.

2.2 Quantification, isolation, speciation

Samples from Day 0 were used as the baseline, and samples from Day 21 were considered the maximum treatment effect time period for analysis (IBC #2017-049 and #2017-021). 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, USA) 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, USA) onto MacConkey agar (Difco, Becton Dickinson Sparks, MD) for quantification of fecal coliforms; specifically, we counted only magenta-colored colonies indicating lactose fermentation and therefore presumptive *Escherichia coli.* This dilution also was spiral plated to MacConkey agar supplemented with tetracycline (Sigma Aldrich, Merck, St. Louis, MO, USA) at 16 milligrams per liter (mg/L) as well as MacConkey agar supplemented with ceftriaxone (Sigma Aldrich, Merck, St. Louis, MO, USA)at 4 mg/L. This same dilution also was spiral-plated to m-*Enterococcus* agar (Difco, Becton Dickinson Sparks, MD) 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 (Sigma Aldrich, Merck, St. Louis, MO, USA) 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, USA).

Two colonies from each plain (i.e., non-antibiotic) agar plate were selected and streaked to tryptic soy agar (TSA) agar with 5% sheep blood (Difco, Becton Dickinson Sparks, MD) 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, USA). 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, USA) 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, USA) was added to each well, including all the sample wells, BTS wells, the 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 (Bruker Daltonik GmbH., Billerica, MA, USA) for reading, using MBT Compass v1.4 software (Bruker

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Daltonik GmbH., Billerica, MA, USA). After confirmation of genus and species, these same confirmed isolates were used for phenotypic susceptibility testing.

2.3 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, USA) to determine minimum inhibitory concentrations (MIC) to arrays of antibiotics suited to gram-negative and gram-positive bacteria. 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(TREK, Thermo Scientific Microbiology, Oakwood Village, OH, USA); finally, 50 µl of the broth culture was inoculated to each well of the U.S. National Antimicrobial Resistance Monitoring System (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, USA). Antibiotics on the CMV3AGPF plate included: chloramphenicol, ciprofloxacin, daptomycin, erythromycin, gentamicin, kanamycin, lincomycin, linezolid, nitrofurantoin, penicillin, quinupristin/dalfopristin, streptomycin, tetracycline, tigecycline, tylosin, and vancomycin (see range of concentrations in 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 (see range of concentrations in Table 2). Three positive control wells and one negative control well 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 with each new serial number or batch of plates. Plates were read using a Sensititre OptiRead™ (TREK, Thermo Scientific Microbiology, Oakwood Village, OH, USA) device. The results were interpreted as susceptible, intermediate, or resistant in accordance with CLSI M100 document guidelines [33], or using the NARMS consensus breakpoints when a CLSI breakpoint was unavailable, via the SWIN software (TREK, Thermo Scientific Microbiology, Oakwood Village, OH, USA) (Table 1, Table 2); later, intermediate results were recoded as susceptible for binary outcome statistical analyses purposes. Minimum inhibitory concentrations (MICs) were plotted using Excel and 95% exact confidence intervals for each of the proportion of resistance among isolates was calculated using Stata® version 16.1 (StataCorp LLC, College Station, TX) to create an integrated table of data and an illustrative figure, known as a "squashtogram".

Antibiotic	Class	Range	Breakpoint
Gentamicin	Aminoglycoside	128-1024	≥500
Kanamycin	Aminoglycoside	128-1024	≥1024
Streptomycin	Aminoglycoside	512-2048	>1000
Vancomycin	Glycopeptide	0.25-32	≥32
Tigecycline	Glycylcycline	0.015-0.5	≥0.5
Lincomycin	Lincosamide	1-8	≥8
Daptomycin	Lipopeptide	0.25-16	≥8
Erythromycin	Macrolide	0.25-8	≥8
Tylosin	Macrolide	0.25-32	≥32
Nitrofurantoin	Nitrofuran	2-64	≥128
Linezolid	Oxazolidinone	0.5-8	≥8
Penicillin	Penicillin	0.25-16	≥16

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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 resistance) for the NARMS gram positive plate (CMV3AGP), 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	0.12/2.38-4/76	≥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 inhibitor combination	1/0.5-32/16	≥32/16

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

2.4 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 the Day 0 sampling was completed.

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 multi-drug resistance count (an integer variable), which also was

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graphed by day and treatment group. This variable was then used to determine multi-drug 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 estimated and plotted by sample day with a 95% confidence interval.

3. Results

3.1 Descriptive statistics

A total of 160 samples, 80 from each sample day, was plated to previously described agars. A total of 320 presumptive *E. coli* isolated from plain MacConkey agar, 160 from Day 0 and 160 from 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 was 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. The single sample which exhibited no growth on tetracycline-supplemented agar was from the combined zinc and menthol group on Day 21. 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. In comparison, concerning the results of growth on tetracycline-supplemented MacConkey agar both the menthol and the combined menthol and zinc groups exhibited a statistically significant ($P < 0.05$) decrease in $\log_{10}$ CFU per gram of feces from 6.06 (95% CI of 5.69 to 6.43) on Day 0 for the menthol group and 6.05 (95% CI of 5.68 to 6.42) for the combined menthol and zinc group, to 5.04 (95% CI of 4.67 to 5.41) for the menthol group and 4.84 (95% CI of 5.68 to 6.42) for the combined zinc and menthol group on Day 21, Additionally, both the control and zinc group decreased, though not significantly ($P> 0.05$) (Figure 1B) over the same period.

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 number. 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 should be interpreted as an increase in the levels of resistance.

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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. These decreases in the difference between plain and tetracycline supplemented agar indicated an increase in resistance during the study period. 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 Day 0 to Day 21, suggesting a decrease in levels of resistance; however, again these results were not significant ($P > 0.05$).

Additionally, growth on ceftriaxone-supplemented MacConkey agar exhibited no significant effects ($P > 0.05$) for day or treatment group. All $\log_{10}$ CFU per gram of feces tended to decrease from Day 0 to Day 21. The samples which did not grow on ceftriaxone-supplemented agar were spread equally 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. 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. There was a tendency across treatment groups for decreased $\log_{10}$ CFU from Day 0 to Day 21, however these were not significant (Figure 1D). 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 the two sampling days to varying degrees, 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 also not significant ($P > 0.05$).

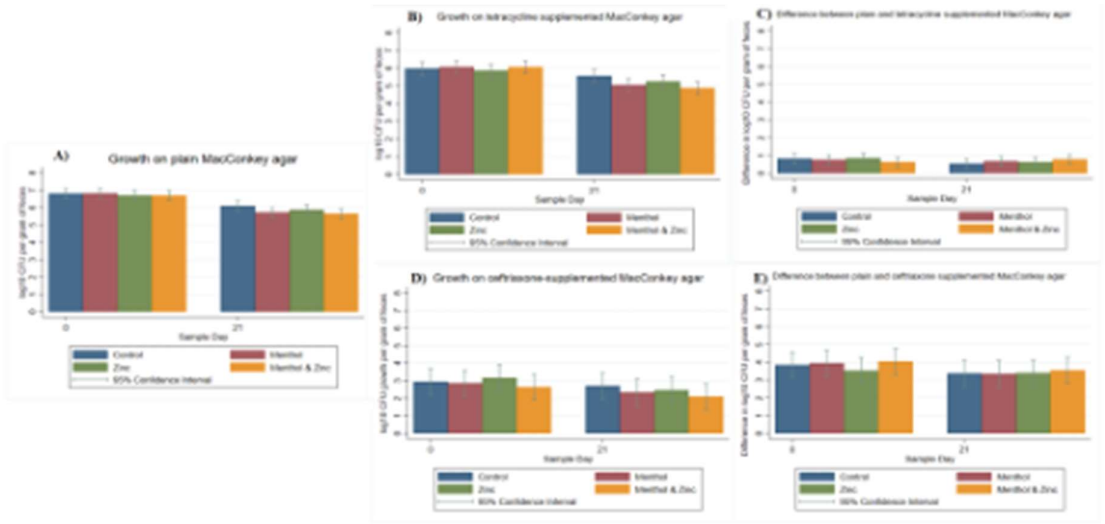

Figure 1 A) $\log_{10}$ CFU per gram of feces on plain MacConkey agar, B) $\log_{10}$ CFU per gram of feces on tetracycline-supplemented (16 mg/L) MacConkey agar, C) Difference in $\log_{10}$ CFU between plain and tetracycline-supplemented (16 mg/L) MacConkey agar, D) $\log_{10}$ CFU per gram of feces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

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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. The treatment groups at Day 21 were not significantly different than their baselines at Day 0. The $\log_{10}$ CFU per gram of feces grown 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 grown on tetracycline-supplemented agar on Day 0, however, this was not significantly different ($P > 0.05$) from the other treatment groups (Figure 2B). By Day 21, there were still no significant differences among treatment groups, and no significant differences compared to Day 0 ($P > 0.05$).

There was a tendency for the difference between plain and tetracycline supplemented agar to decrease from Day 0 to Day 21, thus, proportion of resistance had increased (Figure 2C). The menthol group tended to increase, indicating a decrease in the proportion of resistance, however the changes observed were not significant ($P > 0.05$). 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 significant ($P > 0.05$) 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 and were 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.

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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 an approximate ten-fold increase in erythromycin resistance during that 21-day period. 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 maintained an experiment-wise significant ($P< 0.05$) decrease in difference from Day 0 to Day 21, indicating that an increase in resistance occurred for this group.

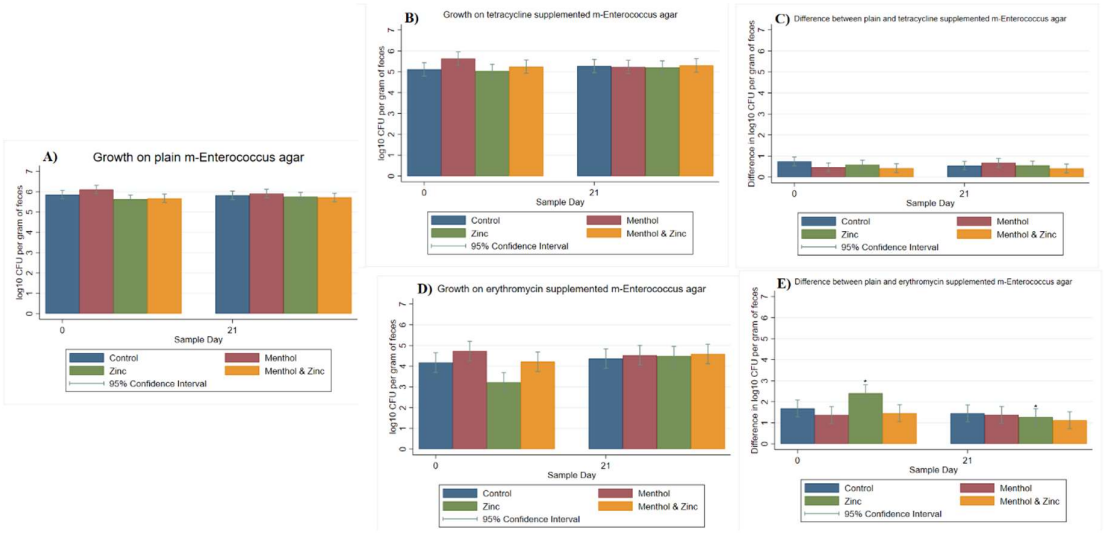

Figure 2 A) $\log_{10}$ CFU per gram of feces on plain m-*Enterococcus* agar, B) $\log_{10}$ CFU per gram of feces on tetracycline-supplemented m-*Enterococcus* agar, C) Difference in $\log_{10}$ CFU between plain and tetracycline-supplemented m-*Enterococcus* agar, D) $\log_{10}$ CFU per gram of feces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

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 versus resistant *E. coli* for gentamicin and nalidixic acid.

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	# 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

2 **Figure 3 Number and percentage (with 95% CI) of *E. coli* isolates that were resistant and the distribution of isolates (%) across the observed minimum inhibitory concentrations (MIC) for each antibiotic. Black vertical lines indicate the human medical interpretive breakpoint CLSI (or, NARMS). Grey boxes indicate unmeasured values above and below highest and lowest limit of assayed antibiotic concentrations, respectively. Isolates which exceeded growth at the highest antibiotic concentration were placed into the next highest MIC column.**

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Similarly, the resistance of *E. coli* isolates to each antibiotic class (Figure 4) by sample day and treatment showed that aminoglycoside resistance (collapsing gentamicin and streptomycin) 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 than for the control group. The menthol group increased from 42.5% isolates resistant on Day 0 to 52.5% on Day 21, 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.

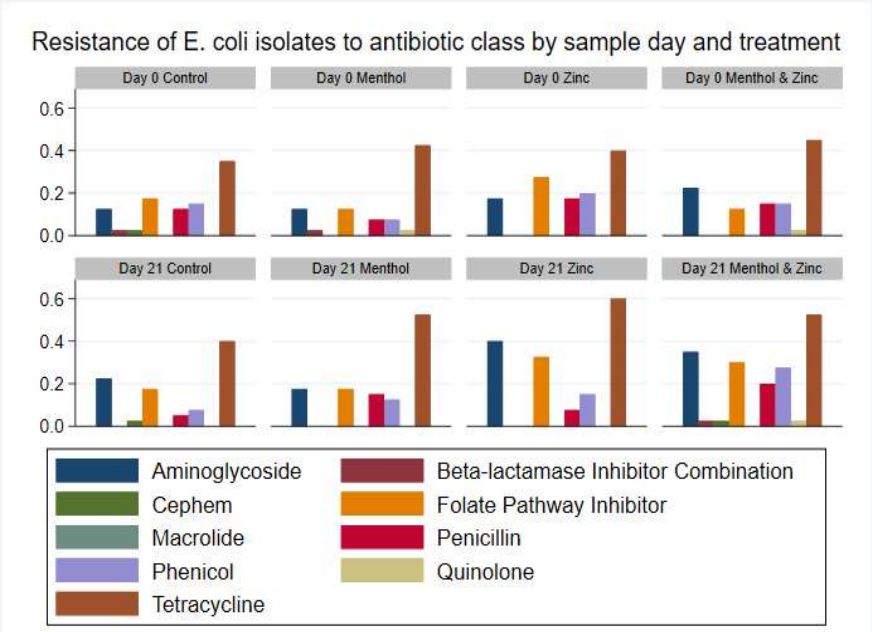

Figure 4 Resistance (proportion) of *E. coli* isolates to each antibiotic class by sample day and treatment

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 Day 0 to Day 21 for the zinc group; however, this increase was not significant (Figure 5. The combined menthol and zinc group also showed an increase in the predicted prevalence of aminoglycoside resistant isolates on Day 0 to Day 21, but these increases were also not significant. The temporal effect of sample day alone showed an increase from Day 0 to Day 21; however, this increase was not significant (P> 0.05).

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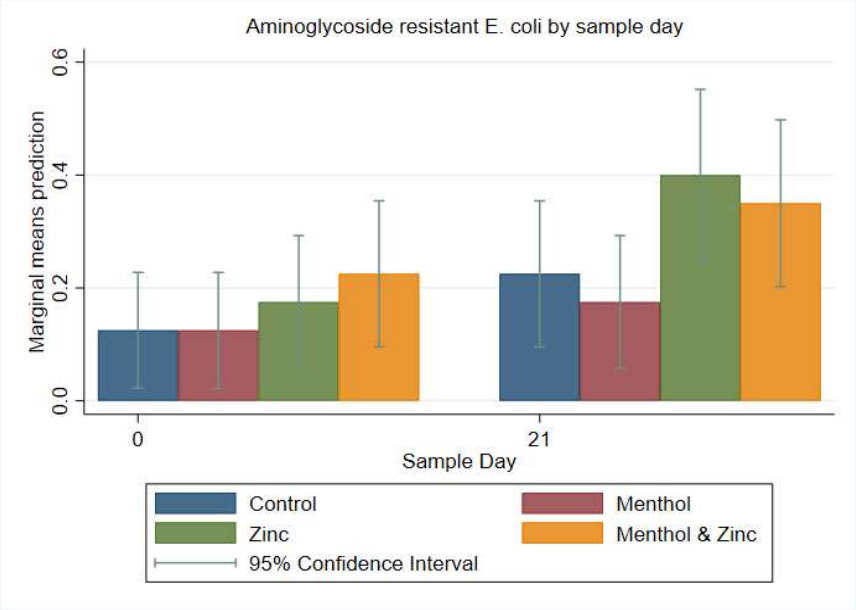

Figure 5 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of aminoglycoside

19 Multi-level mixed effect logistic regression modeling was also performed on the binary outcome of
20 resistance to tetracycline class antibiotics. The predicted prevalence of tetracycline-resistant E. coli
21 tended to increase from Day 0 to Day 21 among all groups. Most notably, the zinc group increased
22 in the predicted prevalence of tetracycline resistant E. coli, from Day 0 to Day 21; however, this
23 increase was not significant. Additionally, sample day alone was not significant in the predicted
24 prevalence of tetracycline resistant E. coli (Figure 6).

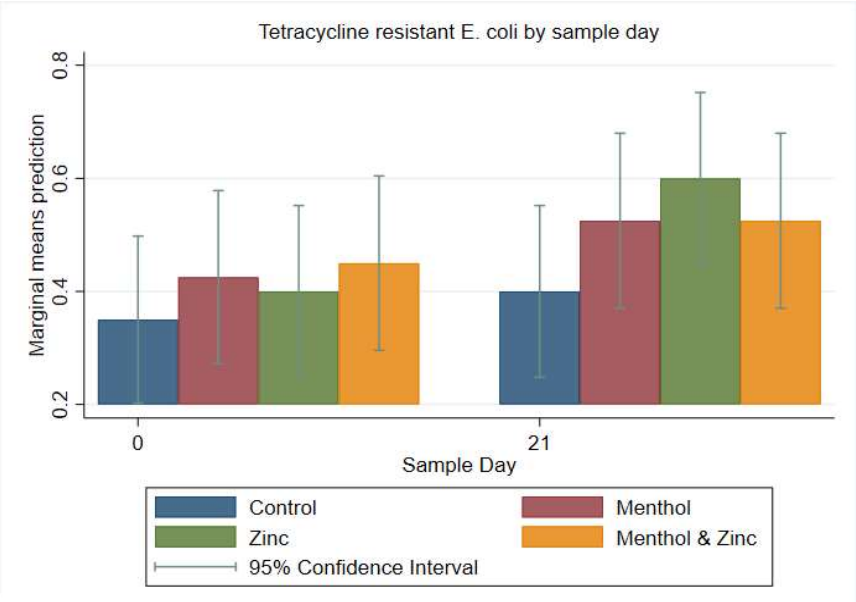

Figure 6 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of tetracycline

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Additionally, the resistance to the number of antibiotic classes by sample day and treatment (Figure 7) showed a trend towards increasing multi-drug resistance in the zinc group from a total of 27.5% isolates multi-drug resistant on Day 0 to 32.5% on 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 multi-drug 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 multi-drug 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.

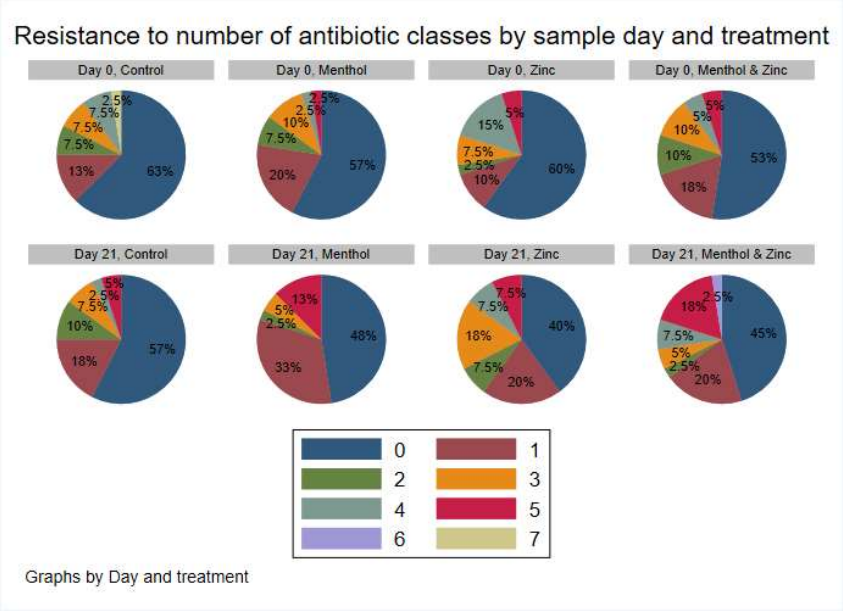

Figure 7 Resistance (%) of *E. coli* isolates to number of antibiotic classes by sample day and treatment.

Additionally, the zinc and menthol group did not have any isolates resistant to 6 antibiotic classes on Day 0; however, on Day 21 2.5% of isolates were resistant. Most of this gain in resistance prevalence came at the expense of the proportion of isolates that were initially 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 statistically tested for significance using a multi-level mixed logistic regression.

Multi-level mixed effect logistic regression modeling was performed on the binary outcome of multi-drug resistant (i.e., resistance to ≥ 3 antibiotic classes) *E. coli* isolates (Figure 8). There were no significant differences among treatment groups. The control group exhibited a decrease in the predicted prevalence of MDR isolates from Day 0 to Day 21, however, this decrease was not significant ($P > 0.05$). The menthol group, the zinc group, and the combined zinc and menthol group increased from Day 0 to Day 21, 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 Day 0 to Day 21 in multi-drug resistance for sample day alone; again, this was also not significant ($P > 0.05$).

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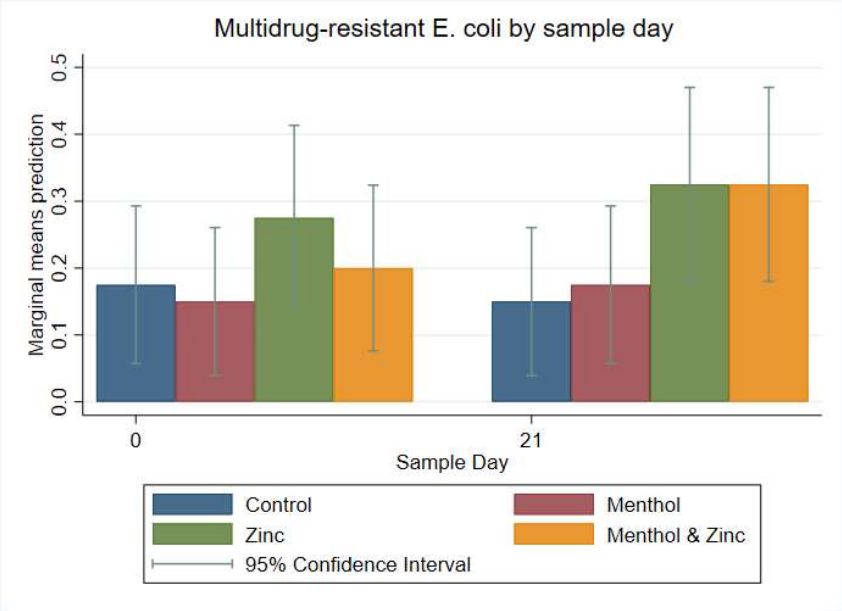

Figure 8 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of multi-drug resistant (resistant to ≥3 classes of antibiotics) *E. coli*

54 For phenotypic resistance of *Enterococcus* spp. (Figure 9), all isolates were susceptible to gentamicin,
55 tigecycline, and vancomycin. Nearly all isolates (91.25%) were resistant to lincomycin, while
56 approximately a third were resistant to quinupristin/dalfopristin and tetracycline. Not surprisingly,
57 resistance to erythromycin and tylosin (both macrolides) was nearly equal, at 17.81% and 18.44%,
58 respectively. Less than 1% of isolates were resistant to chloramphenicol, kanamycin, linezolid,
59 penicillin, or streptomycin. It should be noted that a bimodal distribution across isolates appeared
60 in regard to the MICs of both tetracycline and lincomycin, corresponding to their distinct
61 categorization as either susceptible or resistant to these two drugs.

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	# 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 9 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.

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Additionally, resistance of *Enterococcus* spp. isolates to antibiotic class and by sample day (Figure 10), 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 exhibit decreased macrolide resistance, from 30% on Day 0 to 17.5% on Day 21.

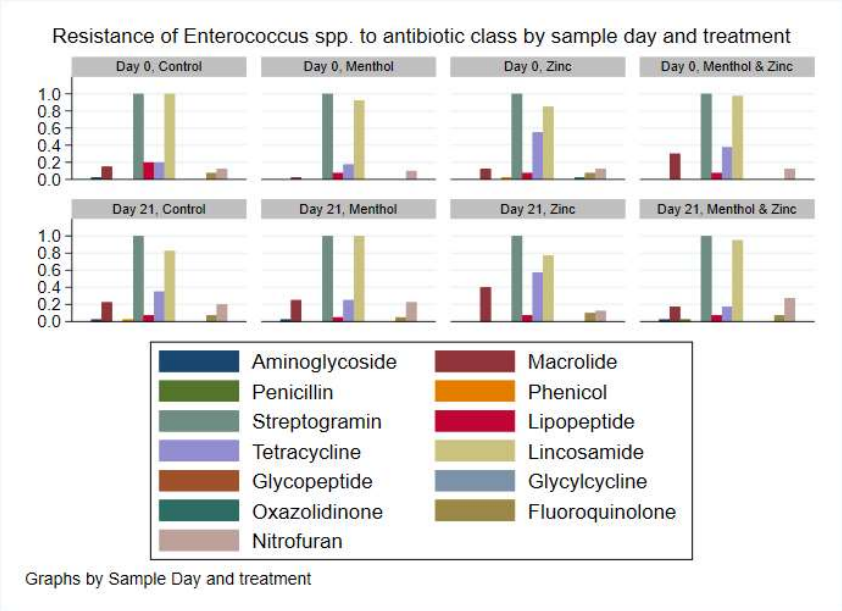

Figure 10 Resistance (proportion) of *Enterococcus* spp. isolates to each antibiotic class by sample day and treatment.

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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 Day 0 to 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 Day 0 to 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 11).

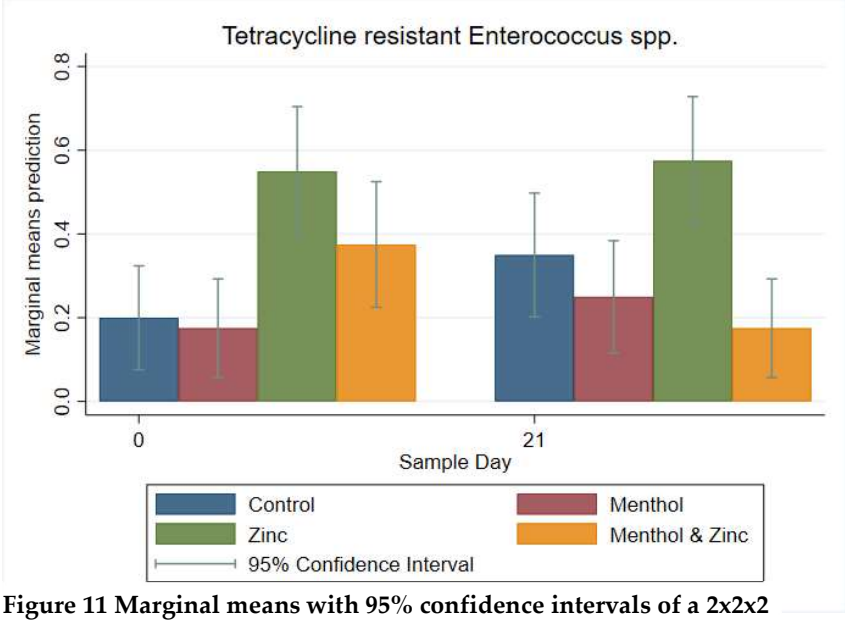

Figure 11 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of tetracycline resistant *Enterococcus* spp

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 (Figure 12). Day 0 to Day 21 comparisons showed 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 was performed, which showed the increase in macrolide resistant enterococci in the menthol group was significant ($P < 0.05$). In contrast, the combination of menthol and zinc treatment groups tended 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).

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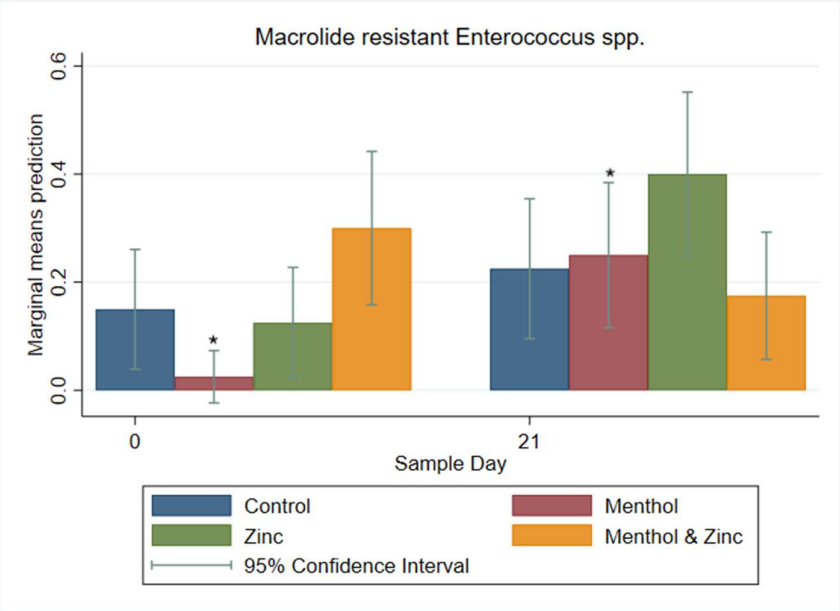

Figure 12 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of macrolide resistant *Enterococcus* spp

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

The percentage of isolates resistant to the number of antibiotic classes by sample day and treatment (Figure 13) showed that all *Enterococcus* spp. isolates were resistant to at least one class of antibiotic, with no pan-susceptible isolates observed. The menthol group showed an increase in the percentage of multi-drug 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 multi-drug 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, while on Day 21 10% of isolates were resistant to only 1 antibiotic class. The percentage of multi-drug 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.

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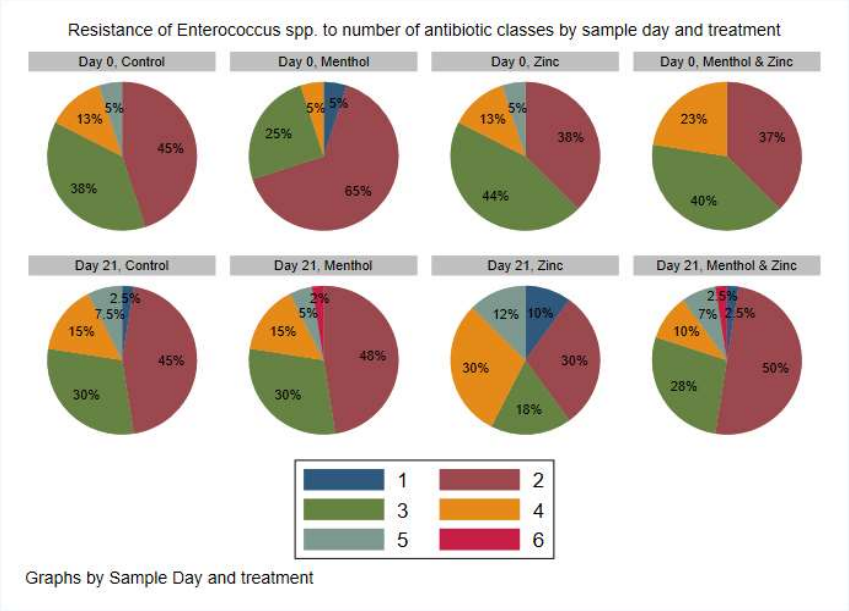

Figure 13 Resistance (%) of *Enterococcus* spp. isolates to number of antibiotic classes by sample day and treatment.

The results of a multi-level mixed effect logistic regression model on the binary outcome of multi-drug resistant (i.e., resistance to ≥ 3 antibiotic classes) *Enterococcus* spp. isolates (Figure 14) 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, 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 Day 0 to Day 21; however, this decrease was not significant ($P > 0.05$).

4. Discussion

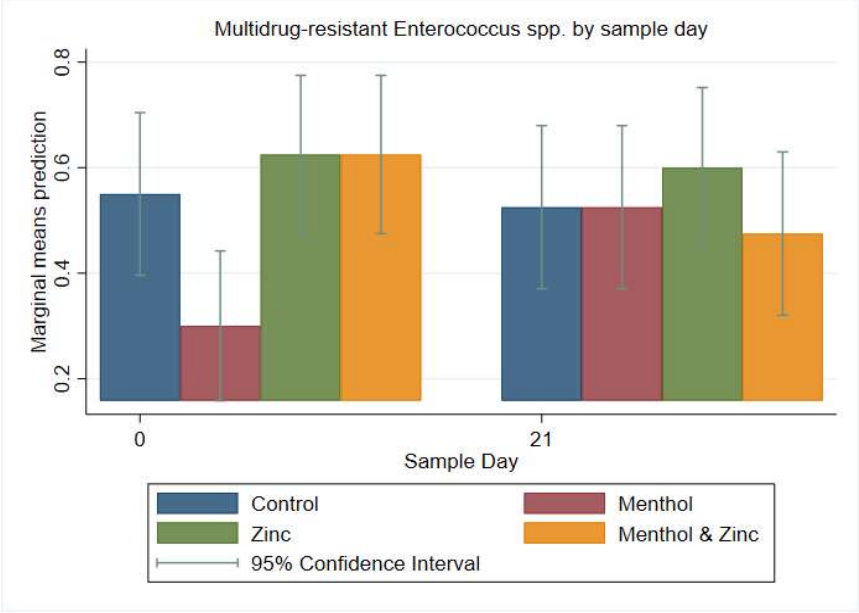

Figure 14 Marginal means with 95% confidence intervals of a 2x2x2 multi-level mixed logistic regression model, using factors of zinc, menthol, and sample day on the binary outcome of multi-drug resistant (resistant to ≥3 classes of antibiotics) *Enterococcus* spp

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This randomized controlled trial demonstrated that there were some trends towards increasing resistance, but little to no significant effects of zinc or menthol supplementation as alternatives to antibiotics on *E. coli*; in contrast, several significant effects of zinc and menthol supplementation were observed to be present among enterococci. The $\log_{10}$ CFU per gram of feces on plain MacConkey agar was significantly affected by sample day (study period). 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* in cattle [30]. However, *mentha piperita* (peppermint) essential oil and menthol have been shown to inhibit quorum sensing, which regulates the expression of certain genes, and biofilm formation of gram-negative organisms including *E. coli* [34].

Our data showed no noticeable effects of sample day or treatment group 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 [35]. 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 [36] [37]. Yet, another more recent study also conducted in swine cautioned against this observation and stated that tolerance to zinc was not associated with multi-drug resistance [38]. At the time of our study among beef cattle in the United States there appears to be little evidence for co-selection of antimicrobial resistance among fecal bacteria from animals fed supranutritional levels of zinc (300 ppm) or menthol at 0.3%.

Among enterococci, menthol tended to result in decreased 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 when advancing from Day 0 to Day 21. Additionally, the menthol group tended to be associated with an increase in 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 [39]. 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 [40]. While tea tree oil has been licensed for the past 100 years, clinical resistance has yet to be reported [41]. However, after several generations of methicillin-resistant *Staphylococcus aureus* (MRSA) were exposed to tea tree oil, a resistant subpopulation emerged [42]. It is therefore plausible that after repeated exposure to menthol, in addition to antibiotic exposure, a multi-drug 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 [43].

In our study 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 [20]. 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 [44].

5. Conclusions

Although no significant treatment effects were present for *E. coli*, these trial data suggest that there are 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 found in the literature, 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.

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