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Antimicrobial resistance and genomic epidemiology of enteric bacteria on the farm-to-fork interface

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Antimicrobial Resistance and Genomic Epidemiology of Enteric Bacteria on the Farm-to-Fork Interface

By

KATIE LEE

DISSERTATION

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Table of Contents

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Antimicrobial resistance and genomic epidemiology of enteric bacteria on the farm-to-fork interface

Katie Lee

University of California, Davis, 2024

Antimicrobial resistance (AMR) is one of the most serious public health threats of the century. Resistant bacteria and AMR genes (ARGs) can spread through human and animal populations through pathways such that selective pressures in one population inextricably impacts others on the One Health continuum. This dissertation takes a farm-to-fork approach on AMR by evaluating the distribution and risk factors for AMR in retail products, AMR co-selection in food-producing animals, and genomic profiles of these bacteria.

In Chapter 1, a cross-sectional study was conducted to assess the distribution and AMR profiles of *Salmonella* from retail meat products in California. From multivariable logistic regression, season of purchase and meat type were significantly associated with the isolation of *Salmonella*. Whole genome sequencing (WGS) characterized *Salmonella* isolates into 14 distinct serotypes corresponding to 17 MLST patterns. Diverse ARGs including those of high public health significance and putative plasmids were identified. The IncFIB(pN55391) replicon previously reported in connection to the worldwide dissemination of pESI-like mega plasmid carriage in an emerged *S.* Infantis clone was detected in four of the six multidrug-resistant (MDR) isolates.

In Chapter 2, *Escherichia coli* from samples in Chapter 1 were assessed to gain further insight on the clinical and epidemiologic risks associated with AMR in retail meat products from California. Phenotypic resistance to ampicillin, gentamicin, streptomycin, and tetracycline were significantly associated with meat type, with poultry counterparts (chicken or ground turkey) exhibiting higher odds for resistance compared to non-poultry meats (beef and pork). Clustering analysis and co-occurrence networks revealed that genomic AMR determinants of *E. coli* from retail meat were highly heterogeneous with sparsity of shared gene networks and minimally driven by retail-level factors of meat type, season of purchase, packaging, and antibiotic label claims.

In Chapter 3, the impact of dietary zinc supplementation in pre-weaned dairy calves on phenotypic AMR of fecal *Enterococcus* spp. and *E. coli* was investigated. Accelerated failure time (AFT) models were constructed to determine the association between zinc treatment and AMR, with exponentiated coefficients adapted for minimum inhibitory concentration (MIC) values instead of time representing the degree of change in AMR (MIC Ratio, MR). Zinc supplementation did not significantly alter the MIC in *Enterococcus* spp. for 13 tested antimicrobials and in *E. coli* for azithromycin and ceftriaxone. However, a significant reduction in *E. coli* MIC values was observed for ciprofloxacin (MR= 0.17, 95% CI 0.03–0.97) and nalidixic acid (MR= 0.28, 95% CI 0.15–0.53) for zinc-treated compared to placebo-treated calves.

In Chapter 4, whole-genome comparative analysis was conducted to investigate the host-microbe interface of MDR *E. coli* from dairy calves. The pangenome of *E. coli* was open, with all-by-all genome similarity comparisons clustering primarily by sequence type (ST) rather than host factors of diarrheal disease, zinc supplementation, and antimicrobial exposure. *E. coli* lacked the typical virulence factors of diarrheagenic strains, however virulence factors overlapping with those in major pathotypes were identified, with the most prevalent genes corresponding to iron acquisition. Dietary zinc exposure was not associated with the selection of individual ARGs, however significant associations between the occurrence of certain ARGs and metal resistance genes were identified.

Collectively, this dissertation provides greater insight into the epidemiology of AMR in enteric bacteria of public health significance. This improved understanding of the distribution and drivers of AMR in food products and food-producing animals will inform future AMR monitoring and control strategies by supporting more targeted approaches to mitigate AMR from farm-to-fork.

Introduction

The Antimicrobial Resistance Crisis

Antimicrobial resistance (AMR) is one of the most serious public health threats faced in this century, with the occurrence of drug-resistant organisms augmented by the ever-growing range of infections, limited progress towards discovery of additional antimicrobial agents, and the imminent development of resistance to currently available drugs (Michael et al., 2014). In 2019, the global burden of bacterial AMR was estimated to directly account for 1.27 million deaths and contribute to 4.95 million deaths (Murray et al., 2022). Recent estimates suggest that mortalities caused by antimicrobial resistance (AMR) will rise to 10 million people by 2050 (Kraker et al., 2016). The emergence and spread of AMR are attributed to use of antimicrobials in human and veterinary medicine, environmental contamination, and the impacts of anthropogenic activities that drive the proliferation of these microbial hazards across the One Health interface (McEwen and Collignon, 2018; Larsson and Flach, 2022). There is growing recognition that selective pressures imparting microbial adaptations in one sector inextricably impacts other One Health sectors, highlighting the need to better understand where AMR is emerging, what is driving its development, and how to best mitigate its dissemination.

Consequences of AMR

The occurrence of AMR has multifaceted consequences, in which one of the immediate impacts is poor health outcomes from difficult or untreatable infections with existing antimicrobial agents. Reduced efficacy of antimicrobial drugs contribute to increased risk of disease dissemination, illness severity, and death (Michael et al., 2014; National Academies of Sciences et al., 2021). In tandem, greater disease burden from longer patient recovery time and additional treatment and diagnostics for patient care exacerbate economic costs of AMR in health systems (Dadgostar, 2019). On a global scale, AMR has impacts on poverty and employment, with the World Bank research indicating that AMR imposes greater comparable impact on low-income countries. Drug-resistant infections also extend to animal populations

and plants, with the occurrence of AMR in food and food-producing animals compromising animal health through sickness and mortality, reducing productivity and profits, and threatening food security (National Academies of Sciences et al., 2021). While antimicrobial drugs are considered the cornerstone of modern medicine, the progression towards a "post-antibiotic" era of untreatable, deadly infections marks the arms race against microbes to combat AMR (Kwon and Powderly, 2021), particularly for priority pathogens – e.g. ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. – that are in urgent need of new antibiotic treatment options (De Oliveira et al., 2020; Murugaiyan et al., 2022).

Defining and Measuring AMR

AMR is a naturally occurring process from genetic changes in microbes, however its emergence and dissemination can be accelerated by the use and overuse of antimicrobials (Bennani et al., 2020). Bacterial AMR frequently occurs through acquired resistance through chromosomal gene mutations and acquisition of exogenous resistance genetic determinants from mobile genetic elements and horizontal gene transfer (Partridge et al., 2018; Salam et al., 2023). Bacteria can also exhibit AMR through intrinsic resistance, which refers to naturally occurring resistance from mechanisms that do not involve mutations or acquisition of genetic elements; frequently, these involve efflux pumps and reduced permeability (Cox and Wright, 2013; Salam et al., 2023). AMR can be defined in various contexts, in which clinical resistance is presented in patients with an infection that does not respond to an antibiotic expected to resolve the infection. In laboratory settings, microbiological resistance is determined for bacterial strains that are unaffected by and able to grow in the presence of defined thresholds of antibiotics. Lastly, molecular resistance is defined as the presence of genetic elements (e.g. genes or mutations) that have been shown to confer or be associated with resistance (Chandler, 2019). AMR can be additionally characterized through phenotypic and genotypic resistance. Phenotypic resistance is evaluated through antimicrobial susceptibility testing and by determining the minimum inhibitory concentration (MIC), or the lowest concentration of an antimicrobial drug that inhibits visible growth of a bacterial strain after

incubation (Andrews, 2001). Using the MIC, bacterial isolates are then typically classified based on interpretive criteria or breakpoints as resistant, intermediate resistance, or susceptible (Kowalska-Krochmal and Dudek-Wicher, 2021). Multidrug resistance (MDR) is defined based on these categorization of MICs, with a common definition being resistance to at least one drug in three or more antimicrobial classes (Magiorakos et al., 2012; Glossary of Terms Related to Antibiotic Resistance | NARMS | CDC, 2019). Genotypic resistance is determined through detection of mutations and/or genes that cause drug resistance, with commonly used methods including polymerase chain reaction (PCR) or more comprehensive approaches such as whole genome sequencing (WGS) (Feldgarden et al., 2019).

An Integrated Approach to Advancing Knowledge on AMR

The World Health Organization describes a One Health approach as "an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems" (One health, n.d.). Although there are exceptions and restrictions for certain species, drugs from the majority of antimicrobial classes are used across both human and veterinary medicine such that resulting emergence of AMR in one population can lead to AMR or compromised health outcomes from resistant bacteria in another population. Addressing AMR necessitates an integrated approach with consideration of the interconnected transmission pathways and shared reservoirs across these human, animal, and environmental interfaces (Salam et al., 2023). In the United States, the National Antimicrobial Resistance Monitoring System (NARMS) monitors AMR in enteric and foodborne bacteria from various sources, with the Food and Drug Administration (FDA) assessing retail meats, the Center for Disease Control and Prevention (CDC) assessing ill persons, and the United States Department of Agriculture (USDA) assessing ceca and regulatory samples (Karp et al., 2017; Medicine, 2024). Based on principles of a One Health approach, strategic goals of the program which extend to general efforts to address AMR include but are not limited to monitoring trends across populations to understand the distribution of AMR, timely dissemination of data to support cross-collaborative interventions for AMR mitigation and outbreak investigations, and advancement in epidemiologic and microbiologic AMR research (Karp et al., 2017). A

core component to tackling AMR based on the One Health framework across these three domains (humans, animals, and environment) is the expansion and refinement of sample collection (e.g. geographical location, sample types, and bacterial species), development and use of novel and robust methods for the evaluation of AMR, and the timely, accessible deployment of improved methodologies to involved agencies and partners. Critically, these efforts need to encompass capacity building for sample collection, testing of bacterial isolates, implementing advanced technologies such as next-generation sequencing approaches, and harmonized protocols for data collection, analysis, and sharing.

Dissertation Objectives and Summary

This dissertation focuses on AMR on the farm-to-fork interface, with overall goals to advance knowledge on AMR and the genomic epidemiology of enteric bacteria. Chapter one and two investigate the distribution and risk of foodborne AMR in pathogenic and indicator commensal bacteria from retail meat products, using the first data from expanded NARMS monitoring in California. Chapter three evaluates the impact of non-antimicrobial modulators of AMR using novel analytic approaches. Lastly, Chapter four evaluates the host-microbe interface of MDR bacteria through comprehensive and comparative analysis of genome diversity and composition. Collectively, this dissertation provides greater insight into the epidemiology of AMR in enteric bacteria of public health significance. This improved understanding of the distribution and drivers of AMR in food products and food-producing animals will inform future AMR monitoring and control strategies by supporting more targeted approaches to mitigate AMR

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Chapter 1: Antimicrobial resistance profiles of non-typhoidal *Salmonella* from retail meat products in California, 2018

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Antimicrobial Resistance Profiles of Non-typhoidal *Salmonella* From Retail Meat Products in California, 2018

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Non-typhoidal *Salmonella* remains a leading cause of foodborne illness in the United States, with food animal products serving as a key conduit for transmission. The emergence of antimicrobial resistance (AMR) poses an additional public health concern warranting better understanding of its epidemiology. In this study, 958 retail meat samples collected from January to December 2018 in California were tested for *Salmonella*. From multivariable logistic regression, there was a 6.47 (90% CI 2.29–18.27), 3.81 (90% CI 1.29–11.27), and 3.12 (90% CI 1.03–9.45) higher odds of contamination in samples purchased in the fall, spring, and summer than in winter months, respectively, and a 3.70 (90% CI 1.05–13.07) higher odds in ground turkey compared to pork samples. Fourteen distinct serotypes and 17 multilocus sequence types were identified among the 43 isolates recovered, with *S.* Kentucky (25.58%), *S.* Reading (18.60%), *S.* Infantis (11.63%), and *S.* Typhimurium (9.30%) comprising the top serotypes. High prevalence of resistance was observed in retail chicken isolates for streptomycin (12/23, 52.17%) and tetracycline (12/23, 52.17%), in ground turkey isolates for ampicillin (8/15, 53.34%), and in ground beef isolates for nalidixic acid (2/3, 66.67%). Fourteen (32.56%) were susceptible to all antimicrobials tested, 11 (25.58%) were resistant to one drug, and 12 (27.91%) were resistant to two drugs. The remaining six isolates (13.95%) were multidrug-resistant (MDR, ≥3 drug classes) *S.* Infantis (n = 4), *S.* Reading (n = 1), and *S.* Kentucky (n = 1). Whole-genome sequencing (WGS) identified 16 AMR genes and 17 plasmid replicons, including $bla_{CTX-M-65}$ encoding ceftriaxone resistance and a D87Y mutation in *gyrA* conferring resistance to nalidixic acid and reduced susceptibility to ciprofloxacin. The IncFIB(pN55391) replicon previously identified in connection to the worldwide dissemination of pESI-like mega plasmid carriage in an emerged *S.* Infantis clone was detected in four of the six MDR isolates. Genotypes from WGS showed high concordance with phenotype with overall sensitivity

and specificity of 95.31% and 100%, respectively. This study provides insight into the AMR profiles of a diversity of *Salmonella* serotypes isolated from retail meat products in California and highlights the value of routine retail food surveillance for the detection and characterization of AMR in foodborne pathogens.

Keywords: non-typhoidal *Salmonella enterica* (NTS), antimicrobial resistance, retail meat, phenotype, whole-genome sequencing (WGS), resistance genes, plasmid, public health surveillance

INTRODUCTION

Salmonella enterica is a Gram-negative, facultative anaerobic bacteria part of the *Enterobacteriaceae* family and a pathogen imparting significant global health burdens (Andino and Hanning, 2015). In the United States, non-typhoidal *Salmonella* (NTS) is a leading cause for foodborne illness and responsible for 1.35 million cases, 26,500 hospitalizations, and 420 deaths each year (Centers for Disease Control and Prevention [CDC], 2021). While infections are typically self-limiting, they can progress to systemic infection requiring clinical treatment particularly in infants, older individuals, and immunocompromised people (Foley and Lynne, 2008; Antunes et al., 2016). Furthermore, the emergence of antimicrobial-resistant *Salmonella* underscores a significant public health concern, with drug-resistant infections resulting in increased morbidities and mortalities stemming from longer duration and severity of disease and reduced treatment efficacy (Kurtz et al., 2017; Nair et al., 2018; Jajere, 2019).

While *Salmonella* is present in a large diversity of natural reservoirs, the vast majority of human salmonellosis infections arise from handling and consumption of contaminated food animal products (Callaway et al., 2008; Foley and Lynne, 2008; Andino and Hanning, 2015), attributed by poultry and livestock serving as major sources (Crump et al., 2015; Heredia and García, 2018). Emerged resistance to traditional antimicrobial agents such as ampicillin and trimethoprim-sulfamethoxazole has reduced treatment options and led to the empirical use of critically important antimicrobial drugs (Frasson et al., 2016). Fluoroquinolones (e.g., ciprofloxacin) and third-generation cephalosporins (e.g., ceftriaxone) are currently the primary treatment options for invasive salmonellosis, with the latter being an important alternative first-line treatment for pediatric infections due to the limited number of FDA-approved indications for fluoroquinolones in children (Jackson et al., 2016). Notably, antibiotics in these same drug classes are employed in veterinary medicine for treatment of food animals. While fluoroquinolone drugs have been withdrawn for use in poultry (Food and Drug Administration [FDA], 2017), enrofloxacin is currently approved for use in cattle and swine commonly for the treatment of respiratory diseases (Food and Drug Administration [FDA], 2021a). Additionally, ceftiofur—a veterinary third-generation cephalosporin drug—is used for treatment of respiratory disease in various livestock, bacterial infections in poultry, and for treatment of subclinical and clinical mastitis in dairy cattle (Food and Drug Administration [FDA], 2021a). While antimicrobial agents vastly improve health outcomes in human and veterinary medicine alike, the ubiquity and magnitude of their usage have raised concerns on the consequences of selective pressures imposed for the emergence and dissemination of multidrug-resistant (MDR) pathogens. Antimicrobial resistance (AMR) in food animals has long been implicated as a source for resistant infections in humans, and it has become a priority public health effort to monitor the persistence and dissemination of drug-resistant pathogens such as NTS and their carriage of associated genetic determinants on the farm-to-fork continuum (Karp et al., 2017).

In the United States, the National Antimicrobial Resistance Monitoring System (NARMS) monitors AMR in enteric bacteria from animals, food, and humans (Food and Drug Administration [FDA], 2020a), including retail meat, which serves as a major conduit for MDR *Salmonella*. The epidemiology of AMR is dynamic and complex; with respect to resistance in NTS, it has been observed to be variable on a multitude of factors including serotype, source, and geographic location (Hoelzer et al., 2010; Hong et al., 2016; Nyirabahizi et al., 2020; Yin et al., 2021). The objective of this study was to characterize and assess the AMR profiles of *Salmonella* isolates recovered from fresh retail chicken, ground turkey, ground beef, and pork chop samples purchased in California over a 1-year period as part of routine NARMS surveillance. This study also utilized whole-genome sequencing (WGS) with the goal to identify the diversity of AMR genes conferring drug resistance and the carriage of genetic elements of significant public health concern.

MATERIALS AND METHODS

Study Area and Sampling

Samples in this study were collected as part of the routine NARMS retail meat testing program. From January to December 2018, a total of 958 fresh samples consisting of 478 skin-on/bone-in chicken, 240 ground turkey, 120 pork chop, and 120 ground beef were purchased from retail stores in California twice each month. Sampling locations were selected based on randomization of grocery stores by zip codes in northern (City and County of San Francisco, Contra Costa County, and Alameda County) and southern California (West Los Angeles, East Los Angeles, Ontario, and Irvine). A variety of meat types and cuts from different brands were purchased at each store. Packaging of samples in this study included modified atmospheric packaging (MAP), plastic bag, vacuum sealed, chub, paper wrapped, and plastic film packaging. Samples were transported on ice to the laboratory, refrigerated, and processed within 72 h of purchase.

Sample Processing and Bacterial Isolation

Samples were processed per the NARMS Retail Meat Surveillance protocol. Briefly, 25 g of each sample in 250 ml buffered peptone water (Becton Dickinson, Franklin Lakes, NJ, United States) was hand massaged for 3 min or placed on a mechanical shaker at 200 rpm for 15 min. Fifty milliliters of rinsate was added to 50 ml of double-strength lactose broth (Becton Dickinson, Franklin Lakes, NJ, United States) and incubated at 35°C for 24 h. After overnight enrichment, 0.1 ml of lactose broth was transferred to 9.9 ml Rappaport-Vassiliadis R10 (RVR10) broth (Becton Dickinson, Franklin Lakes, NJ, United States) and incubated at 42°C for 16–20 h. The RVR10 enrichments were then streaked onto XLT-4 (Remel, Lenexa, KS, United States) and Hektoen Enteric (Becton Dickinson, Franklin Lakes, NJ, United States) agars and incubated at 35°C for 18–24 h. Up to two suspect *Salmonella* colonies based on colony morphology from each selective agar were then streaked to purity on blood agar plates. Isolates were shipped on dry ice to the FDA's Center for Veterinary Medicine (CVM) for antimicrobial susceptibility testing and WGS.

Antimicrobial Susceptibility Testing

Salmonella isolates were tested using a broth microdilution method for 14 antimicrobial drugs using the NARMS Gram-negative plates (Thermo Fisher Scientific, Waltham, MA, United States) per standard protocols (Food and Drug Administration [FDA], 2016). Minimum inhibitory concentration (MIC) values for each drug were used to classify isolates based on the Clinical and Laboratory Standards Institute (CLSI) guidelines. NARMS consensus interpretive criteria were used for streptomycin and azithromycin, due to unavailability of CLSI breakpoints for these two drugs (Food and Drug Administration [FDA], 2021b). Breakpoints used to classify resistant isolates for each antimicrobial drug were as follows: amoxicillin/clavulanate (≥32/16 μg/ml), ampicillin (≥32 μg/ml), azithromycin (≥32 μg/ml), cefoxitin (≥32 μg/ml), ceftriaxone (≥4 μg/ml), chloramphenicol (≥32 μg/ml), ciprofloxacin (≥1 μg/ml), gentamicin (≥16 μg/ml), meropenem (≥4 μg/ml), nalidixic acid (≥32 μg/ml), streptomycin (≥32 μg/ml), sulfisoxazole (≥512 μg/ml), tetracycline (≥16 μg/ml), and trimethoprim-sulfamethoxazole (≥4/76 μg/ml). Phenotypic resistance was presented as resistant isolates, with intermediate and susceptible isolates grouped together for analysis. Multidrug resistance was defined as resistance to ≥1 drug in ≥3 antimicrobial classes (Magiorakos et al., 2012). Due to the significance of ciprofloxacin for salmonellosis treatment and the expansion of CLSI criteria for its intermediate susceptibility range, reduced susceptibility to ciprofloxacin was also noted (≥0.12 μg/ml) (Food and Drug Administration [FDA], 2021b).

Whole-Genome Sequencing

Salmonella isolates were streaked to blood agar plates, and pure colonies were extracted from overnight cultures per manufacturer's protocol using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, United States). DNA purity and quantification was assessed using the NanoDrop and Qubit fluorometer, respectively. Libraries were prepared using the Illumina Nextera XT kit per manufacturer's protocol with quality control and quantification done on the Bioanalyzer and Qubit. Final libraries were sequenced using v2 chemistry for 2 × 250-bp paired end reads on the Illumina MiSeq platform. Sequences were demultiplexed, and adapters were removed using MiSeq Reporter. Read trimming and assembly were conducted as previously described (Tyson et al., 2015), with *de novo* assembly done using the CLC Genomics Workbench and genome annotation using NCBI's Prokaryotic Genome Automated Pipeline (Tyson et al., 2015). Species confirmation and serotyping were determined from WGS data per the FDA NARMS Manual of Laboratory Methods (Food and Drug Administration [FDA], 2016); SeqSero1 and SeqSero2 were used for serotyping with any discrepant isolates additionally serotyped according to the Kauffmann–White scheme (Food and Drug Administration [FDA], 2016; Zhang et al., 2019). Serotypes used for analysis correspond to the final serotype determinations submitted to NCBI as attributes with whole-genome sequences, which are deposited under BioProject PRJNA292661 (**Supplementary Table 1**).

Identification of Resistance Genes, Quinolone Resistance-Determining Region Mutations, and Plasmid Replicons

Resistance genes were identified from assemblies by methods previously described (Tyson et al., 2015), with Perl scripts used to identify hits (≥85% amino acid identity and ≥50% sequence length) from a reference database of compiled genes from the ResFinder (Center for Genomic Epidemiology, DTU), ARG-ANNOT (IHU Méditerranée Infection), and CARD (McMaster University) public databases. Additionally, quinolone resistance-determining region (QRDR) mutations were assessed through extraction and analysis of the *gyrA*, *gyrB*, *parC*, and *parE* genes using ClustalW in MEGA (McDermott et al., 2016). Plasmid replicons were identified using PlasmidFinder (Center for Genomic Epidemiology), with hits determined as having ≥95% identity and ≥60% coverage.

Multilocus Sequence Typing and Minimum Spanning Trees

To assess the relationship between *Salmonella* isolates in this study, the PubMLST database[1] was used to determine the sequence type (ST) from WGS data for each isolate based on the seven-gene legacy multilocus sequence typing (MLST) loci for *Salmonella*: *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*. MLST data was then used to generate and visualize minimum spanning trees using the Global Optimal eBURST (goeBURST) algorithm (Francisco et al., 2009) with PHYLOViZ (Francisco et al., 2012).

Data Analysis

A total of 43 *Salmonella* isolates from 41 retail meat samples were included in the analysis. Two isolates from a ground turkey and two from a ground beef sample were included due to more

[1]https://pubmlst.org/

than one unique AMR phenotype profile recovered from each of these samples. Descriptive statistics (prevalence of *Salmonella*, distribution of covariates, and antimicrobial susceptibility testing results) and logistic regression models were conducted using SAS 9.4. The outcome binary variable for logistic regression was designated as the presence or absence of *Salmonella*, and the covariates included region of sample collection (northern and southern California), season, meat type, packaging, and label claim (conventional and reduced antibiotic use). Categorization of reduced antibiotic use included samples with packaging claims of organic and/or no antibiotic usage. All other samples with absence of organic or antibiotic claims were categorized as conventional. Univariate logistic regression was performed to determine the crude associations between the outcome and each covariate. A multivariable logistic regression model was then fitted using candidate variables with $p < 0.25$ from univariate analysis. The significance of all two-way interactions was tested, and the final model was selected based on the lowest Akaike's Information Criterion (AIC). Variable selection for the final model was also guided by the literature where associations between *Salmonella* and factors such as meat source have been previously substantiated. Given the smaller sample sizes present in this study, a significance level of $\alpha = 0.10$ was used to reduce the probability of a type II error. Genotype was considered concordant with phenotype when an isolate with phenotypic resistance to a drug had known resistance genes or mutations conferring resistance to the corresponding drug (true positive, TP) or when an isolate with phenotypic susceptibility to a drug had absence of resistance genes or mutations conferring resistance to the corresponding drug (true negative, TN). False negatives (FN) were defined as isolates that were phenotypically resistant but genotypically susceptible, and false positives (FP) were defined as isolates that were phenotypically susceptible but genotypically resistant. Sensitivity was calculated as TP/(TP + FN) and specificity as TN/(TN + FP). Matrices were created for phenotypic antimicrobial testing results and the presence/absence of resistance genes and plasmid replicons. A heatmap and hierarchical clustering were performed using the heatmap3 package in R, with dissimilarity matrices constructed using the Manhattan distance algorithm and clustered using the UPGMA method.

RESULTS

Isolation of *Salmonella* From Raw Retail Meat Products in California

Out of 958 retail meat products, *Salmonella* was isolated from 41 (4.28%) samples, with the highest recovery in ground turkey (14/240, 5.83%) followed by chicken (23/478, 4.81%), ground beef (2/120, 1.67%), and pork chops (2/120, 1.67%) (**Table 1**).

Factors Associated With *Salmonella* Contamination of Raw Retail Meat Products in California

Region of sample purchase (northern and southern CA), packaging type, and label claim were not significantly associated with the recovery of *Salmonella*, with the final multivariable logistic regression model including season and meat type as significant covariates. Odds of *Salmonella* isolation was 3.70 (90% CI 1.05–13.07) times higher in ground turkey when compared to pork chops, adjusting for season. Adjusting for meat type, samples collected in the fall, spring, and summer months had a 6.47 (90% CI 2.29–18.27), 3.81 (90% CI 1.29–11.27), and 3.12 (90% CI 1.03–9.45) times higher odds of *Salmonella* contamination compared to those collected in the winter months, respectively (**Table 1**).

Distribution of *Salmonella* Serotypes and Multilocus Sequence Typing Profiles

From serotyping and MLST analysis, 14 distinct serotypes and 17 STs were identified (**Table 2**). The most frequently isolated serotypes were *S.* Kentucky (11/43, 25.58%) and *S.* Reading (8/43, 18.60%), with all *S.* Kentucky isolates recovered from chicken samples and all *S.* Reading isolates recovered from ground turkey. The remaining 12 serotypes displayed distinctive source trends, with exceptions of *S.* Infantis being recovered from three different meat types—chicken, ground turkey, and ground beef—and *S.* Schwarzengrund recovered from chicken and ground turkey (**Table 2**). Each serotype was associated with one ST, with the exception of *S.* Kentucky isolates, which were distributed across four different STs (**Figure 1A**). By source, ST32 isolates were recovered across different retail meats (**Figure 1B**). The greatest serotype and ST diversity was observed in isolates from chicken samples (**Figure 1B**), though the wide distribution of isolates overall is indicative of a high degree of diversity in genetic profiles across all *Salmonella* isolates recovered in this study (**Figure 1**).

Phenotypic Antimicrobial Resistance Profiles

All 43 of the *Salmonella* isolates in this study were susceptible to azithromycin, meropenem, and trimethoprim-sulfamethoxazole; 32.56% (14/43) of isolates were susceptible to all antimicrobials tested, 25.58% (11/43) were resistant to one drug, 27.91% (12/43) to two drugs, and 13.95% (6/43) to three or more antimicrobial drugs tested. The highest resistance was observed for tetracycline (17/43, 39.53%), followed by streptomycin (15/43, 34.89%) and ampicillin (10/43, 23.26%). Resistance to aminoglycoside drugs—gentamicin and streptomycin—were observed in chicken and ground turkey isolates, with over half of the isolates from chicken samples displaying resistance to streptomycin (12/23, 52.17%). Resistance to sulfonamides—sulfisoxazole—was only detected in chicken and ground turkey isolates (**Table 3**).

Resistance to all three beta-lactam combination agent drugs tested (amoxicillin-clavulanic acid, cefoxitin, and ceftriaxone) was observed in one chicken isolate. This was a *S.* Kentucky isolate resistant to beta-lactam/combination, aminoglycoside, and penicillin drug classes and one of the six MDR isolates identified in this study. The other five MDR isolates included four *S.* Infantis isolates recovered from a ground turkey ($n = 2$), a ground beef ($n = 1$), and a chicken ($n = 1$) sample and a *S.* Reading isolate from a ground turkey sample. Notably, four of the six MDR isolates (all *S.* Infantis) displayed resistance to

TABLE 1 | Prevalence and logistic regression models of risk factors for *Salmonella* in retail meat products from California.

Variable	Univariate models			Multivariable model	
	***Salmonella* positive *n/N* (%)**	**OR (90% CI)**	***P*-value**	**OR (90% CI)**	***P*-value**
Region					
Northern CA	17/478 (3.56%)	0.70 (0.41–1.19)	0.272	–	–
Southern CA	24/480 (5.00%)	1.00			
Season					
Spring	11/240 (4.58%)	3.80 (1.29–11.20)	0.043[a]	3.81 (1.29–11.27)	0.042[b]
Summer	9/238 (3.78%)	3.11 (1.03–9.39)	0.092[a]	3.12 (1.03–9.45)	0.091[b]
Fall	18/240 (7.50%)	6.41 (2.27–18.07)	0.003[a]	6.47 (2.29–18.27)	0.003[b]
Winter	3/240 (1.25%)	1.00		1.00	
Meat type					
Chicken	23/478 (4.81%)	2.98 (0.88–10.15)	0.142[a]	3.01 (0.88–10.27)	0.140
Ground turkey	14/240 (5.83%)	3.66 (1.04–12.85)	0.090[a]	3.70 (1.05–13.07)	0.088[b]
Ground beef	2/120 (1.67%)	1.00 (0.19–5.25)	1.000	1.00 (0.19–5.27)	1.000
Pork chop	2/120 (1.67%)	1.00		1.00	
Packaging type					
MAP (modified atmospheric packaging)	25/554 (4.51%)	1.31 (0.61–2.80)	0.563	–	–
Plastic bag	7/95 (7.37%)	2.20 (0.86–5.64)	0.168[a]	–	–
Other (vacuum, chub, or paper)	3/137 (2.19%)	0.62 (0.19–2.01)	0.504	–	–
Plastic film	6/172 (3.49%)	1.00			
Label claim					
Conventional	29/624 (4.65%)	1.31 (0.74–2.33)	0.444	–	–
Reduced antibiotic claim	12/334 (3.59%)	1.00			

[a]*A p = 0.25 cut-off from univariate analysis was used for selection of candidate variables for multivariable analysis.*
[b]*Statistically significant at α = 0.10.*

nalidixic acid and reduced susceptibility to ciprofloxacin, and resistance to ceftriaxone was only observed in MDR isolates (**Tables 3**, **4**).

Five distinct antibiogram profiles were observed in MDR isolates, with two *S.* Infantis isolates displaying the tetra-resistant pattern ampicillin, ceftriaxone, tetracycline, and nalidixic acid in addition to reduced susceptibility to ciprofloxacin. In non-MDR isolates, the most common resistance patterns observed were streptomycin and tetracycline (STR-TET, $n = 6$) followed by sulfisoxazole and tetracycline (FIS-TET, $n = 4$) (**Table 4**).

Detection of Antimicrobial Resistance Genes and Plasmid Replicons

Among the 43 *Salmonella* isolates, 16 distinct antimicrobial genes and 17 plasmid replicons were identified. Resistance genes encoding all three types of aminoglycoside-modifying enzymes (AMEs)—acetyltransferases *(aac(6')-Iaa* and *aac(3')-IVa)*, nucleotidyltransferases *(ant(3')-Ia)*, and phosphotransferases *(aph(3')-Ib*, *aph(6')-Id, aph(3')-Ia*, and *aph(4')-Ia)*—were detected in this study. Beta-lactamase genes detected included bla_{TEM-1C} and extended spectrum beta-lactamase (ESBL) $bla_{CTX-M-65}$ from class A and AmpC-type bla_{CMY-33} from class C. Quinolone resistance-mediating genes detected included a mutation of the QRDR of *gyrA* (D87Y). No plasmid-mediated quinolone resistance (PMQR) genes were detected. Other genes detected included those encoding resistance to tetracycline (*tetA* and *tetB*), sulfonamide (*sul1* and *sul2*), and florfenicol-chloramphenicol (*floR*). Eighty-six percent (37/43) of isolates carried at least one plasmid replicon, 55.8% (24/43) carried two or more, and 39.5% (17/43) carried three or more. The most commonly detected plasmids were ColpVC (16/43, 37.21%), IncX1 (12/43, 27.91%), IncI1 (10/43, 23.26%), and IncFIB(AP001918) (9/43, 20.93%). The distribution of all AMR genes and plasmid replicons is presented in **Figure 2**.

Correlation Between Genotype and Phenotype

In this study, resistance genes identified from WGS correlated with phenotypic testing results with an overall sensitivity and specificity of 95.31% and 100%, respectively. Sensitivity was not calculated for azithromycin, meropenem, trimethoprim-sulfamethoxazole, and ciprofloxacin, due to the absence of resistant isolates to these drugs. Discrepancies were present in aminoglycosides, in which two of the three gentamicin-resistant isolates and one of the 15 streptomycin-resistant isolates did not carry resistance-conferring genes, resulting in a sensitivity of 33.33% and 93.33% respectively, for these drugs (**Table 5**).

Hierarchical Clustering of *Salmonella* Isolates by Phenotype, Genotype, and Plasmid Replicon Profiles

Hierarchical clustering of *Salmonella* isolates depicts the co-occurrence of specific AMR profiles and plasmid replicon

TABLE 2 | Distribution of serotypes and multilocus sequence typing patterns for *Salmonella* isolates (n = 43).

Serotype	Retail meat types (no. of isolates)					MLST pattern							
	Chicken (n = 23)	Ground turkey (n = 15)	Ground beef (n = 3)	Pork (n = 2)	Total n/N (%)	*aroC*	*dnaN*	*hemD*	*hisD*	*purE*	*sucA*	*thrA*	ST
S. Agona	0	1	0	0	1/43 (2.33%)	3	3	7	4	3	3	7	13
S. Albany	0	2	0	0	2/43 (4.65%)	104	100	54	78	104	9	48	292
S. Berta	0	1	0	0	1/43 (2.33%)	2	2	3	124	2	2	6	435
S. Braenderup	2	0	0	0	2/43 (4.65%)	12	2	15	14	11	14	16	22
S. Enteritidis	2	0	0	0	2/43 (4.65%)	5	2	3	7	6	6	11	11
S. Infantis	1	2	2	0	5/43 (11.63%)	17	18	22	17	5	21	19	32
S. Kentucky	8	0	0	0	11/43 (25.58%)	62	53	54	60	5	53	54	152
	1	0	0	0		62	53	54	60	636	53	54	3,169
	1	0	0	0		76	14	3	77	64	64	67	198
	1	0	0	0		62	53	54	60	508	53	54	2,132
S. Newport	0	0	1	0	1/43 (2.33%)	2	57	15	14	15	20	12	132
S. Reading	0	8	0	0	8/43 (18.60%)	11	10	25	13	10	58	4	412
S. Rissen	0	0	0	1	1/43 (2.33%)	92	107	79	156	64	151	87	469
S. Schwarzengrund	2	1	0	0	3/43 (6.98%)	43	47	49	49	41	15	3	96
S. Thompson	1	0	0	0	1/43 (2.33%)	14	13	18	12	14	18	1	26
S. Typhimurium	4	0	0	0	4/43 (9.30%)	10	7	12	9	5	9	2	19
S. Uganda	0	0	0	1	1/43 (2.33%)	147	13	15	123	15	19	17	684

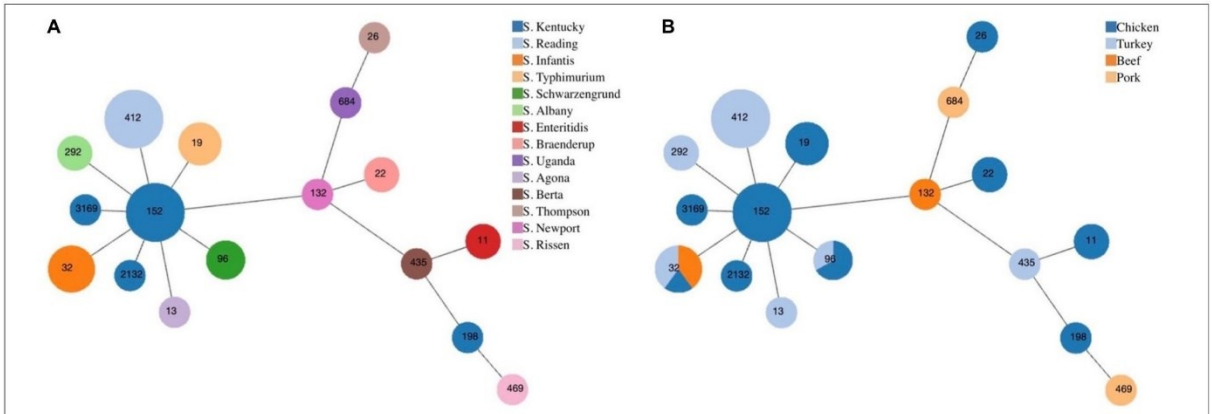

FIGURE 1 | Minimum spanning tree based on multilocus sequence typing of seven housekeeping genes for *Salmonella* isolates from retail meat, by panel **(A)** serotype and **(B)** meat type. Each circle represents one sequence type and is indicated by the number in the circle. The size of each circle corresponds to the number of isolates.

types by serotype and source of isolates. The row dendrogram produced three notable clusters based on isolate-specific profiles. Cluster A corresponds to over half of the *S.* Reading isolates in this study, with the five isolates in this cluster all ampicillin resistant through carriage of a bla_{TEM-1C} gene and displaying intermediate resistance to amoxicillin-clavulanic acid. Three of the four *S.* Typhimurium isolates in this study were presented in cluster B, sharing chicken source and phenotypic resistance to sulfisoxazole and tetracycline conferred through *sul2* and *tetA* genes, respectively. Cluster C included the *S.* Kentucky isolates resistant to streptomycin and tetracycline (n = 7), with resistance conferred through *aph(3')-Ib* (*strA*) and *aph(6')-Id* (*strB*), and the other tetracycline gene detected in this study, *tetB* (**Figure 3**).

Main clusters from the column dendrogram depict the co-occurrence of phenotypes, AMR genes, and plasmid replicons. IncI1, IncX1, and IncFIB(AP001918) replicons were detected primarily in tetracycline (*tetB*) and streptomycin (*aph(6')-Id* and *aph(3')-Ib*) resistant *S.* Kentucky isolates (cluster D). The Col440II and ColpVC replicons were detected in ampicillin-resistant and amoxicillin-clavulanic acid intermediate-resistant *S.* Reading isolates with carriage of bla_{TEM-1C} (cluster E). IncC and ColpHAD28 replicons were detected in tetracycline (*tetA*)

TABLE 3 | Percentage of *Salmonella* isolates resistant to antimicrobials from phenotypic susceptibility testing, by retail meat type.

CLSI class	Antimicrobial rank[a]	Antimicrobial agent	Chicken (*n* = 23)	Ground turkey (*n* = 15)[b]	Ground beef (*n* = 3)[c]	Pork (*n* = 2)	Total *n*/*N* (%)
Aminoglycosides	1	GEN	1 (4.35%)	2 **(13.33%)**	0	0	3/43 (6.88%)
	1	STR	12 **(52.17%)**	3 (20.00%)	0	0	15/43 (34.89%)
B-lactam combination agents	1	AMC	1 (4.35%)	0	0	0	1/43 (2.33%)
Cephems	2	FOX	1 **(4.35%)**	0	0	0	1/43 (2.33%)
	1	AXO	1 (4.35%)	2 (13.33%)	1 **(33.33%)**	0	4/43 (9.30%)
Folate pathway antagonists	2	FIS	5 **(21.74%)**	2 (13.33%)	0	0	7/43 (16.28%)
	2	COT	0	0	0	0	0/43 (0%)
Macrolides	1	AZI	0	0	0	0	0/43 (0%)
Penems	1	MER	0	0	0	0	0/43 (0%)
Penicillins	1	AMP	1 (4.35%)	8 **(53.34%)**	1 (33.33%)	0	10/43 (23.26%)
Phenicols	2	CHL	1 **(4.35%)**	0	0	0	1/43 (2.33%)
Quinolones	1	NAL	1 (4.35%)	2 (13.33%)	2 **(66.67%)**	0	5/43 (11.63%)
	1	CIP[d]	2 (8.70%)[d]	2 (13.33%)[d]	2 **(66.67%)**[d]	0	6/43 (13.95%)[d]
Tetracyclines	2	TET	12 **(52.17%)**	3 (20.00%)	1 (33.33%)	1 (50.0)	17/43 (39.53%)

Meat type with the highest percentage of resistant isolates for each drug is rendered in bold.
[a]Rank based on the WHO categorization of antimicrobials of critical importance to human medicine.
[b]Two isolates with different phenotypic profiles were included from one ground turkey sample.
[c]Two isolates with different phenotypic profiles were included from one ground beef sample.
[d]Results presented for ciprofloxacin (CIP) are for intermediate susceptibility.
GEN, gentamicin; STR, streptomycin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; AXO, ceftriaxone; FIS, sulfisoxazole; COT, trimethoprim-sulfamethoxazole; AZI, azithromycin; MER, meropenem; AMP, ampicillin; CHL, chloramphenicol; NAL, nalidixic acid; CIP, ciprofloxacin; TET, tetracycline.

and sulfisoxazole-resistant (*sul2*) *S.* Typhimurium isolates (cluster F). Lastly, the IncFIB(pN55391) plasmid replicon was detected in MDR *S.* Infantis isolates exhibiting third-generation cephalosporin (ceftriaxone, $bla_{CTX-M-65}$) and quinolone (nalidixic acid, *gyrA* mutation) resistance and fluoroquinolone reduced susceptibility (ciprofloxacin, *gyrA* mutation) (cluster G).

DISCUSSION

Despite improvements to regulatory and farm-to-fork practices in biosecurity, animal husbandry, and HACCP standards, NTS remains a leading pathogen responsible for foodborne illness in the United States. Control and elimination of *Salmonella* in retail meat products are challenging, with food animals serving as perpetual vectors and reservoirs through clinical disease and as asymptomatic carriers, contamination of farm environments, and vertical and horizontal transmission (Jajere, 2019). Though a high proportion of *Salmonella* isolates from food animal origins are pan susceptible, the presence of multiple drug-resistant phenotypes in these isolates remains comparably high relative to that in human clinical isolates, where resistance has remained relatively stable in the past decade (Food and Drug Administration [FDA], 2020b). The persisting recurrence of *Salmonella* outbreaks traced to food animal products and evidence for the emergence, evolution, and dissemination of drug-resistant strains emphasize the importance of active surveillance of AMR in foodborne pathogens. Here, we provide an insight on the presence of *Salmonella* in retail meat products purchased in California and the corresponding AMR phenotypic and genotypic profiles.

Salmonella Contamination of Retail Meat Products

In this study, we observed similar frequencies of *Salmonella* contamination in fresh retail meat products purchased in California (4.3%) compared to the national average from routine NARMS surveillance in the same year (4.0%, 2018) (Food and Drug Administration [FDA], 2020b). A comparison of prevalence findings from different studies should be assessed with caution, as variability in sample collection methods, location, time, and isolation protocols may affect results. Nevertheless, our results here are consistent with previous findings of poultry products being more frequently contaminated with *Salmonella* than other meat types (Jørgensen et al., 2002; Hyeon et al., 2011; Kim et al., 2012; Li et al., 2020) due to the high-frequency colonization and carriage of *Salmonella* in the microbiome of healthy poultry animals (Antunes et al., 2016). The highest recovery of *Salmonella* in our study was in ground turkey (14/240, 5.83%) followed by chicken (23/478, 4.81%) samples, likely due to ground poultry counterparts requiring additional processing steps, which increase opportunities for cross-contamination.

The highest recovery of *Salmonella* from retail meats was observed in fall months in this study. While a previous study also found greater prevalence in fall months (Xu et al., 2020), overall findings have been inconsistent with respect to seasonality trends (Zhao et al., 2001; Zdragas et al., 2012; Wang et al., 2014; Erickson et al., 2018). It has been suggested that temporal trends may occur by year as opposed to season (Sivaramalingam et al., 2013)

TABLE 4 | Antimicrobial resistance (AMR) patterns of *Salmonella* serotypes resistant to one or more antimicrobial drugs from retail meat in California, 2018[a–d].

Serotype		Antimicrobial pattern (no. of isolates)
Name	**No. of isolates *n/N* (%)[b]**	
S. Albany	2/43 (4.65%)	GEN (*n* = 1)
		GEN-STR (*n* = 1)
S. Infantis	5/43 (11.63%)	NAL-CIP[c] (*n* = 1)[e]
		AMP-AXO-NAL-TET-CIP[c] (*n* = 2)[e,f]
		AMP-AXO-NAL-STR-FIS-TET-CIP[c] (*n* = 1)[f]
		CHL-GEN-NAL-STR-FIS-TET-CIP[c] (*n* = 1)
S. Kentucky	9/43 (20.93%)	STR (*n* = 1)
		STR-TET (*n* = 6)
		STR-TET-CIP[c] (*n* = 1)
		AMC-AMP-FOX-AXO-STR (*n* = 1)
S. Reading	6/43 (13.95%)	AMP (*n* = 5)
		AMP-STR-FIS-TET (*n* = 1)
S. Rissen	1/43 (2.33%)	TET (*n* = 1)
S. Schwarzengrund	2/43 (4.65%)	STR (*n* = 2)
S. Typhimurium	4/43 (9.30%)	FIS-TET (*n* = 4)
Total	29/43 (67.44%)	–

Multidrug-resistant isolates are rendered in bold.
[a]Isolates from the following serotypes not listed were susceptible to all 14 antibiotics tested: S. Agona (n = 1), S. Berta (n = 1), S. Braenderup (n = 2), S. Enteritidis (n = 2), S. Kentucky (n = 2), S. Newport (n = 1), S. Reading (n = 2), S. Schwarzengrund (n = 1), S. Thompson (n = 1), and S. Uganda (n = 1).
[b]Percentages calculated as number of isolates resistant to one or more antimicrobial drugs to the total number of Salmonella isolates in the study.
[c]Ciprofloxacin (CIP) in antimicrobial patterns indicates intermediate susceptibility.
[d]Multidrug resistance (MDR) was defined as resistance to ≥1 drug in ≥3 antimicrobial classes.
[e]Isolates were recovered from the same ground beef sample.
[f]Isolates were recovered from the same ground turkey sample.
GEN, gentamicin; STR, streptomycin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; AXO, ceftriaxone; FIS, sulfisoxazole; COT, trimethoprim-sulfamethoxazole; AZI, azithromycin; MER, meropenem; AMP, ampicillin; CHL, chloramphenicol; NAL, nalidixic acid; CIP, ciprofloxacin; TET, tetracycline.

and that the discordances in observed seasonal contamination of *Salmonella* in food products are likely confounded by other contributing factors from production, processing, and distribution processes that serve as primary drivers of pathogen contamination and proliferation. From a food safety perspective, this contrasts with the seasonality trends observed in incidences of human salmonellosis, which has been associated with factors such as temperature and oscillations in human activity (e.g., increased consumption of meat products during certain times of the year) rather than differences in the frequency of retail meat contamination itself (Ravel et al., 2010).

Distribution of *Salmonella* Serotypes and Antimicrobial Resistance Profiles

An important facet to *Salmonella* epidemiology is the fluctuating significance, distribution, and AMR profiles of serotypes over time. *Salmonella* serotypes by nature display host specificity and varied pathogenicity for human and animal hosts depending on their degree of adaptation (Jajere, 2019). Common serotypes that have been associated with foodborne disease have been broad-spectrum host adapted such as *S.* Enteritidis, *S.* Typhimurium, and the monophasic *S.* Typhimurium variant *S.* 1,4,[5],12:i:- (Yang et al., 2015; Antunes et al., 2016; Jajere, 2019; Mandilara et al., 2021).

The two *S.* Enteritidis and four *S.* Typhimurium isolates recovered in this study were all from retail chicken products, of which both *S.* Enteritidis isolates were susceptible to all 14 antibiotics tested and the four *S.* Typhimurium isolates displayed resistance to two drugs, tetracycline and sulfisoxazole. Tetracycline and sulfonamides are two major classes of antibiotics that have been conventionally utilized for prophylactic and therapeutic treatment of food animals (Granados-Chinchilla and Rodríguez, 2017). Tetracycline is a broad-spectrum bacteriostatic agent that was traditionally widely administered to poultry flocks through drinking water and feed (Chopra and Roberts, 2001). As of January 2017, its application in feed has been limited to therapeutic use through a requirement of the Veterinary Feed Directive (Granados-Chinchilla and Rodríguez, 2017). Despite this restriction, the highest frequency of resistance in our study was to tetracycline (17/43, 39.5%), driven by the proportionately large number of resistant chicken isolates (12/23, 52.2%). We observe a similar trend in streptomycin (15/43, 34.9%) due to the high level of resistance across all serotypes from chicken isolates (12/23, 52.2%). Streptomycin is another drug that is historically used in food-producing animals and serves as both an indicator for aminoglycoside resistance in the food supply chain (McDermott et al., 2016; Wang et al., 2019) and an epidemiologic marker for presence of penta resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline (ACSSuT), a pattern observed in widely disseminated virulent and MDR strains like *S.* Typhimurium DT104 and U302 (Yu et al., 2008). The third most frequent resistant drug observed in our study was ampicillin (10/43, 23.3%) attributed by ground turkey isolates (8/15, 53.3%), which is consistent to the routinely higher level of ampicillin resistance detected through NARMS surveillance in retail turkey isolates (35.5%, 2018) relative to those from retail chicken, beef, and pork (Food and Drug Administration [FDA], 2020b, 2021c; Singer et al., 2020). Lastly, the detection of four MDR *S.* Infantis isolates from retail chicken, ground turkey, and ground beef products in our study mirrors NARMS surveillance findings from the last few years in which the rise in MDR *Salmonella* from retail meats is attributed by a marked increase in MDR *S.* Infantis superseding other leading resistant serotypes (Food and Drug Administration [FDA], 2021c; Tyson et al., 2021).

Whole-Genome Sequencing for Prediction of Antimicrobial Resistance

Increasing affordability and improved turnaround time for WGS have vastly improved the resolution of foodborne bacteria profiling and allowed for its integration in routine surveillance efforts as done here in this study. In particular, its utility for identification of resistance mechanisms provides the genotypic basis for *in silico* predictions of phenotypic resistance including

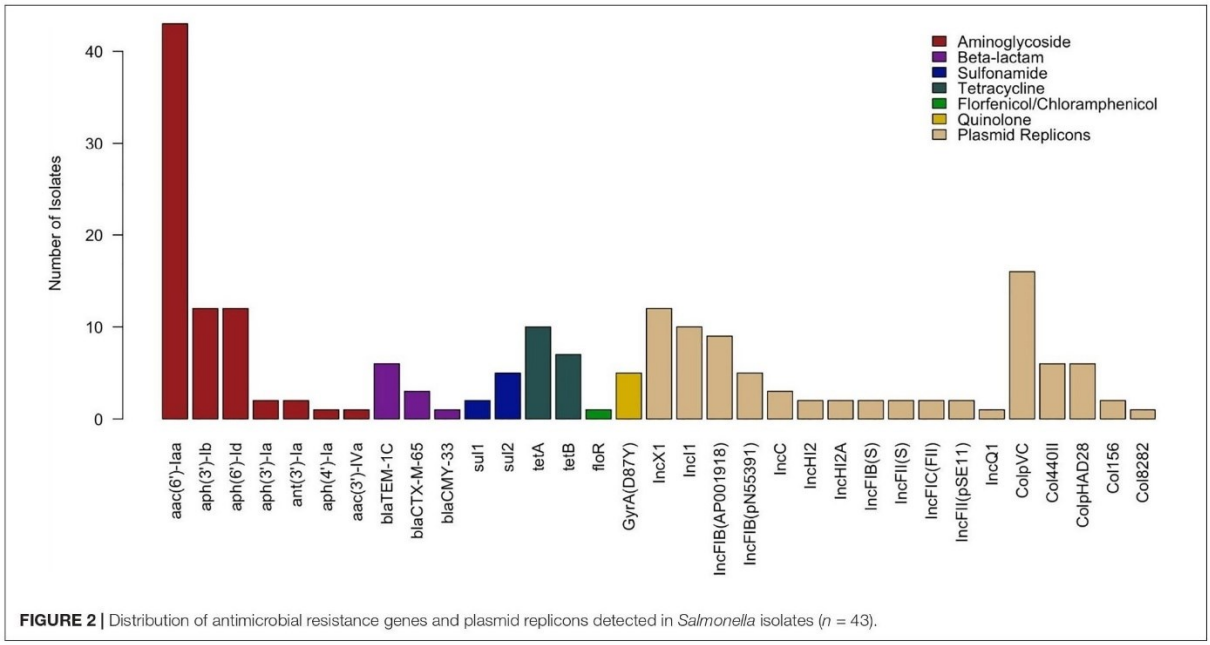

FIGURE 2 | Distribution of antimicrobial resistance genes and plasmid replicons detected in *Salmonella* isolates (n = 43).

TABLE 5 | Evaluation of genotype prediction of phenotypic resistance.

CLSI class	Antimicrobial agent	Phenotype: susceptible (no. of isolates)		Phenotype: resistant (no. of isolates)		Sensitivity[b] (%)	Specificity[c] (%)
		Genotype: resistant (FP)[a]	Genotype: susceptible (TN)[a]	Genotype: resistant (TP)[a]	Genotype: susceptible (FN)[a]		
Aminoglycosides	GEN	0	40	1	2	33.33	100
	STR	0	28	14	1	93.33	100
B-lactam combination agents	AMC	0	42	1	0	100	100
Cephems	FOX	0	42	1	0	100	100
	AXO	0	39	4	0	100	100
Folate pathway antagonists	FIS	0	36	7	0	100	100
	COT	0	43	0	0	N/A[d]	100
Macrolides	AZI	0	43	0	0	N/A[d]	100
Penems	MER	0	43	0	0	N/A[d]	100
Penicillins	AMP	0	33	10	0	100	100
Phenicols	CHL	0	42	1	0	100	100
Quinolones	NAL	0	38	5	0	100	100
	CIP	0	43	0	0	N/A[d]	100
Tetracyclines	TET	0	26	17	0	100	100
Overall		0	538	61	3	95.31	100

[a]*FP, false positive; TN, true negative; TP, true positive; FN, false negative.* [b]*Sensitivity was calculated as TP/(TP + FN).* [c]*Specificity was calculated as TN/(TN + FP).* [d]*Sensitivity could not be calculated because none of the isolates were resistant to these drugs. GEN, gentamicin; STR, streptomycin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; AXO, ceftriaxone; FIS, sulfisoxazole; COT, trimethoprim-sulfamethoxazole; AZI, azithromycin; MER, meropenem; AMP, ampicillin; CHL, chloramphenicol; NAL, nalidixic acid; CIP, ciprofloxacin; TET, tetracycline.*

resistance for drugs that are not included in routine testing (McDermott et al., 2016; Su et al., 2019; NIHR Global Health Research Unit on Genomic Surveillance of AMR, 2020). Despite the small number of isolates in this study and the large proportion that are susceptible, our findings here affirm results from previous studies that demonstrated the robust capacity of WGS for prediction of phenotypic resistance in *Salmonella* and other bacterial species (Tyson et al., 2015, 2018; McDermott et al., 2016; Neuert et al., 2018). For the 43 *Salmonella* isolates here, WGS data predicted phenotypic

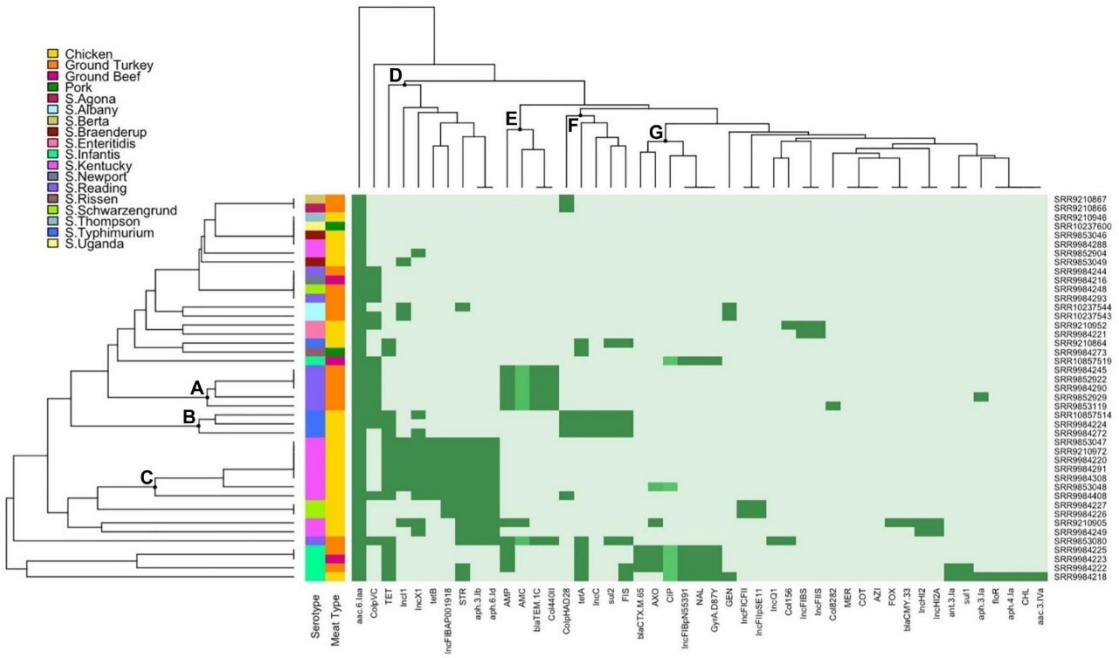

FIGURE 3 | Heatmap and hierarchical clustering of *Salmonella* isolates by phenotypic antimicrobial resistance (AMR), antimicrobial genes, and plasmid replicons. Dark green represents resistant phenotype, presence of gene, or presence of plasmid replicon. Medium green indicates reduced susceptibility for ciprofloxacin or intermediate resistance for amoxicillin-clavulanic acid and ceftriaxone. Light green indicates susceptible phenotype, absence of gene, or absence of plasmid replicon. Letters (A–G) indicate the main clusters discussed in the text. GEN, gentamicin; STR, streptomycin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; AXO, ceftriaxone; FIS, sulfisoxazole; COT, trimethoprim-sulfamethoxazole; AZI, azithromycin; MER, meropenem; AMP, ampicillin; CHL, chloramphenicol; NAL, nalidixic acid; CIP, ciprofloxacin; TET, tetracycline.

results with an overall sensitivity and specificity of 95.31% and 100%, respectively.

The three discordant isolates resulting in lowered sensitivity were observed for gentamicin and streptomycin, attributed by phenotypic resistance in the absence of detected resistance genes. One potential explanation is that genes may not have been present when a colony was sequenced at a different time from when phenotypic testing was performed, but moreover, the imperfect correlation observed here presents a few important considerations. First, concordant genotypic prediction of phenotypic resistance is reliant on the recommended breakpoints used for classification of isolates. False classification of phenotypic results can occur when MIC values fall just below or above a breakpoint and/or in instances where interpretation of MICs is based on alternative guidelines like NARMS consensus interpretative criteria in the absence of available CLSI criteria. This is a likely explanation for the discordant streptomycin results observed here and previous studies (Tyson et al., 2015, 2018; McDermott et al., 2016; Neuert et al., 2018; Pornsukarom et al., 2018), as streptomycin is a drug traditionally used for food animals but not in the treatment of enteric infections and therefore lacks defined CLSI clinical breakpoints (McDermott et al., 2016; Wang et al., 2019). Secondly, in instances where interpretative criteria are available, alternative use of different breakpoints for classification can also affect concordance of WGS results with phenotype. For instance, specificity for ciprofloxacin in this study was fully concordant only when using CLSI breakpoints for resistance (≥1 μg/ml). The CLSI breakpoint for reduced ciprofloxacin susceptibility (≥0.12 μg/ml) is currently used as an alternative criterion for classification of resistance to capture emerging fluoroquinolone resistance (Food and Drug Administration [FDA], 2021b). Traditionally, resistance to fluoroquinolones is conferred through one or more chromosomal mechanisms mediated by mutations in the QRDRs of target enzymes DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) (Hooper and Jacoby, 2016). For ciprofloxacin, resistance is observed to be conferred in combinations of mutations within both *gyrA* and *parC* (Redgrave et al., 2014; Neuert et al., 2018). Five isolates in this study exhibited reduced susceptibility, but classification of these isolates as resistant would have resulted in lowered specificity—88.37% (38/43) instead of 100%—as these isolates only carried a single *gyrA* mutation. Lastly, WGS predictions of phenotypic resistance are as robust as our ability to identify the genetic determinants that confer resistance. This highlights the impact of reference database(s) selection, importance of active curation and inclusion of novel genes to ensure database

comprehensiveness, and an unavoidable caveat of relying on WGS approaches when unknown resistance mechanisms cannot be detected.

Co-occurrence of Resistance Profiles and Plasmid Replicons by Serotype and Source

Although the significance of the associations between serotypes and the presence/absence of AMR genes and plasmid replicons could not be assessed due to the small number of isolates recovered for each serotype, results here are congruent with other studies that have reported co-occurrence of certain plasmid(s) and AMR genes with serotype (Mather et al., 2013; Pornsukarom et al., 2018; Zhao et al., 2020). It should be noted that while the presence of a plasmid replicon is likely indicative of the corresponding plasmid type, it is possible that the replicon type may have been chromosomally integrated or co-integrated on plasmids with multiple replicons (Johnson et al., 2007; McMillan et al., 2020). Such occurrences are considered rare, and the detection of plasmid replicons as done here presents a quick and efficient way to screen for the presence of putative plasmids.

In this study, we identified several plasmid replicons previously detected in *Salmonella* that are associated with resistance. IncX1 and IncI1 are two incompatibility groups frequently distributed in *Enterobacteriaceae*, with the latter frequently found in *Salmonella* from food animal sources (Kaldhone et al., 2019), as evident through the *S.* Kentucky isolates from chicken samples in this study. IncC (formerly grouped as IncA/C) plasmids are also frequently prevalent in pathogenic *Enterobacteriaceae* and are associated with bla_{CMY} and bla_{NDM} genes (Hancock et al., 2017; Ambrose et al., 2018), though here we observed their co-occurrence with *sul2* and *tetA* genes in *S.* Typhimurium isolates from chicken. In *S.* Reading turkey isolates from this study, we detected the Col440II replicon and bla_{TEM-1C}. Carriage of bla_{TEM-1C} on Col440II was first detected in a *S.* Hadar turkey isolate in 2007, where thereafter detection of this plasmid with bla_{TEM-1C} was reported in *S.* Reading in 2014, also from a turkey source (Miller et al., 2020). Recently, *S.* Reading isolates with this plasmid were identified in an emerged clade linked to United States and Canadian outbreaks from live turkeys and raw turkey products, including one which occurred during our 2018 study period (November 2017 to March 2019) (Centers for Disease Control and Prevention [CDC], 2019; Hassan et al., 2019; Miller et al., 2020). The genomic investigation of isolates from clinical, meat product, environmental, and animal sources from Miller et al. indicated that a novel clone of *S.* Reading emerged and disseminated across North America in parallel to expansion of commercial turkey production, likely through vertical transmission from a common source (Miller et al., 2020). Detection of turkey isolates with the distinguishing carriage of Col440II and bla_{TEM-1C} gene here supports their findings and highlights the value of integrated surveillance in detection and elucidation of emerging microbial hazards in the food supply chain.

Another public health concern to note from this study is the detection of the IncFIB(pN55391) plasmid replicon among four MDR *S.* Infantis isolates. The IncFIB(pN55391) plasmid was first detected in MDR, ESBL-producing *S.* Infantis strains in Israel, where thereafter rapid clonal expansion resulted in its worldwide dissemination (Franco et al., 2015; Hindermann et al., 2017; Alba et al., 2020; García-Soto et al., 2020). To date, MDR ESBL-producing *S.* Infantis has been reported across the United States in humans, food animals, and—as evidenced here and in previous studies—retail meats (Tate et al., 2017; Brown et al., 2018; Alba et al., 2020; M'ikanatha et al., 2021; Tyson et al., 2021). In the past few years, NARMS surveillance has reported a rise in resistant *Salmonella* isolates from retail meats, which is attributed to increasing numbers of MDR *S.* Infantis (Tyson et al., 2021). Dissemination of *S.* Infantis with ESBL carriage on a large conjugative plasmid as indicated by four of the five isolates carrying $bla_{CTX-M-65}$ in this study is concerning due to its potential to disseminate resistance genetic elements to other pathogens and the challenges in treating infections exhibiting resistance to penicillins, extended-spectrum cephalosporins, monobactams, and other drugs conferred through MDR status (Tate et al., 2017; M'ikanatha et al., 2021).

CONCLUSION

Despite the relatively low frequency of *Salmonella* contamination observed in retail meat products in this study, the diversity of serotypes and AMR profiles present in the isolates recovered highlights the risk of retail meat products as reservoirs and conduits for drug-resistant NTS. Findings here also demonstrate the complementary role of WGS with phenotypic testing for the high-resolution profiling of foodborne pathogens. Moreover, this study sheds light on the importance of surveillance for the assessment of emerging and circulating AMR hazards and the need to continue these efforts to best guide intervention measures for AMR mitigation across farm-to-fork interfaces.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

KLe, EA, MP, and XL conceptualized the study. KLe, AH, MR, MS, and MH-F performed the laboratory analysis. KLe, AH, KLa, MR, MS, and MH-F curated the data. KLe analyzed the data and contributed in the writing—original draft preparation. KLe, EA, and XL performed the methodology. EA, MP, and XL gathered resources. MP, EA, AH, KLa, XL, MR, MS, and MH-F contributed in the writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.835699/full#supplementary-material

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Chapter 2: Assessment of prevalence and diversity of antimicrobial resistant *Escherichia coli* from retail meats in southern California

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Assessment of Prevalence and Diversity of Antimicrobial Resistant *Escherichia coli* from Retail Meats in Southern California

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Abstract: Retail meat products may serve as reservoirs and conduits for antimicrobial resistance, which is frequently monitored using *Escherichia coli* as indicator bacteria. In this study, *E. coli* isolation was conducted on 221 retail meat samples (56 chicken, 54 ground turkey, 55 ground beef, and 56 pork chops) collected over a one-year period from grocery stores in southern California. The overall prevalence of *E. coli* in retail meat samples was 47.51% (105/221), with *E. coli* contamination found to be significantly associated with meat type and season of sampling. From antimicrobial susceptibility testing, 51 isolates (48.57%) were susceptible to all antimicrobials tested, 54 (51.34%) were resistant to at least 1 drug, 39 (37.14%) to 2 or more drugs, and 21 (20.00%) to 3 or more drugs. Resistance to ampicillin, gentamicin, streptomycin, and tetracycline were significantly associated with meat type, with poultry counterparts (chicken or ground turkey) exhibiting higher odds for resistance to these drugs compared to non-poultry meats (beef and pork). From the 52 *E. coli* isolates selected to undergo whole-genome sequencing (WGS), 27 antimicrobial resistance genes (ARGs) were identified and predicted phenotypic AMR profiles with an overall sensitivity and specificity of 93.33% and 99.84%, respectively. Clustering assessment and co-occurrence networks revealed that the genomic AMR determinants of *E. coli* from retail meat were highly heterogeneous, with a sparsity of shared gene networks.

Keywords: *Escherichia coli*; antimicrobial resistance (AMR); retail meat; phenotype; whole-genome sequencing (WGS); resistance genes; public health surveillance

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1. Introduction

The emergence and dissemination of antimicrobial resistance (AMR) is of worldwide public health concern [1]. As a habitant of the endogenous microbiota of both humans and animals, *Escherichia coli* is both a commensal enteric bacterium and a pathogen responsible for various nosocomial, foodborne, and waterborne infections [2–12]. The increasing global incidence of multidrug resistant *E. coli*, particularly those resistant to therapeutically important drugs such as cephalosporins and fluoroquinolones, and last resort antibiotics such as carbapenem and colistin, prompts the need for integrated initiatives to monitor and reduce the spread of resistant organisms and their AMR genetic determinants [13–16]. Enhancing our understanding of AMR in *E. coli* is important as their ubiquity and genomic plasticity enables high frequency of AMR mobilization, which promotes the acquisition and transfer of resistance to other bacterial species [17,18]. Hence, commensal *E. coli* is also frequently utilized as indicator organisms of AMR for the broader microbial community [19–21].

Antimicrobial use in human and veterinary medicine are perceived as key drivers of AMR emergence [22,23], with selective pressures imposed amongst food animals comprising one avenue of public health concern [24,25]. Raw foods of animal origin such as meat products may serve as reservoirs and conduits for AMR [26,27], and have been included in

integrated monitoring efforts due to their epidemiological linkage to foodborne outbreaks involving zoonotic pathogens [28,29] and the need to better understand the maintenance and dissemination of AMR along the food chain [30]. In the United States, the National Antimicrobial Resistance Monitoring System (NARMS) monitors AMR in various foodborne bacteria—including commensal *E. coli*—from humans, food-producing animals, and retail meat [31]. The distribution of AMR has been suggested to vary geographically [1,32,33], with human activity and movement of food and animals contributing to the evolution of bacterial populations [34–37].

In this study, we present the first available data on *E. coli* from retail meats in southern California as part of expanded surveillance coverage of NARMS retail meat sampling in 2018. The objective of this study was to determine the prevalence, the distribution of AMR and associated genetic determinants, and the potential drivers of AMR variability in *E. coli* from retail meat. This study aims to enhance our understanding of AMR in foodborne *E. coli*, and to provide insight on the clinical and epidemiologic risks associated with retail meat products in California.

2. Results

2.1. Risk Factors Associated with the Presence of E. coli in Retail Meat Products

Escherichia coli isolates were recovered from 47.51% (105/221) of samples, with the highest frequency observed in ground turkey (70.37%, 38/54), followed by chicken (67.86%, 38/56), pork chop (32.14%, 18/56), and ground beef (20.00%, 11/55) (Table 1).

Table 1. Risk factors for presence of *E. coli* in retail meat products from southern California.

Risk Factor	*E. coli* Positive n/N (%)	Univariable Models		Multivariable Model	
		OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
Meat Type					
Chicken	38/56 (67.86%)	8.44 (3.55, 20.09)	<0.0001	9.43 (3.84, 23.21)	<0.0001
Ground Turkey	38/54 (70.37%)	9.50 (3.93, 22.95)	<0.0001	11.00 (4.39, 27.54)	<0.0001
Pork Chop	18/56 (32.14%)	1.90 (0.80, 4.51)	0.15	1.93 (0.79, 4.71)	0.15
Ground Beef	11/55 (20.00%)	1.00	-	1.00	-
Season					
Spring	22/60 (36.67%)	0.30 (0.13, 0.69)	0.0046	0.22 (0.083, 0.55)	0.0015
Summer	28/60 (46.67%)	0.45 (0.20, 1.03)	0.0592	0.36 (0.14, 0.91)	0.031
Autumn	28/60 (46.67%)	0.45 (0.20, 1.03)	0.0592	0.36 (0.14, 0.91)	0.031
Winter	27/41 (65.85%)	1.00	-	1.00	-
Packaging type					
Modified atmosphere packaging (MAP)	55/85 (64.71%)	3.40 (1.55, 7.48)	0.0023	-	-
Plastic film	26/64 (40.63%)	1.27 (0.56, 2.88)	0.57	-	-
Other (Vacuum, chub, paper)	10/32 (31.25%)	0.84 (0.31, 2.27)	0.74	-	-
Plastic bag	14/40 (35.00%)	1.00	-	-	-
Label Claim					
Reduced antibiotic claim	27/37 (72.97%)	3.67 (1.68, 8.02)	0.0011	-	-
Conventional	78/184 (42.39%)	1.00	-	-	-
Presence of *Salmonella*					
Yes	8/11 (72.73%)	3.11 (0.80, 12.04)	0.10	-	-
No	97/210 (46.19%)	1.00	-	-	-
Overall prevalence	105/221 (47.51%)	-	-	-	-

From multivariable logistic regression, risk factors which were significantly associated with the presence of *E. coli* in retail meat products included meat type and season. The odds of *E. coli* isolation were 9.43 (95% CI 3.84–23.21) and 11.00 (95% CI 4.39–27.54) times higher in skin-on/bone-in chicken and ground turkey compared to ground beef products, respectively. Samples purchased in the spring, summer, and autumn had a 0.22 (95% CI 0.083–0.55), 0.36 (95% CI 0.14–0.91), and 0.36 (95% CI 0.14–0.91) times odds of *E. coli* isolation compared to those purchased in the winter. (Table 1).

2.2. Phenotypic Antimicrobial Resistance of E. coli from Retail Meat Products

From antimicrobial susceptibility testing (AST) of 105 *E. coli* isolates, 51 (48.57%) were susceptible to all antimicrobials tested, 54 isolates (51.43%) were resistant to at least 1 drug, 39 (37.14%) to 2 or more, and 21 (20.00%) to 3 or more drugs. All isolates were susceptible to azithromycin and meropenem, and 15 (14.29%) were multidrug resistant (MDR). Of the MDR isolates, 10 (66.67%) were from ground turkey, 3 (20%) were from chicken, and 2 (13.33%) were from pork chops.

E. coli isolates exhibited the highest overall frequency of resistance to tetracycline (43.81%), followed by streptomycin (30.48%), ampicillin (20.95%), and gentamicin (16.19%) (Table 2). From exact logistic regression, isolates from ground turkey had a significantly higher odds of resistance to ampicillin (OR 4.94, 95% CI 1.17–30.11), streptomycin (OR 3.81, 95% CI 1.10–15.56), and tetracycline (OR 5.24, 95% CI 1.65–18.55) compared to isolates from non-poultry meat types (beef and pork). Isolates from chicken and ground turkey products also exhibited higher odds of resistance to gentamicin compared to those from non-poultry origin; however, the association was only significant for isolates from chicken (OR 8.47, 95% 1.05–394.18) (Table 3). Collectively, *E. coli* isolates in this study had high diversity of phenotypic AMR patterns, with a total of 21 unique antibiogram patterns identified amongst the 54 non-susceptible isolates. Contributing to this diversity in distribution of antibiogram patterns were three isolates—two MDR isolates from pork chops and one non-MDR from ground turkey—with decreased susceptibility to ciprofloxacin (Table 4).

Table 2. Distribution of phenotypic antimicrobial resistance in *E. coli* isolates from retail meat samples (n = 105).

CLSI Class	Antimicrobial Agent	Number of Isolates Resistant to Antimicrobial Drugs (%)				
		Chicken (n = 38)	Ground Turkey (n = 38)	Pork Chop (n = 18)	Ground Beef (n = 11)	All Samples (n = 105)
Aminoglycosides	STR	10 (26.32%)	17 (44.74%)	5 (27.78%)	0 (0%)	32 (30.48%)
	GEN	9 (23.68%)	7 (18.42%)	1 (5.56%)	0 (0%)	17 (16.19%)
Beta-lactam combination agents	AMC	1 (2.63%)	2 (5.26%)	0 (0%)	0 (0%)	3 (2.86%)
Cephems	FOX	1 (2.63%)	1 (2.63%)	0 (0%)	0 (0%)	2 (1.90%)
	AXO	1 (2.63%)	1 (2.63%)	0 (0%)	0 (0%)	2 (1.90%)
Folate pathway antagonists	COT	0 (0%)	2 (5.26%)	0 (0%)	0 (0%)	2 (1.90%)
Macrolides	AZI	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Penems	MER	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Penicillins	AMP	5 (13.16%)	14 (36.84%)	3 (16.67%)	0 (0%)	22 (20.95%)
Phenicols	CHL	0 (0%)	2 (5.26%)	0 (0%)	0 (0%)	2 (1.90%)
Quinolones	NAL	0 (0%)	1 (2.63%)	1 (5.56%)	0 (0%)	2 (1.90%)
	CIP	0 (0%)	1 (2.63%)	0 (0%)	0 (0%)	1 (0.95%)
Tetracycline	TET	15 (39.47%)	24 (63.16%)	7 (38.89%)	0 (0%)	46 (43.81%)

STR: streptomycin; GEN: gentamicin; AMC: amoxicillin/clavulanic acid; FOX: cefoxitin; AXO: ceftriaxone; COT: trimethroprim-sulfamethoxazole; AZI: azithromycin; MER: meropenem; AMP: ampicillin; CHL: chloramphenicol; NAL: nalidixic acid; CIP: ciprofloxacin; TET: tetracycline.

Table 3. Association between retail meat type and phenotypic antimicrobial resistance.

Meat Type	Antimicrobial Drug (Abbreviation)							
	Ampicillin (AMP)		Gentamicin (GEN)		Streptomycin (STR)		Tetracycline (TET)	
	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
Chicken	1.31 (0.23, 9.20)	1.00	8.47 (1.05, 394.18)	0.042	1.70 (0.45, 7.27)	0.56	2.03 (0.63, 7.08)	0.29
Ground turkey	4.94 (1.17, 30.11)	0.025	6.18 (0.72, 294.92)	0.13	3.81 (1.10, 15.56)	0.032	5.24 (1.65, 18.55)	0.003
Non-poultry (beef, pork)	1.00	-	1.00	-	1.00	-	1.00	-

Table 4. Distribution of phenotypic antibiogram patterns in *E. coli* isolates (n = 105).

Antibiogram Pattern	Number of Isolates (n/N %)	Multidrug Resistant
Susceptible to all drugs in MIC panel	51 (48.57%)	No
TET	12 (11.43%)	No
STR-TET	8 (7.62%)	No
AMP-TET	5 (4.76%)	No
GEN-STR-TET	5 (4.76%)	No
GEN-STR	4 (3.81%)	No
AMP	2 (1.90%)	No
AMP-GEN-STR-TET	2 (1.90%)	Yes
AMP-GEN-TET	2 (1.90%)	Yes
AMP-STR-TET	2 (1.90%)	Yes
AMC-AMP-FOX-AXO-GEN-STR-TET	1 (0.95%)	Yes
AMC-AMP-FOX-AXO-GEN-TET	1 (0.95%)	Yes
AMC-AMP-STR-TET	1 (0.95%)	Yes
AMP-CHL-STR-TET	1 (0.95%)	Yes
AMP-CHL-STR-TET-COT	1 (0.95%)	Yes
AMP-GEN-NAL-STR-TET-CIP	1 (0.95%)	Yes
AMP-GEN-STR	1 (0.95%)	No
AMP-NAL-STR-TET-CIP^{dsc} *	1 (0.95%)	Yes
AMP-STR-TET-CIP^{dsc} *	1 (0.95%)	Yes
STR	1 (0.95%)	No
STR-TET-CIP^{dsc}	1 (0.95%)	No
STR-TET-COT	1 (0.95%)	Yes

* CIP^{dsc} denotes decreased susceptibility to ciprofloxacin. STR: streptomycin; GEN: gentamicin; AMC: amoxicillin/clavulanic acid; FOX cefoxitin: AXO ceftriaxone; COT: trimethroprim-sulfamethoxazole; AZI: azithromycin: MER: meropenem; AMP: ampicillin; CHL: chloramphenicol; NAL: nalidixic acid; CIP: ciprofloxacin; TET: tetracycline.

2.3. Genetic Determinants of AMR in E. coli from Retail Meat Products

Whole-genome sequencing (WGS) was conducted on a random subset of *E. coli* isolates in this study (n = 52). The genomes of these 52 *E. coli* isolates were screened for AMR genetic determinants including point mutations conferring quinolone resistance. A total of 27 AMR determinants were identified, including *mdf(A)*, which was detected in all isolates, and other commonly occurring genes corresponding to tetracycline (*tet(A)*, *tet(B)*, and *tet(C)*) and sulfonamide (*sul1* and *sul2*) resistance. The largest diversity of genes was observed for aminoglycoside resistance (nine genes encoding for various acetyltransferases, nucleotidyltransferases, or phosphotransferases) followed by those for beta-lactam resistance (bla_{CMY-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{HERA-3}, and $bla_{SHV-187}$). Notably, a variant of a colistin resistance gene, *mcr-9*, was detected in one MDR *E. coli* isolate from a chicken sample. Quinolone resistance genetic determinants were detected in two isolates including (1) a plasmid-mediated quinolone resistance (PMQR) gene, *qnrB19*, from an MDR isolate from pork chop; and (2) two chromosomal mutations in *gyrA* encoding S83L and D87N

amino acid substitutions from an MDR isolate from ground turkey. Other resistance genes detected included those conferring resistance to macrolide (*ere(A)*), phenicol (*floR*), and folate synthesis inhibitor (*dfrA14*) drug(s) (Figure 1a).

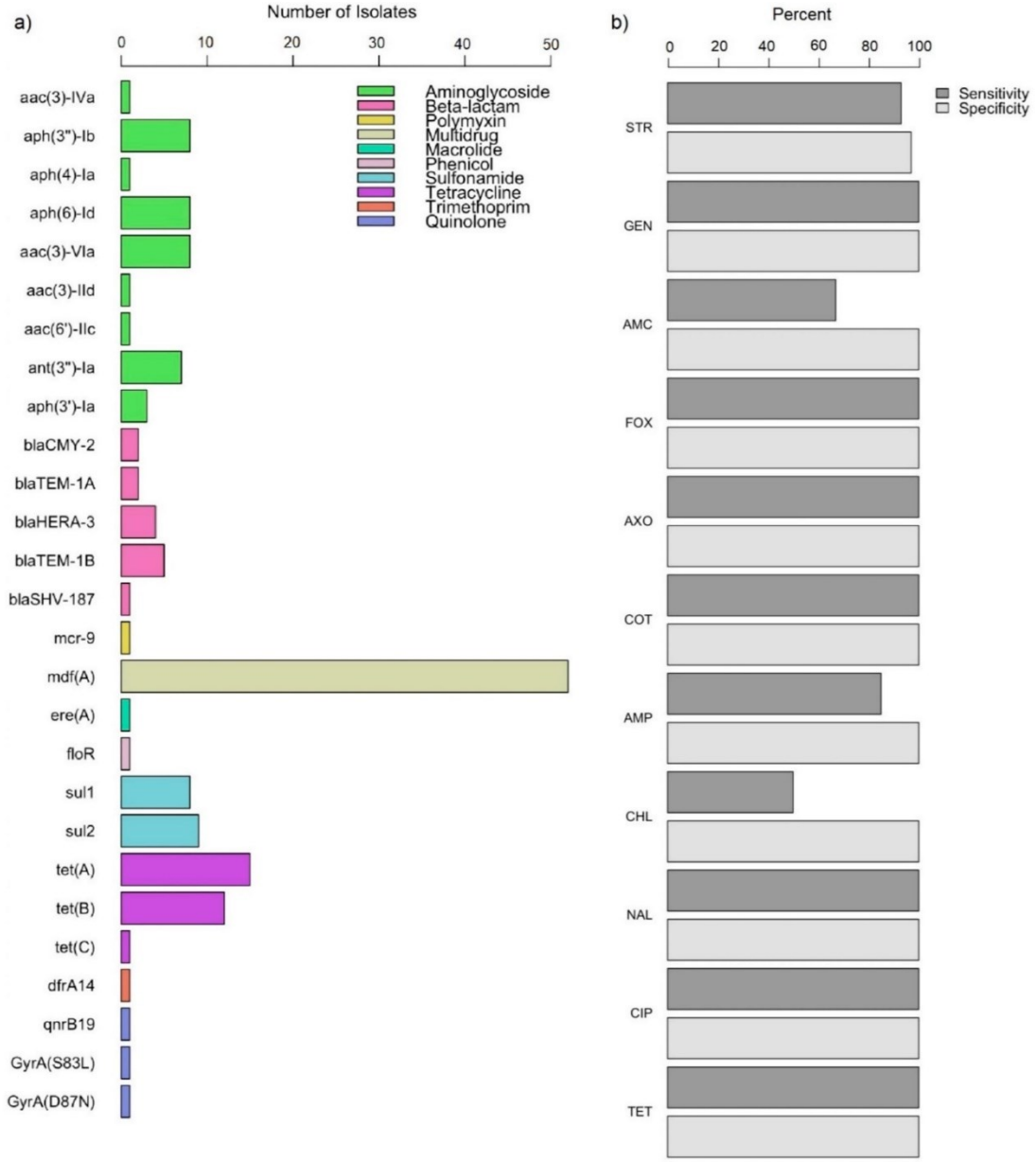

Figure 1. Genomic antimicrobial resistance in *E. coli* isolates from retail meat (n = 52), by (**a**) distribution of antimicrobial resistance genes and (**b**) concordance with phenotypic antimicrobial resistance. STR: streptomycin; GEN: gentamicin; AMC: amoxicillin/clavulanic acid; FOX: cefoxitin; AXO: ceftriaxone; COT: trimethroprim-sulfamethoxazole; AMP: ampicillin; CHL: chloramphenicol; NAL: nalidixic acid; CIP: ciprofloxacin; TET: tetracycline.

2.4. Concordance of AMR Phenotype and Genotype

Based on the 52 *E. coli* isolates that underwent both WGS and AST, the drug-specific and overall sensitivity and specificity was determined to assess WGS predictions of resistant and susceptible AMR phenotypes, respectively. In this study, AMR genetic determinants identified through WGS predicted phenotypic AMR with an overall sensitivity of 93.33% and specificity of 99.84%. The greatest discordance for sensitivity was observed for chloramphenicol (50%), followed by amoxicillin/clavulanic acid (66.67%), ampicillin (84.62%), and streptomycin (93.33%). Discordance for specificity was observed only for streptomycin (97.30%) (Figure 1b, Table S3).

2.5. Clustering Analysis of E. coli Isolates

From the presence and absence of AMR genetic determinants, *E. coli* isolates of retail meat origin in this study did not differ significantly by meat type, season, packaging type, and label claim (PERMANOVA and ANOSIM $p > 0.05$; Figure 2 and Table 5). Tests for differences in *E. coli* AMR genetic compositions indicated that dispersion differences were present among retail meat types (PERMDISP2 $p < 0.05$; Table 5). The results of PERMANOVA and/or ANOSIM by the grouping factor of meat type should thus be interpreted with care due to the assumption of equal variance among meat types not being met. By season, packaging type, and label claim, the compositional variance among the groups for each of these factors was not significantly different (PERMDISP2 $p > 0.05$; Table 5). Overall, the grouping factors assessed in this study each accounted for a relatively low proportion of the variance in *E. coli* AMR genetic composition (PERMANOVA R^2 0.0094–0.088), with indications of even distributions of ranks within and between groups for packaging types (ANOSIM R = 0.038) and greater dissimilarities in the average of ranks within-group than those of between-groups for meat type, season, and label claim (ANOSIM R < 0, Table 5) [38].

Table 5. Results of PERMDISP2, PERMANOVA, and ANOSIM tests based on the presence and absence of AMR genetic determinants. All tests were performed using a Jaccard distance metric and 10,000 permutations.

Grouping Factor	PERMDISP2 *p*-Value (F)	PERMANOVA *p*-Value (R^2)	ANOSIM *p*-Value (R)
Meat type	0.010 (4.25)	0.10 (0.088)	0.89 (−0.059)
Season	0.41 (0.97)	0.73 (0.044)	0.80 (−0.022)
Packaging type	0.78 (0.36)	0.55 (0.053)	0.24 (0.038)
Label claim	0.39 (0.78)	0.80 (0.0094)	0.87 (−0.11)

2.6. Co-Occurrence Networks of AMR Genetic Determinants in E. coli Isolates

Despite the diversity in AMR genetic determinants present in *E. coli* isolates in this study (Figure 3a), the co-occurrence network of AMR genes was sparse, with the most commonly co-occurring genes being *mdf(A)* with *tet(A)* and *mdf(A)* with *tet(B)* at a co-occurrence frequency of 15 (28.85%) and 12 (23.08%) genomes, respectively (Figure 3c). At a lower co-occurrence threshold of ≥5 isolates, the networks of *mdf(A)* with *tet(A)* and/or *tet(B)* genes co-occurred with either a gene cluster comprised of *aph(3″)-Ib* and *aph(6)-Id* or another including *aac(3)-VIa*, *ant(3″)-Ia*, and *sul1* (Figure 3b).

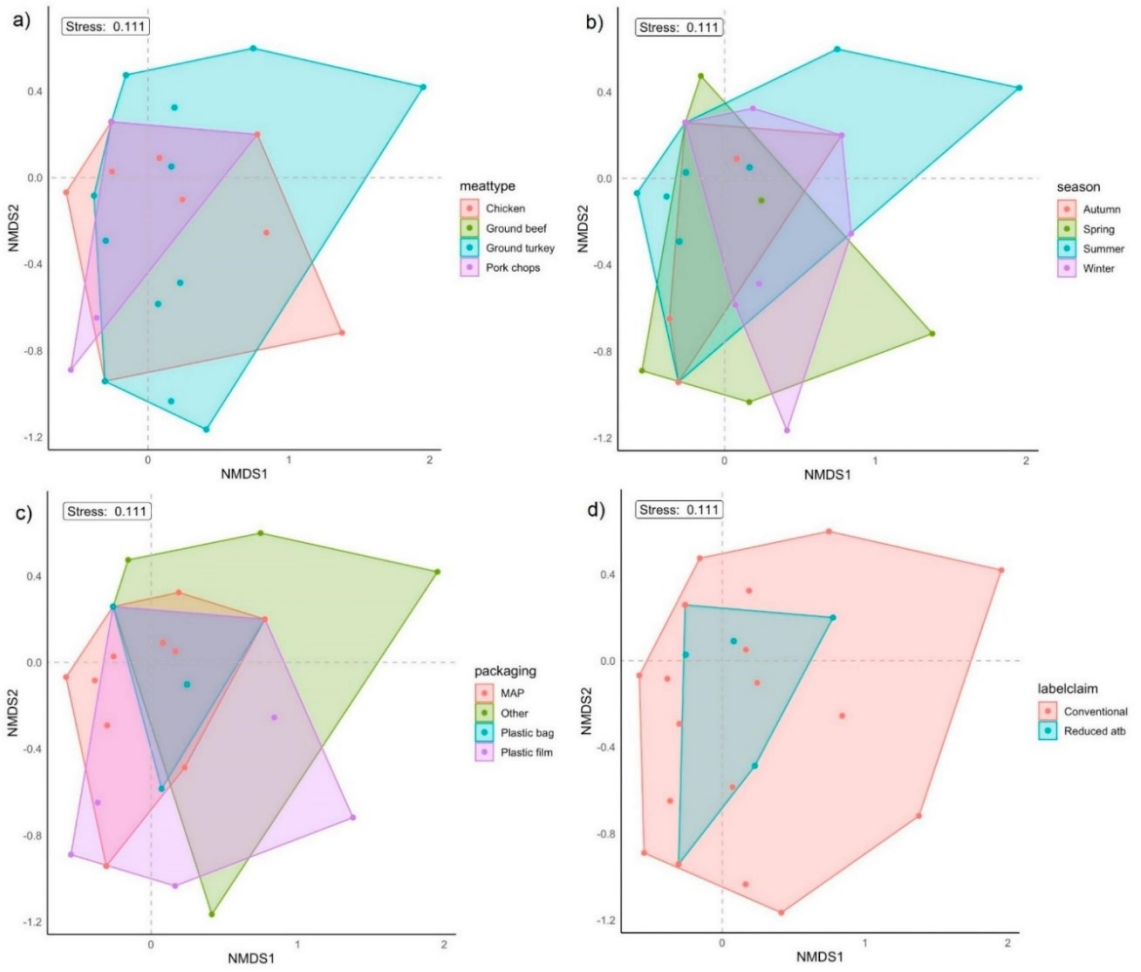

Figure 2. Non-metric multidimensional scaling (NMDS) performed using presence and absence of antimicrobial resistance genes from *E. coli* isolates from retail meat (n = 52). Individual points represent an isolate, with convex hulls displaying grouping factors of (**a**) meat type; (**b**) season; (**c**) packaging type; and (**d**) label claim.

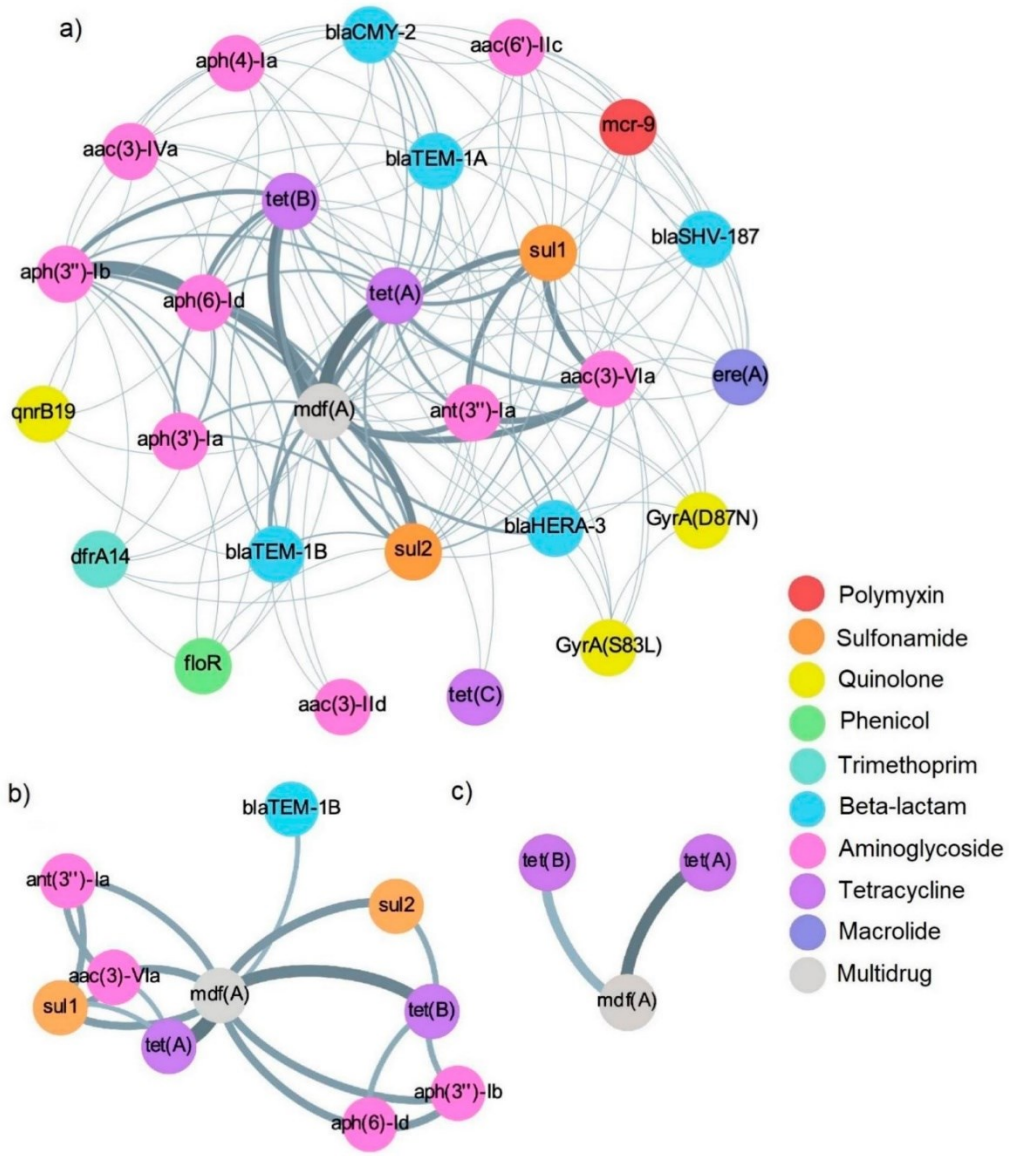

Figure 3. Co-occurrence networks of antimicrobial resistance genes in *E. coli* isolates (n = 52) by frequency thresholds of (**a**) 1 isolate; (**b**) 5 isolates; and (**c**) 10 isolates. Each node represents a gene and is color coded by antimicrobial class. Edges indicate frequency of co-occurrence, with a light to dark gradient representing low to high co-occurrence, respectively.

3. Discussion

The data presented in this study were collected from raw meat products from retail stores in southern California using the same sampling and processing protocols from FDA NARMS, thus enabling comparisons between our data and previous/concurrent NARMS data collected. No NARMS data have been available on *E. coli* from retail meats in California, as only certain pathogens of interest (*Salmonella* and *Campylobacter*) have been included in routine AMR assessment for California prior to this study.

Of the 221 retail meat products collected from southern California in 2018 and assessed in this study, *E. coli* was isolated from around half of the samples. Compared to the national average for NARMS retail testing for the same year, isolation of *E. coli* from samples in California was higher overall (47.51% vs. national average 28.75%) with higher recovery of *E. coli* in our study for chicken (67.86% vs. national average 19.22%), ground turkey (70.37% vs. national average 45.65%), and pork chop (32.14% vs. national average 25.82%), and lower recovery in ground beef (20.00% vs. national average 28.54%) [39].

Previous studies assessing the presence of enteric bacteria in meat products have found substantial variability in the prevalence of *E. coli* [26,40–42], which could be due to differences in sampling methodology, sample composition and origin, and processing protocols. Nevertheless, the significant association between *E. coli* contamination and meat type observed in our study is congruent with findings from studies conducted in multiple countries, where chicken and/or turkey products were found to have comparably higher prevalence of *E. coli* relative to non-poultry meat types such as beef and pork [26,41–43]. Additionally, season of retail meat purchase in our study was also significantly associated with the presence of *E. coli*, which could be explained by season serving as a surrogate for other unmeasured but correlated variables such as temperature, climate, and other temporal factors that more directly reflect the abiotic and biotic drivers of enteric bacteria persistence and proliferation in meat products [44]. This is substantiated by findings from a previous study which evaluated the relationship between weather variables and zoonotic foodborne pathogen contamination of meat products along the food chain in Canada. Although no definite seasonal trend was identified in their study for generic *E. coli*, Smith et al. found correlations between increased total precipitation and increased average/monthly temperature and *E. coli* in retail beef and pork, respectively [45].

The elevated frequencies of ampicillin, streptomycin, and tetracycline resistance in *E. coli* from ground turkey and gentamicin resistance in chicken compared to non-poultry meats (beef and pork) in our study are consistent with previous data collected in the United States [26,46,47]. While *E. coli* resistance to certain antimicrobials appears to be globally ubiquitous—for instance, high tetracycline resistance in *E. coli* from retail meats documented in Canada, India, Korea, and China—the overall heterogeneity across global frequencies of resistance is most likely attributed to differences in antimicrobial usage and the variability in selective pressures imposed across different countries and food animal production sectors [41,42,48,49]. For instance, a previous study reported 75.7% of *E. coli* from poultry meat in Korea being resistant to nalidixic acid, which, while consistent with national data of veterinary antibiotics sold for use in Korean poultry production [50], is in contrast to findings in our study of low nalidixic acid (1.90%) and ciprofloxacin (0.95%) resistance that are likely reflective of the ban of fluoroquinolone use in poultry production and restrictions in fluoroquinolone use for other food animal species in the US by the Food and Drug Administration [51,52].

The detection of *mcr-9*—a gene encoding a putative phosphoethanolamine transferase that reduces affinity for colistin—from an MDR *E. coli* isolate from retail chicken in this study is noteworthy as colistin is not used for treatment of food animals in the United States [53,54]. Colistin is a polymyxin antimicrobial and one of the few last-resort drugs available to treat life-threatening multidrug drug-resistant (MDR) and extensively drug-resistant (XDR) infections caused by Gram-negative bacteria such as carbapenem resistant *E. coli*, *P. aeruginosa*, and *K. pneumoniae* [55,56]. It was previously believed that colistin resistance was solely mediated by chromosomal genes (*phoPQ*, *pmrAB*, and *mgrB*) until the plasmid-mediated *mcr-1* gene from China was first reported in 2015 [57], and *mcr-1* variants (*mcr-2* through *10*) were subsequently identified in over two dozen bacterial species across six continents [58–60]. In the United States, *mcr-9* was first identified in a *Salmonella enterica* serotype Typhimurium clinical isolate in 2019 [61]. Since then, analysis of previously collected bacterial isolates from routine NARMS retail meat surveillance has traced *mcr-9* in isolates collected as far back as 2002, with findings of its occurrence in a pronounced proportion of *Salmonella* isolates in the US (28.6%, 2002–2019)—particularly

S. Saintpaul from ground turkey—and also in a few *E. coli* isolates from samples collected in 2018 and 2019 [54]. Epidemiologically, *mcr-9* has not been linked to clinical resistance of colistin [54,61], which is consistent with findings here of *mcr-9* carriage in a colistin-susceptible *E. coli* isolate. It has, however, been shown that the presence of sub-inhibitory levels of colistin is sufficient to induce *mcr-9* expression through mediation by the two-component regulatory system *qseBC*, resulting in elevated MIC levels [53]. This highlights the importance of bacterial genomic surveillance efforts as disseminated *mcr* genes and other resistant determinants of high public health concern can remain undetected and/or unexpressed in non-resistant isolates until induced by antimicrobial exposure [53].

Consistent with the diversity of phenotypic AMR profiles of *E. coli* isolates, whole-genome sequencing identified several other AMR genetic determinants, including those mediating resistance to other drugs of high clinical importance such as fluoroquinolones and cephalosporins. Genetic characterization of fluoroquinolone resistance has been well documented to occur through combinations of chromosomal mutations within DNA gyrase (e.g., *gyrA*) and/or topoisomerase IV genes (e.g., *par*C) [62], as observed in the MDR ciprofloxacin resistant isolate from ground turkey in this study (*gyrA* mutations S82L and D87N). Plasmid-mediated quinolone resistance determinants (e.g., *qnrB19* from an MDR isolate from pork chop with decreased susceptibility to ciprofloxacin in this study) confer low-level resistance but nevertheless still impart concerns due to the reported frequencies of their of co-occurrence with other AMR genetic determinants—for instance, in ESBL-producing *E. coli*—and their high propensity to simultaneously disseminate multiple resistances [63–66]. To this point, we observed low prevalence of cephalosporin resistant *E. coli* in our study, which was mediated by AmpC-type beta-lactamase gene, $bla_{\text{CMY-2}}$, but these isolates were MDR with carriage of over eight other AMR genes.

Previous studies have evaluated the ability of WGS to predict *E. coli* phenotypic resistance, with findings of imperfect but high overall concordance between genotypic and phenotypic resistance [20,67,68], as also observed in this study. The discrepancies in sensitivity for amoxicillin/clavulanic acid and chloramphenicol in our study could be attributed to the very small number of resistant isolates for these drugs which the analysis was based on. Moreover, the concordance of WGS with AMR phenotype is heavily dependent on the categorization of susceptible and resistant isolates, with grouping of intermediate isolates potentially affecting results, alongside any ambiguity in breakpoints used. An example of the latter is the lack of Clinical and Laboratory Standards Institute (CLSI) criteria for streptomycin, a veterinary drug which exhibited both imperfect sensitivity and specificity here and discordance in prior studies [67,69–71]. Additionally, AMR genetic determinants conferring less definitive phenotypic resistances such as those encoding multidrug efflux pump genes (e.g., *mdf*(*A*) in this study) add complexity to both data analysis and interpretation. Other considerations have been detailed previously and include the impacts of technical processes in conducting AST/WGS, database selection, and thresholds used for determining the presence or absence of AMR genetic determinants [72]. Lastly, it should be noted that our study evaluated a small number of isolates, all of which were derived from retail meats limited in one geographic region. Thus, while this study finds WGS to be a robust tool for phenotypic AMR predictions in foodborne *E. coli*, its utility for other bacteria from different sources should be considered with caution and supplemented with phenotypic testing to ensure comprehensive AMR assessment.

E. coli isolates in this study were genomically heterogeneous with respect to AMR, with meat type, season, packaging, and label claim accounting for very little of the variability in AMR genetic determinants and a sparsity of shared gene networks observed. Our results suggest that AMR acquisition in *E. coli* from retail meat exhibits greater complexities that could not be fully explained by the retail-level factors assessed and/or by the data collected, as the small number of isolates in this study are likely not representative of the diversity of *E. coli* as a whole from retail meats. Moreover, a limitation of retail level surveillance conducted in this study is that other factors along the farm-to-fork continuum which could potentially contribute to AMR could not be evaluated. Nevertheless, our findings

from AMR gene co-occurrence networks reflect certain intricacies of AMR dynamics. For instance, we observe elevated co-occurrence of gene networks corresponding to a broad multidrug transporter (*mdf*(*A*)) and to tetracycline, sulfonamide, and aminoglycoside genes which confer resistance to antimicrobials that are conventionally used in food animal production in the US. The higher frequencies of co-occurrence of these genes (*tet*(*A*), *tet*(*B*), *aph*(*3″*)-*Ib*, *aph*(*6*)-*Id*, *aac*(*3*)-*VIa*, *ant*(*3″*)-*Ia*, *sul1*, and *sul2*) could result from direct exposure to the corresponding antimicrobials at some point along the food chain, or persistence as a result of co-selection from these genes occurring on the same mobile genetic element (e.g., plasmids). We did not assess AMR gene carriage on plasmids in this study due to the limited capacity of short-read sequencing data to fully resolve plasmid structures [73]; however, other studies employing long-read sequencing have confirmed the occurrence of these genes on the same plasmid(s) in *E. coli* [74–76]. Lastly, the diversity of AMR genetic determinants identified in this study—including those corresponding to antimicrobials not used in food animals in the US (e.g., *mcr-9*)—suggests that the accumulation of AMR reservoirs could occur even in the absence of direct selective pressures, with the acquisition and loss of certain AMR genes in *E. coli* possibly attributed to the presence or absence of fitness costs that are associated with the maintenance of these genes [77,78].

4. Materials and Methods

4.1. Study Area and Sampling

Samples in this study were collected as part of routine NARMS retail meat surveillance in 2018, when the program expanded geographical coverage to include southern California. From January to December 2018, a total of 480 fresh retail meat samples consisting of 240 skin-on/bone-in chicken, 120 ground turkey, 60 ground beef, and 60 pork chops were purchased from retail grocery stores in southern California twice each month. Sampling locations were selected based on the NARMS retail store sampling plan through random selection of grocery stores within zip codes corresponding to West Los Angeles, East Los Angeles, Ontario, and Irvine. Samples were transported on ice to the laboratory, refrigerated, and processed within 72 h of purchase.

4.2. Sample Processing and E. coli Isolation

A random selection of 221 samples (56 chicken, 54 ground turkey, 55 ground beef, and 56 pork chops) was processed for isolation of *E. coli* per the 2018 NARMS Retail Meat Surveillance protocol [79]. Briefly, 25 g of each sample was placed in Whirl-Paks containing 250 mL buffered peptone water (Becton Dickinson, Franklin Lakes, NJ, USA) and hand massaged for 3 min. A total of 50 mL of rinsate was then added to 50 mL double-strength MacConkey broth (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated at 35 °C for 24 h. Following overnight enrichment, a loopful (10 µL) was streaked to a MacConkey plate and incubated at 35 °C for 24 h. One suspect *E. coli* colony based on typical colony morphology was streaked to purity on blood agar plates and incubated overnight at 35 °C. Isolates were confirmed as *E. coli* using biochemical tests (indole positive and oxidase negative), and banked in Brucella broth with 15% glycerol, frozen, and shipped on dry-ice to the FDA's Center for Veterinary Medicine (CVM) for antimicrobial susceptibility testing and whole-genome sequencing.

4.3. Antimicrobial Susceptibility Testing

E. coli isolates were tested using a broth microdilution method for 14 antimicrobial drugs using the NARMS Gram-negative plates (Thermo Fisher Scientific, Waltham, MA, USA) per standard protocols [80]. NARMS breakpoints were used to classify isolates into susceptible, intermediate, and resistant categories based on the minimum inhibitory concentration (MIC) values for each drug; these breakpoints are based on the CLSI guidelines with the exception of streptomycin and azithromycin, where NARMS consensus interpretive criteria were used due to absence of available CLSI breakpoints for these two drugs (Table S2) [81]. Due to the limited range of dilutions in the drug panel, resistance could not

be determined for azithromycin and sulfisoxazole and only susceptible classification were determined for these two drugs. For analysis, intermediate and susceptible isolates were grouped together. Decreased susceptibility (DSC) to ciprofloxacin (≥0.12 μg/mL) was also noted in descriptive analyses due to the expanded definition from CLSI for its intermediate susceptibility MIC range [81]. Multidrug resistance was defined as resistance to one or more drugs in three or more antimicrobial classes [82].

4.4. Whole-Genome Sequencing and Identification of Resistance Genes

A subset of *E. coli* isolates (n = 52) was randomly selected to undergo whole-genome sequencing (WGS) by short-read sequencing on the Illumina MiSeq using v2 or v3 chemistry for 2 × 250-bp paired end reads, and identification of resistance genes was conducted as previously described [67]. Briefly, genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA), and libraries were prepared using the Illumina Nextera XT kit per manufacturer's protocols. Sequences were demultiplexed using MiSeq Reporter and assembled using the CLC Genomics Workbench. The ResFinder database (Center for Genomic Epidemiology, DTU) was used to identify resistance gene hits (≥85% amino acid identity and ≥50% sequence length), and Perl scripts were used to extract and analyze the *gyrA* gene at amino acid position 83 and 87 to assess chromosomal mutations associated with quinolone resistance.

4.5. Data Analysis

Descriptive statistics (prevalence of *E. coli*, distribution of predictor variables, antimicrobial susceptibility testing results, and prevalence of antimicrobial resistance genes) and binary logistic regression models were conducted using SAS On-Demand for Academics. Predictor variables evaluated in this study include meat type (ground turkey, chicken, pork chop, and ground beef), time of year of sample purchase, packaging type (modified atmosphere packaging, plastic film, vacuum, chub, paper, and plastic bag), label claims, packaging type, and presence of *Salmonella*. For label claims, reduced antibiotic use included samples labeled organic or reduced/no antibiotic usage; all other samples with absence of such label claims were classified as conventional. Retail meat samples in this study were concurrently processed for isolation of *Salmonella*, with data on the presence of *Salmonella* obtained through methods detailed previously [69].

The association between the presence of *E. coli* in retail meat samples and predictor variables were evaluated using logistic regression models. Univariable logistic regression models were used to evaluate the crude association between each predictor variable and the outcome binary variable, which was designated as the presence or absence of a recovered *E. coli* isolate from the retail meat sample. A multivariable logistic regression model was then fitted based on the retention of significant variables, assessment of collinearity, testing of all two-way interactions, and best model fit as determined by the lowest Akaike's Information Criterion (AIC). The association between predictor variables and whether an *E. coli* isolate from retail meat was multidrug resistant or not, and whether it was resistant or not to ampicillin, gentamicin, streptomycin, and tetracycline, were individually evaluated using exact logistic regression models. These four drugs were selected for evaluation due to the higher frequency of observed resistance.

Prediction of phenotypic antimicrobial resistance (AMR) from antimicrobial susceptibility testing based on genotypic AMR from the presence of antimicrobial resistance genes was evaluated as previously described [69]. Briefly, phenotype and genotype concordance for each drug included true positives (TP: resistant isolate with corresponding AMR genetic determinants) and true negatives (TN: susceptible isolates with absence of corresponding AMR genetic determinants). Discordance included false negatives (FN: resistant isolates with absence of corresponding AMR genetic determinants) and false positives (FP: susceptible isolates with presence of corresponding AMR genetic determinants). Sensitivity and specificity were then calculated as TP/(TP+FN) and TN/(TN+FP), respectively. Due to the absence of phenotypically resistant isolates to sulfisoxazole, azithromycin, and meropenem,

sensitivity could not be assessed for them, so these three drugs were omitted from the overall calculation for sensitivity. Multidrug resistance gene, *mdf(A)*, was omitted from the concordance analysis due to ambiguity in its AMR phenotype conferral.

To assess the collective AMR gene profiles of *E. coli* isolates, clustering based on the presence and absence of AMR genetic determinants was evaluated using functions in the vegan package [83] in R by grouping factors of meat type, season of retail meat purchase, packaging type, and label claim. Non-metric multidimensional scaling (NMDS) was performed using the metaMDS function with a Jaccard distance metric and in two dimensions. The permutest and betadisper functions were used to conduct a PERMDISP2 procedure to evaluate if dispersions of groups for each of the grouping factors were homogenous [84,85]. Permutational analysis of variance (PERMANOVA) was conducted to test the equivalence of centroids of groups for each grouping factor using the adonis2 function. Additionally, analysis of similarity (ANOSIM) was performed to evaluate for each grouping factor whether the average of ranks within-group distances was greater or equal to that of between-group distances [38]. The aforementioned tests (PERMDISP2, PERMANOVA, and ANOSIM) were performed using 10,000 permutations and a Jaccard distance metric.

From the presence and absence data of AMR determinants in *E. coli* isolates, a pairwise co-occurrence matrix was constructed by transforming the binary data. The resulting co-occurrence data were then visualized as networks of co-occurring genes using Gephi [86], with nodes representing genes and edges representing the frequency of co-occurrence. Networks were evaluated by frequency of co-occurrence thresholds based on $\geq$1 genome (all *E. coli* isolates), $\geq$5 genomes, and $\geq$10 genomes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics12040782/s1, Table S1: List of *E. coli* isolates in this study; Table S2: NARMS breakpoints used to classify minimum inhibitory concentrations of *E. coli* isolates; Table S3: Genotypic prediction of phenotypic resistance in *E. coli* isolates from retail meat (n = 52).

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Data Availability Statement: Whole-genome sequences of *E. coli* isolates in this study are deposited under BioProject PRJNA292663.

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Chapter 3: Impact of zinc supplementation on phenotypic antimicrobial resistance of fecal commensal bacteria from pre-weaned dairy calves

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Impact of zinc supplementation on phenotypic antimicrobial resistance of fecal commensal bacteria from pre-weaned dairy calves

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The objective of this study was to evaluate the impact of dietary zinc supplementation in pre-weaned dairy calves on the phenotypic antimicrobial resistance (AMR) of fecal commensal bacteria. A repository of fecal specimens from a random sample of calves block-randomized into placebo (n = 39) and zinc sulfate (n = 28) groups collected over a zinc supplementation clinical trial at the onset of calf diarrhea, calf diarrheal cure, and the last day of 14 cumulative days of zinc or placebo treatment were analyzed. Antimicrobial susceptibility testing was conducted for *Enterococcus* spp. (n = 167) and *E. coli* (n = 44), with one representative isolate of each commensal bacteria tested per sample. Parametric survival interval regression models were constructed to evaluate the association between zinc treatment and phenotypic AMR, with exponentiated accelerated failure time (AFT) coefficients adapted for MIC instead of time representing the degree of change in AMR (MIC Ratio, MR). Findings from our study indicated that zinc supplementation did not significantly alter the MIC in *Enterococcus* spp. for 13 drugs: gentamicin, vancomycin, ciprofloxacin, erythromycin, penicillin, nitrofurantoin, linezolid, quinupristin/dalfopristin, tylosin tartrate, streptomycin, daptomycin, chloramphenicol, and tigecycline (MR = 0.96–2.94, $p > 0.05$). In *E. coli*, zinc supplementation was not associated with resistance to azithromycin (MR = 0.80, $p > 0.05$) and ceftriaxone (MR = 0.95, $p > 0.05$). However, a significant reduction in *E. coli* MIC values was observed for ciprofloxacin (MR = 0.17, 95% CI 0.03–0.97) and nalidixic acid (MR = 0.28, 95% CI 0.15–0.53) for zinc-treated compared to placebo-treated calves. Alongside predictions of MIC values generated from these 17 AFT models, findings from this study corroborate the influence of age and antimicrobial exposure on phenotypic AMR.

Antimicrobial resistance (AMR) is one of the most significant public health threats faced in this century[1]. The global challenge to address AMR has prompted studies to better ascertain its distribution across diverse host populations and environments, to infer the pathways of its spread, and to identify the risk factors associated with its emergence and persistence. Comparably, understanding of specific factors that modulate AMR and the extent to which they promote or decrease resistance, particularly in food-producing animals, remains limited.

The occurrence of AMR in food-producing animals not only limits therapeutic options that compromises animal health, but also raises concerns of the potential for its dissemination through animal populations, environmental matrices, and the food chain[2]. In dairy cattle, it has been well documented that a higher frequency of AMR is typically observed in calves compared to older cattle[3–6]. This observed difference in resistance between younger and older cattle may partially be attributed to factors such as increased susceptibility to disease during early life, dietary changes, and initial exposure to the environment and antimicrobial treatments[7,8]. Improving our understanding of AMR dynamics during early calf life is thus of considerable importance as this period presents an opportunity to reduce AMR acquisition in bovine hosts and their environments.

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Diarrhea is the leading cause of morbidity and mortality in pre-weaned dairy calves, with causative agents including viral (e.g. bovine rotavirus), parasitic (e.g. *Cryptosporidium parvum*), and bacterial pathogens (e.g. *Salmonella enterica*)[9]. In diarrheic calves, the frequency of co-infection with more than one pathogen and increased susceptibility to bacteremia from compromised small intestinal function makes antimicrobial therapy a critical and frequent component of treatment protocols[10,11]; the USDA reported in 2014 that 21.1% of pre-weaned heifers were affected with diarrhea or other digestive disorders, of which 75.9% were treated with antimicrobials[12]. To ensure animal health and mitigate AMR, alternatives such as zinc supplementation has been explored[13,14]. Zinc is a trace mineral with a crucial role in many aspects of cellular metabolism and a key constituent of over 300 structural or catalytic enzymatic functions[15]. It also has an essential role in cellular signal transduction, cell proliferation and differentiation, and regulation of innate and adaptive immunity[15,16]. Deficiency compromises mechanisms involved in pathogen neutralization and alters cytokine production to impact inflammatory responses, resulting in increased risk for disease and infection in hosts[15]. The immunity and gut integrity benefits of zinc makes its application in dairy calves of particular interest to simultaneously reduce incidence of diarrheal diseases, improve calf health and growth, and decrease antimicrobial use[13,17–19].

In a previously conducted double-blind, block-randomized, placebo-controlled clinical trial, zinc supplementation was shown to be effective in delaying diarrheal onset and expediting diarrhea recovery[14]. However, the utility of zinc supplementation may have unintended consequences as heavy metal exposure has been suggested to enhance the spread of AMR due to the genetic and physiological linkages between metal resistance and AMR. Mechanistically, co-selection for metal resistance and AMR could putatively arise through cross-resistance (one gene/mechanism confers both metal resistance and AMR), co-regulation (the expression of transcriptionally linked metal resistance and AMR systems are modulated by a common gene/regulator), and/or co-resistance (metal resistance and AMR genes occur on the same genetic element)[20]. Studies evaluating the relationship between dietary zinc and AMR have primarily focused on swine, with several studies providing supporting evidence for the selection of multidrug resistance and increase in occurrence and abundance of AMR genetic determinants from zinc supplementation[21–24]. In bovine hosts, studies on dietary zinc and AMR have focused on beef cattle with mixed findings of no AMR selection[25,26] or increased AMR to certain antimicrobials in enteric bacteria[27]. Currently, little is known on the effect of dietary zinc supplementation on AMR in calves. Additionally, the influence on AMR from other contributing factors in conjunction with dietary zinc remains unclear.

To address these knowledge gaps, the aim of this study was to evaluate the impact of dietary zinc supplementation in pre-weaned dairy calves on the phenotypic AMR of fecal commensal bacteria. The objectives were to determine the association between zinc supplementation and AMR in *E. coli* and *Enterococcus* spp. and quantify changes in resistance with respect to dietary zinc exposure and calf-level factors.

Materials and methods

The original trial procedures that generated the repository of fecal samples for the current study were approved by the University of California Davis Institutional Animal Care and Use Committee (protocol number 18067 Approved: March 6, 2014) and performed in accordance with relevant guidelines and regulations. The trial that generated the sample repository was a double-blind, block randomized, placebo-controlled clinical trial evaluating different dietary zinc supplementation treatments and their effect on diarrhea prevention and health in pre-weaned calves. The trial was conducted on a single San Joaquin Valley dairy (Kings County, CA, USA). The detailed study and sampling design were previously described[14]. Briefly, healthy Holstein heifer or bull calves from the one dairy were enrolled at birth (24–48 h of age) and block-randomized by time of birth to zinc or placebo treatment groups. Calves were examined by a veterinarian or trained personnel and excluded if exhibiting obvious morbidities or congenital defects[14]. A random sample of enrolled calves (approximately 8–10% of the study population given budgetary constraints for sample testing) were selected for fecal sampling. Calves analyzed for this study included all sampled calves (n = 67) in the zinc sulfate (n = 28) and placebo (n = 39) treatment groups that had onset of diarrhea at some point over the duration of the clinical trial.

Pre-weaned calf management and treatment protocols

All calves were under the same management practices including housing and diet[14]. Throughout the study, calves were housed in individual metal hutches. The respective treatments were administered in the morning milk bottles for 14 days starting from enrollment; calves in the zinc sulfate treatment group received 0.22 g zinc sulfate monohydrate (80 mg of elemental zinc) (Sigma-Aldrich Company, St. Louis, MO, United States) with 0.44 g milk replacer powder, while those in the placebo group received only milk replacer powder (0.44 g). Antimicrobials included in dietary milk for calves 0–25 days of age included chlortetracycline hydrochloride (Pennchlor 64, Pharmgate Animal Health, Omaha, NE, United States) and neomycin sulfate (NeoMed 325, Bimeda, Inc., Le Sueur, MN, United States). Calves 25 days of age to weaning received dietary milk that included oxytetracycline hydrochloride (NT-10G, Agri-Best™, Strauss Feeds LLC, Watertown, WI, United States). Calves from which samples were employed for the current study were all treated for symptoms of diarrhea at some point over the duration of the trial and received the same treatment consisting of an oral mixture of 118.5 mL bismuth subsalicylate (Bismusal Suspension, Durvet, Inc., Blue Springs, MO, United States) and 31.5 mL of spectinomycin (SpectoGard, Bimeda, Inc., Le Sueur, MN, United States)[14].

Fecal sample collection

Fecal samples were collected on the first day of calf diarrhea onset (D1), exit or cure from diarrhea (Dex), and on the last day of the 14-day treatment period (D14). Sampling was conducted only at two time points for calves where onset of diarrhea occurred on the same day as the last day of the treatment period (D1,D14). Sample collection was conducted as previously described; new gloves and sterile lubricant were used to collect fresh feces

(~ 5 g) into 20 mL polypropylene jars (The Cary Company, QAddison, IL) by digital rectal simulation[14]. Fecal samples were stored at − 20 °C until analysis.

Bacterial isolation

Fecal samples were processed for bacterial isolation by enriching a saturated fecal cotton swab in 10 mL of Tryptic Soy Broth (TSB, Becton Dickinson, Franklin Lakes, NJ, United States) for 24 h. Two 1 mL aliquots from each TSB enrichment were transferred to two tubes, one containing 9 mL of MacConkey and another containing 9 mL of Enterococcosel broth and were incubated for 24 h at 35 °C and 45 °C, respectively. A loopful (10 µL) of MacConkey and Enterococcosel broth was streaked to MacConkey and Enterococcosel agar for isolation of *E. coli* and *Enterococcus* spp., respectively. One putative colony of *E. coli* and *Enterococcus* spp. based on typical morphology (pink colony for *E. coli* and beige colony with strong black halo for *Enterococcus* spp.) was randomly selected from each selective agar plate, and streaked to purity by subculturing on respective selective agars then to blood agar plates (Tryptic Soy Agar with 5% sheep blood, Thermo Scientific, Waltham, MA, United States). All incubation steps for agar plates were performed at 35 ± 2 °C for 18–24 h.

Bacterial confirmation

DNA was extracted from pure cultures of *E. coli* and *Enterococcus* spp. on blood agar plates using a boiling method. Briefly, colonies were suspended in 100 µL of molecular grade water in a sterile 1.5 mL microcentrifuge tube, incubated on a heating block at 100 °C for 20 min, and then centrifuged for 10 min at 5000 rpm[28]. Confirmation of isolates was then conducted as previously described using conventional PCR to screen for the presence of universal stress protein *uspA* for *E. coli*[29,30], and 23S rRNA sequence using forward (ECST784F) and reverse (ENC854R) primers for *Enterococcus* spp[31,32]. Primer sequences used are provided in Supplementary Table 1. During the PCR step, a positive control (*E. coli* ATCC 25922 or *Enterococcus faecalis* ATCC 29212) and two negative controls comprised of a molecular grade water and PCR mastermix blank were used. Confirmed *E. coli* and *Enterococcus* spp. isolates were stored in cryovials containing TSB with 15% glycerol at − 80 °C for further testing.

Antimicrobial susceptibility testing (AST)

Antimicrobial susceptibility testing (AST) was conducted on *E. coli* isolates from D14 fecal samples and 167 enterococci isolates from fecal samples collected at all sample collection time points using the NARMS Gram negative (YCMV3AGNF) and Gram positive (YCMV3AGPF) AST panels, respectively[5]. Briefly, 3–5 colonies of fresh, pure culture were inoculated into 5 mL of dH2O. Turbidity of the suspension was adjusted to 0.5 McFarland standard, or the equivalent of a 0.08–0.1 OD at 625 nm. From the suspension, 10 µL was then transferred to 11 mL of Sensititre Mueller–Hinton Cation adjusted broth to yield 1×10^5 CFU/mL of bacterial suspension. Fifty microliters of the Mueller–Hinton inoculum were then transferred into each well of the AST plate and incubated at 35 °C for 24 h. *E. coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as quality controls. Minimum inhibitory concentration (MIC) values were recorded as the lowest concentration of each antimicrobial drug (AMD) which inhibited visible growth of bacteria. The antimicrobial drugs in each panel and their corresponding dilution ranges (µg/mL) are provided in Supplementary Table 2–3.

Statistical analysis

a. All statistical analyses were conducted using Stata 17.0. Data visualization was conducted in RStudio (ggplot2) and BioRender
b. Calf-level factors

Descriptive statistics of enrolled calves for this study and the characteristics of *E. coli* and *Enterococcus* spp.—with each isolate representing the status of a calf from which the fecal isolate was collected from—were determined based on daily assessment records of individual calves. This included treatment group (whether an isolate originated from a fecal sample collected from a placebo or zinc sulfate treated calf), and calf-level factors of age, diarrhea status, and therapeutic spectinomycin exposure at the time of fecal collection. Different specifications of calf-level covariates were evaluated. For the age of the calf, this included age in days and age by week. Diarrhea status was specified as days on or from diarrhea (positive values indicating days of ongoing diarrhea and negative values indicating days post diarrhea recovery), categorical diarrhea status (isolate collected from a calf that was pre-diarrheic, diarrheic, or recovered from diarrhea). Exposure to spectinomycin was specified as the number of days from the most recent treatment, the number of doses received, whether treatment was received or not, and a categorical variable of days from the most recent treatment (*E. coli*: 0 days, 3–5 days, 6–8 days, and 9–10 days; *Enterococcus* spp.: 0 days, 1–3 days, 4–7 days, 8–23 days).

c. Accelerated failure time (AFT) models

To evaluate the association between isolates from zinc/placebo treatment groups and the degree of change in MIC values, an accelerated failure time (AFT) model was constructed for each antimicrobial drug. The AFT model is a parametric survival model that can be represented by:

$$ln(T_i) = \beta_0 + X_i\beta_i + \varepsilon_i$$

For this study, the AFT model was adapted such that the dependent variable T_i, typically representing time-to-event, is specified as left ($\leq AMD_{min}$ µg/mL), right ($> AMD_{max}$ µg/mL), or interval censored (between two twofold tested antimicrobial drug concentrations) MIC data, where AMD_{min} and AMD_{max} represent the lowest and highest antimicrobial drug concentrations tested, respectively. As negative MIC values are not biologically possible, all left-censored MIC values were specified with a lower bound of 0 instead of $-\infty$. The relationship between T_i—the progression in expected concentration to inhibition for an antimicrobial for the *i*th isolate—and a random error term (ε_i) are assumed to follow a specified distribution. Fixed effects are denoted by the intercept (β_0) and $X_i\beta_i$ representing covariates (X_i) and their corresponding regression coefficients (β_i). To account for confounding from the biological influence of age and therapeutic antimicrobial treatment for diarrhea, calf age and exposure to spectinomycin were controlled for by forced inclusion as independent variables in all *Enterococcus* spp. models. Additionally, repeated measures from *Enterococcus* spp. collected at multiple time points were accounted for by calculating robust standard error estimates using a clustered sandwich variance estimator, which relaxed the assumption of independence among observations and allowed for intragroup correlation of isolates from the same calf[33,34]. As all *E. coli* isolates evaluated were recovered from fecal samples collected at the last day of the 14-day zinc or placebo treatments since calf enrollment (1–2 days of age), all *E. coli* isolates corresponded to calves of similar age (14–16 days), and only exposure to spectinomycin was controlled for in all *E. coli* models.

d. AFT model specification

A manual forward model building approach was used, in which a base model with treatment group as the only independent variable was first evaluated based on BIC estimates to specify a best-fitting (lowest BIC) distribution (Weibull, exponential, or generalized gamma) for model building. The final model with addition of confounders (spectinomycin exposure for *E. coli* models and spectinomycin exposure and calf age for *Enterococcus* spp. models) was identified by evaluating the various specifications for calf-level covariates and using the method of change in estimates to assess confounding. Other calf-level covariates (e.g. diarrhea status) were evaluated and retained in the model if improving model fit. The significance of biologically relevant interactions, specifically calf age—which was collinear with the days of zinc/placebo treatment exposure—and treatment group was tested for all *Enterococcus* spp. models to assess if a dose-dependent treatment effect was present. The specified parametric distribution for each model was also re-evaluated after tentative multivariable models were constructed, with Wald tests used to confirm that the appropriate parametric model was selected. Lastly, the final model for each antimicrobial drug was selected on the criteria that it generated estimates within the hypothetical maximum concentration limit for the MIC (1×10^6 µg/mL)[35,36]. In favor of more parsimonious model selection, the Bayesian Information Criteria (BIC) was used to assess competing models, with lower values indicating better model fit. A 5% significance level was used for all models.

Results

Study population and characteristics of *E. coli* and *Enterococcus* spp.

Fecal samples from randomly selected pre-weaned dairy calves (28 calves from the zinc sulfate treatment group and 39 calves from the placebo treatment group) from a previously conducted zinc supplementation trial were assessed in this study[14]. Due to the observational nature of sample collection, fecal collection occurred across four possible time sequences as detailed in the study schematic (Fig. 1). Characteristics of pre-weaned dairy calves whose samples were identified for the current study are summarized in Table 1; no significant differences in sex, attitude score, fecal score, and age were observed between calves in the zinc sulfate and placebo treatment groups from which fecal samples were utilized in the present study.

A total of 44 *E. coli* isolates from D14 fecal samples and 167 *Enterococcus* spp. from fecal samples collected at all of the aforementioned time points were included for antimicrobial susceptibility testing. Due to both budgetary constraints for laboratory testing and lower recovery of *E. coli* isolates from fecal samples collected at other time points, analysis of *E. coli* was restricted to isolates from D14 samples. As one representative isolate was tested per fecal sample, each commensal isolate represents the AMR status of a calf at the corresponding time point of fecal collection. Of the 44 *E. coli* isolates, 18 (40.91%) and 26 (59.09%) were from zinc and placebo treated calves, respectively. The average age of calves that *E. coli* isolates were collected from was 15.7 days (SD 0.51). Of the *E. coli* isolates, 15.91% corresponded to fecal samples from calves which received no spectinomycin treatment, 15.91% received 1 dose, and 68.18% received 2 doses. Further, 25%, 11.36%, and 63.64% of *E. coli* isolates were from healthy, diarrheic, and diarrhea-recovered calves, respectively. For the 167 enterococci isolates collected through repeated sampling of calves, 67 (40.12%) and 100 (59.88%) were from zinc and placebo treated calves, respectively. The average age of calves which enterococci isolates were collected from was 14.34 days (SD 5.47). Further, 28.74%, 17.37%, 51.50%, and 2.4% of enterococci isolates corresponded to calves having received 0, 1, 2, and 3 doses of spectinomycin treatment, respectively; while 6.59%, 31.74%, 39.52%, and 22.16% were from healthy, day of diarrhea diagnosis, diarrheic, and diarrhea-recovered calves, respectively. Characteristics of commensal isolates by treatment group are summarized in Fig. 2.

Accelerated failure time (AFT) models

Due to the high frequency of right-censored antimicrobial susceptibility testing (AST) data, certain antimicrobial drugs were determined to be unsuitable for modelling given data sparseness and omitted from analysis. This

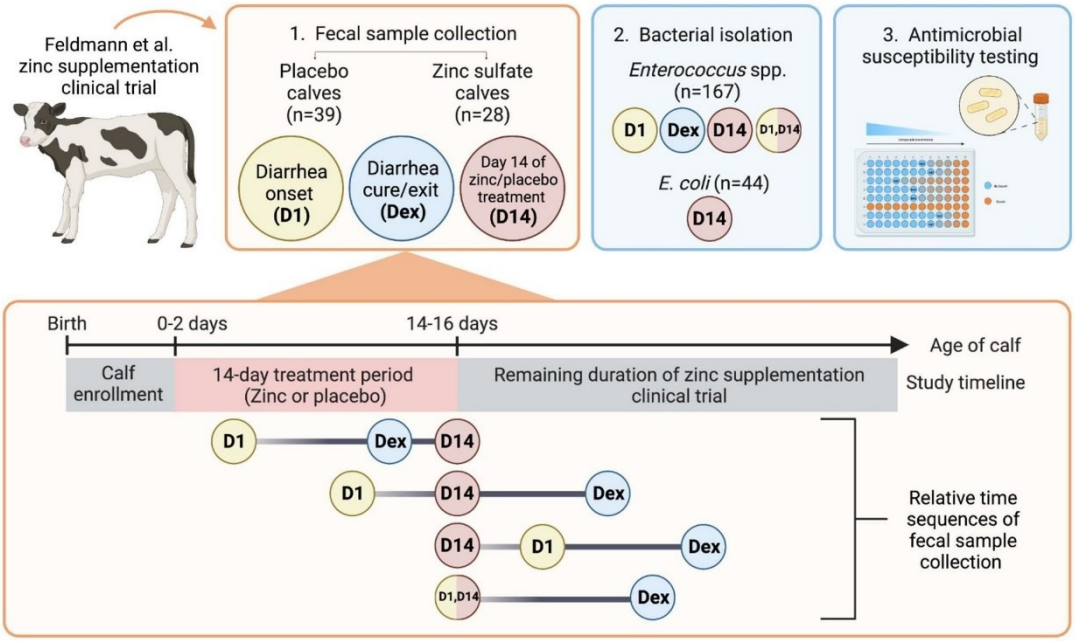

Figure 1. Schematic of fecal collection and analysis of samples collected from pre-weaned dairy calves enrolled in a zinc supplementation clinical trial.

Characteristic	Zinc sulfate (n = 28)	Placebo (n = 39)	Total (n = 67)	*p*-value*
Sex				
Bull	14/28 (50%)	23/39 (59.0%)	37/67 (55.2%)	0.62
Heifer	14/28 (50%)	16/39 (41.0%)	30/67 (44.8%)	
Attitude score				
1 (Bright, alert, readily stood w/stimulation)	16/28 (57.1%)	31/39 (79.5%)	47/67 (70.1%)	0.06
2 (Quiet, alert, and stood only with moderate stimulation)	12/28 (42.9%)	8/39 (20.5%)	20/67 (29.9%)	
Fecal score				
1 (Solid)	21/28 (75%)	31/39 (79.5%)	52/67 (77.6%)	0.89
2 (Semi-formed/loose)	1/28 (3.6%)	2/39 (5.1%)	3/67 (4.5%)	
NS = None seen	6/28 (21.4%)	6/39 (15.4%)	12/67 (17.9%)	
Age at enrollment				
1 day	7/28 (25%)	9/28 (32.1%)	16/67 (23.9%)	1.00
2 days	21/28 (75%)	30/39 (76.9%)	51/67 (76.1%)	

Table 1. Characteristics of Holstein pre-weaned dairy calves from a zinc supplementation clinical trial randomly selected for fecal sample collection and analysis. *Fisher's exact test.

included tetracycline, sulfisoxazole, trimethoprim-sulfamethoxazole, ampicillin, streptomycin, cefoxitin, chloramphenicol, amoxicillin-clavulanic acid, gentamicin, and ceftiofur for *E. coli*; and tetracycline, kanamycin, and lincomycin for enterococci isolates. The distribution of minimum inhibitory concentration (MIC) values and descriptive statistics (MIC_{50} and MIC_{90}) for all tested drugs from AST for *E. coli* and *Enterococcus* spp. isolates are presented in Supplementary Figs. 1–6 and Supplementary Tables 4 and 5.

A total of 17 accelerated failure time (AFT) models, consisting of models for 4 antimicrobial drugs for *E. coli* (Supplementary Table 6–9) and 13 antimicrobial drugs for *Enterococcus* spp. (Supplementary Tables 10–22), were constructed. For *E. coli*, the ciprofloxacin model with spectinomycin exposure specified as the number of doses (BIC 189.5612) was selected as the final model over the competing model with a binary spectinomycin treated or not variable (BIC 188.6194) despite having a lower BIC in the latter given the significant coefficient for the number of doses variable (Supplementary Table 7). For *Enterococcus* spp., the generalized gamma model for streptomycin (BIC 260.1047, Supplementary Table 13) was selected over its Weibull model counterpart (BIC

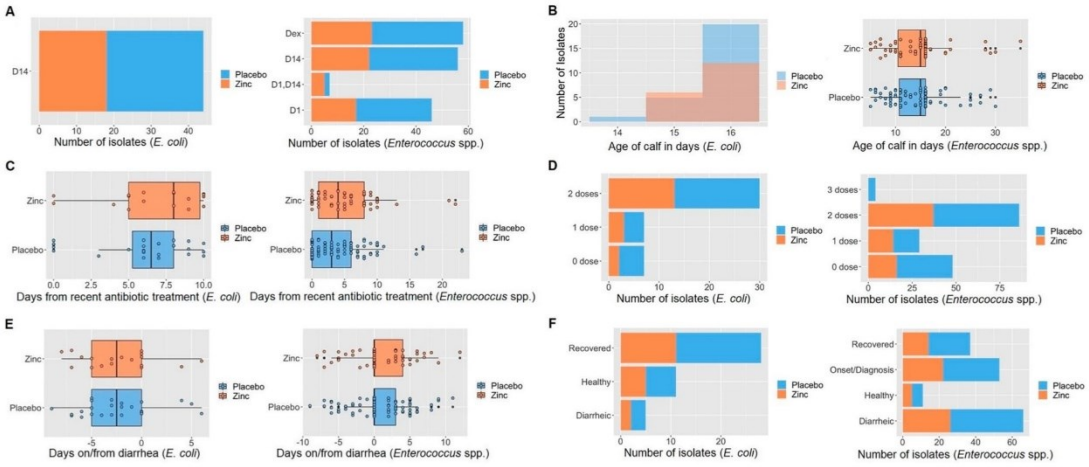

Figure 2. Characteristics of *E. coli* (n = 44) and *Enterococcus* spp. (n = 167) from pre-weaned dairy calves at the time of fecal sample collection by placebo and zinc treatment groups (**A**) Fecal sample type (**B**) Age of calf (**C**) Number of days from recent therapeutic spectinomycin treatment (**D**) Number of therapeutic spectinomycin treatments received (**E**) Days from diarrhea onset (positive numbers) and from diarrheal cure (negative numbers) and (**F**) Diarrhea disease status.

249.5898) due to estimates from the latter model exceeding the hypothetical maximum concentration for MIC; for the same reason, the exponential model for gentamicin (BIC 524.5724, Supplementary Table 22) was selected as the final model over its Weibull model counterpart (BIC 268.565). Lastly, the *Enterococcus* spp. linezolid model with categorical number of days from last spectinomycin treatment (BIC 303.3108) was selected as the final model over the one with a binary spectinomycin (treated or not) variable (BIC 299.7655) given the significant coefficient for the categorical variable (Supplementary Table 16).

Impact of zinc supplementation on AMR of fecal *E. coli* and *Enterococcus* spp.

For each AFT model, the exponentiated coefficient for the primary effect of treatment group (MIC Ratio, or MR) represents the change in MIC values associated with treatment status, in which a MR > 1 indicates that zinc supplementation was associated with an increase in phenotypic AMR whereas a MR < 1 indicates that zinc supplementation was associated with a decrease in phenotypic AMR[35,36]. Overall findings from AFT models indicated that zinc supplementation was not significantly associated with the MIC of *Enterococcus* spp. to 13 drugs: gentamicin, vancomycin, ciprofloxacin, erythromycin, penicillin, nitrofurantoin, linezolid, quinupristin/dalfopristin, tylosin tartrate, streptomycin, daptomycin, chloramphenicol, and tigecycline (MR = 0.96–2.94, $p > 0.05$) (Fig. 3, Table 2, and Supplementary Tables 10–22). In *E. coli*, no significant association was observed between zinc supplementation and resistance of isolates as measured using MIC values for ceftriaxone (MR = 0.95, $p > 0.05$) and azithromycin (MR = 0.80, $p > 0.05$) with the exception of ciprofloxacin (MR = 0.17, 95% CI 0.03–0.97) and nalidixic acid (MR = 0.28, 95% CI 0.15–0.53) where a protective effect against resistance was observed (Fig. 3, Table 3, and Supplementary Tables 6–9). No significant dose-dependent effect of zinc supplementation was observed across all antimicrobial drugs evaluated for *E. coli* and *Enterococcus* spp..

Therapeutic antimicrobial treatment and calf age were significantly associated with AMR

In this study, therapeutic spectinomycin treatment of pre-weaned dairy calves and calf age were significantly associated with AMR for certain antimicrobial drugs in fecal commensal isolates. The effect measures of these confounders represented the corresponding direct effects on AMR when other covariates were held constant; as indirect effects were not accounted for, it should be noted that the magnitude and direction of these associations were not necessarily representative of total effect estimations. Spectinomycin exposure was significantly associated with an increase in MIC for azithromycin, ciprofloxacin, and nalidixic acid in *E. coli* (Supplementary Tables 6, 7, and 8) and tigecycline and linezolid in enterococci isolates (Supplementary Tables 10 and 16). For *Enterococcus* spp., it was also significantly associated with a decrease in MIC for tylosin tartrate, nitrofurantoin, erythromycin, and gentamicin (Supplementary Tables 14, 17, 19, and 22). Calf age at time of sampling was found to be significantly associated with AMR in *Enterococcus* spp. models only; as all *E. coli* isolates were from calves of similar age (14–16 days), age was not evaluated in *E. coli* models. Age was a significant predictor in 5 of the 13 *Enterococcus* spp. models, in which it was significantly associated with a decrease in MIC for tigecycline, nitrofurantoin, penicillin, ciprofloxacin, and gentamicin (Supplementary Tables 10, 17, 18, 20, and 22).

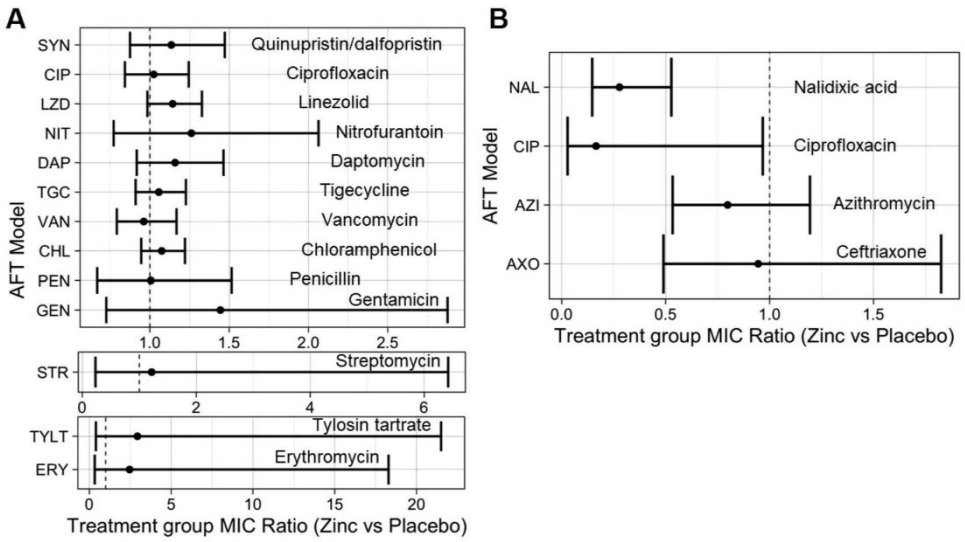

Figure 3. Treatment group minimum inhibitory concentration ratio (MR) estimates and corresponding 95% confidence intervals from accelerated failure time models, after controlling for confounders by (**A**) *Enterococcus* spp. and (**B**) *E. coli*.

Model	Coefficient (SE)	MIC ratio, MR (SE)	*p*-value	95% CI
SYN (quinupristin/dalfopristin)[a]	0.13 (0.13)	1.14 (0.15)	0.340	0.88, 1.47
CIP (ciprofloxacin)[b]	0.024 (0.10)	1.02 (0.10)	0.809	0.84, 1.25
LZD (linezolid)[c]	0.13 (0.076)	1.14 (0.087)	0.079	0.98, 1.33
NIT (nitrofurantoin)[a]	0.23 (0.25)	1.26 (0.32)	0.354	0.77, 2.06
ERY (erythromycin)[b]	0.90 (1.02)	2.46 (2.52)	0.379	0.33, 18.29
TYLT (tylosin tartrate)[b]	1.08 (1.01)	2.94 (2.99)	0.288	0.40, 21.50
DAP (daptomycin)[a]	0.15 (0.12)	1.16 (0.14)	0.216	0.92, 1.46
TGC (tigecycline)[b]	0.055 (0.076)	1.06 (0.081)	0.468	0.91, 1.23
VAN (vancomycin)[a]	−0.039 (0.099)	0.96 (0.095)	0.694	0.79, 1.17
CHL (chloramphenicol)[b]	0.072 (0.065)	1.07 (0.07)	0.270	0.95, 1.22
PEN (penicillin)[a]	0.0061 (0.21)	1.01 (0.21)	0.977	0.67, 1.52
GEN (gentamicin)[d]	0.37 (0.35)	1.44 (0.51)	0.295	0.73, 2.88
STR (streptomycin)[a]	0.20 (0.85)	1.22 (1.03)	0.816	0.23, 6.42

Table 2. Treatment group minimum inhibitory concentration ratio (MR) estimates for *Enterococcus* spp. accelerated failure time models (zinc vs placebo). [a]Model adjusted for age of calf in days and binary spectinomycin treatment variable (received or not). [b]Model adjusted for age of calf in days and days from last spectinomycin treatment. [c]Model adjusted for age of calf in days and categorical days from last spectinomycin treatment (0 days, 1–3 days, 4–7 days, and 8–23 days). [d]Model adjusted for categorical age of calf by week (5–7 days, 8–14 days, 15–21 days, 23–28 days, 29–35 days), and days from last spectinomycin treatment.

Model	Coefficient (SE)	MIC ratio, MR (SE)	*p*-value	95% CI
AZI (azithromycin)[a]	−0.23 (0.21)	0.80 (0.16)	0.273	0.53, 1.19
CIP (ciprofloxacin)[b]	−1.80 (0.90)	0.17 (0.15)	0.046	0.028, 0.97
NAL (nalidixic acid)[b]	−1.28 (0.33)	0.28 (0.09)	0.000	0.15, 0.53
AXO (ceftriaxone)[c]	−0.056 (0.34)	0.95 (0.32)	0.867	0.49, 1.83

Table 3. Treatment group minimum inhibitory concentration ratio (MR) estimates for *E. coli* accelerated failure time models (zinc vs placebo). [a]Model adjusted for categorical days from last spectinomycin treatment (0 days, 3–5 days, 6–8 days, 9–10 days). [b]Model adjusted for number of spectinomycin doses received (0, 1, or 2 doses). [c]Model adjusted for binary spectinomycin treatment variable (received or not) and days on/from diarrhea onset/cure.

Predictions of phenotypic antimicrobial resistance

To further understand the dynamics between zinc supplementation and AMR, predictions of MIC from AFT models for *Enterococcus* spp. (Fig. 4) and *E. coli* isolates (Fig. 5) were assessed. The predicted MIC for enterococci isolates after controlling for calf age was higher in zinc-treated compared to placebo-treated calves across all levels of spectinomycin exposure (Fig. 4, Supplementary Tables 27–39). Predicted MIC decreased after spectinomycin exposure for tylosin tartrate, gentamicin, chloramphenicol, erythromycin, daptomycin, nitrofurantoin, and penicillin and increased for ciprofloxacin, tigecycline, quinupristin/dalfopristin, streptomycin, and vancomycin (Fig. 4). In contrast to predictions from *Enterococcus* spp. models, predictions from *E. coli* models indicated that across all levels of spectinomycin exposure, isolates from placebo-treated calves had higher MIC than those from zinc-treated calves for all antimicrobial drugs (Fig. 5, Supplementary Tables 23–26). Differences in MIC were statistically significant between treatment groups for predicted *E. coli* resistance to nalidixic acid for calves that received 2 doses of spectinomycin treatment; *E. coli* from calves in the placebo group that received 2 doses of spectinomycin treatment on average had 4.76 µg/mL higher MIC for nalidixic acid compared to those from calves in the zinc treatment group that received the same number of spectinomycin doses (Fig. 5, Supplementary Table 25). Following spectinomycin exposure, the predicted *E. coli* MIC increased for ceftriaxone (Fig. 5, Supplementary Table 26). For azithromycin, the predicted MIC for *E. coli* exhibited a cyclical trend in days post spectinomycin treatment in which the MIC increased (3–5 days), decreased (6–8 days) and then increased (9–10 days) (Fig. 5, Supplementary Table 23). Across all models, zinc supplementation and therapeutic spectinomycin were not predicted to increase or decrease the MIC to above or below the CLSI resistance breakpoints of tested antimicrobials.

Discussion

The development of AMR is frequently attributed to use of antimicrobials, but there is increasing recognition that non-antimicrobial agents (e.g. biocides, antiseptics, and heavy metals) may also play a role in the modulation of AMR[37,38]. The large-scale application of these agents in agricultural and livestock production in particular, may pose considerable impact from veterinary medicine, environmental, and public health standpoints; hence, better understanding of non-antimicrobial influences on AMR dynamics in these systems is of great importance. The present study investigated the impact of dietary zinc supplementation in pre-weaned calves on phenotypic AMR using fecal *E. coli* and *Enterococcus* spp. as Gram-negative and Gram-positive indicator organisms, respectively.

In this study, zinc supplementation did not significantly change the MIC to all antimicrobials tested for *E. coli* and *Enterococcus* spp. with the exception of nalidixic acid and ciprofloxacin, in which zinc supplementation was associated with a protective effect against quinolone resistance in *E. coli*. Although the temporality of exposure to zinc preceding resistance cannot be definitively established from our study data due to the cross-sectional nature of evaluating *E. coli* isolates collected at one time point, a previous study evaluating the in vitro selection dynamics of zinc on ciprofloxacin provides evidence in agreement with findings in this study[39]. In laboratory conditions of 0.00625 µg/mL ciprofloxacin and no zinc, ciprofloxacin-resistant *E. coli* was shown to have a significant growth rate advantage relative to a ciprofloxacin-susceptible strain, with this advantage disappearing in the presence of zinc sulfate (0.5 mM and 1.0 mM) at the same ciprofloxacin concentration[39]. Vos et al. further showed through a competition assay that ciprofloxacin-susceptible *E. coli* outcompetes its resistant counterpart under conditions of 0.5 mM zinc sulfate and 0.025 µg/mL ciprofloxacin[39]. While additional work is needed to fully elucidate the role of zinc on quinolone resistance, these congruent findings collectively support the potential antagonistic effects of zinc sulfate on *E. coli* resistance to certain antimicrobial classes.

Limited studies have evaluated the impact of dietary zinc on AMR in calves, with existing zinc supplementation studies conducted in other livestock and evaluating different zinc concentrations. The concentration of zinc fed once per day through milk to pre-weaned dairy calves in this study was 0.22 g zinc sulfate monohydrate, or 80 mg of elemental zinc. Such a non-toxic zinc dose for calves was previously established through a clinical trial[13], which alongside the field trial that contributed samples for the present analysis and other independent studies, collectively found beneficial impacts of zinc supplementation on the growth performance and diarrheal recovery in pre-weaned calves[14,19,40]. The lack of evidence for the promotion of AMR by dietary zinc in this study is consistent with a factorial trial on feedlot cattle that found copper and/or zinc supplementation (30 or 300 mg/kg) over a 32-day period resulted in minimal changes to MICs of copper, zinc, and antibiotics of fecal *E. coli* and *Enterococcus* spp[25]. These results are further corroborated through another feedlot cattle study, where quantified resistance to ceftriaxone and tetracycline among enteric *E. coli* were unaffected by dietary zinc concentrations (0–90 mg/kg)[26]. In contrast, one feedlot cattle study found that dietary zinc was associated with a significant increase in macrolide resistance of fecal enterococci isolates[27]. The relationship between zinc supplementation and AMR has been more extensively studied in swine, where concentrations of > 2000 mg/kg are typically used in feed additives to prevent post-weaning diarrhea and to enhance growth performance[41]. In contrast to findings from dietary zinc in cattle, multiple studies on swine have found that high dietary zinc may promote AMR, with one study finding that dietary zinc oxide (2,103 mg/kg) increased multi-resistant *E. coli* in feces, digesta, and mucosa[21]; and another finding an increased proportion of multi-resistant *E. coli* in ileum and colon digesta of between zinc oxide (18.6%; 2500 mg/kg) and control (0%; 50 mg/kg) groups[22]. The conflicting findings across these studies suggests that the modulation of AMR by zinc may be in part, or in combination with other unidentified factors, affected by: (1) the host site of bacterial isolation, (2) the duration and dose of supplementation, and (3) the form of zinc (organic/inorganic) administered. In cattle and swine, the absorption of trace minerals such as zinc occurs primarily in the small intestine, with endogenous zinc loss mainly occurring through excretion of feces[42,43]. Differences in the bioavailability and dose of administered zinc may result in variable quantities and duration of zinc presence in the intestinal lumen, which could subsequently exert disparate effects on the gut resistome. Zinc absorption and excretion are homeostatically regulated, with a previous study finding that pigs

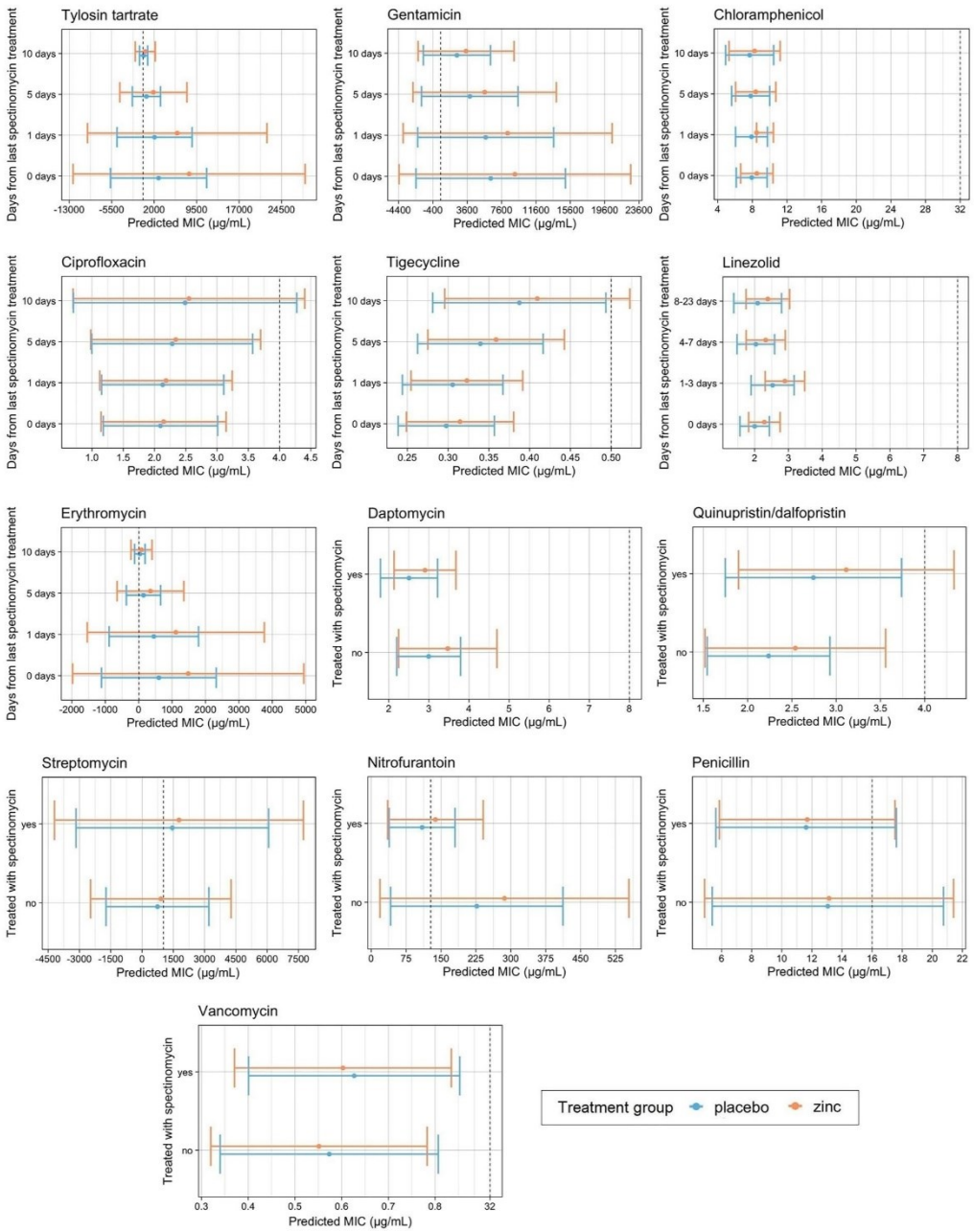

Figure 4. Predicted minimum inhibitory concentrations (MIC, µg/mL) and corresponding 95% confidence intervals for *Enterococcus* spp. accelerated failure time (AFT) models for each antimicrobial drug model and by therapeutic spectinomycin treatment status, controlling for calf age. Dashed lines mark resistant breakpoints based on CLSI criteria for each antimicrobial drug.

fed 2000 ppm zinc did not become zinc loaded and excrete increased quantities of zinc in feces until after 13 to 14 days of supplementation[44,45]. Although the digestive tracts of swine (non-ruminant) and cattle (ruminant) at maturity are structurally different, the gastrointestinal (GI) tract of pre-weaned calves is functionally monogastric

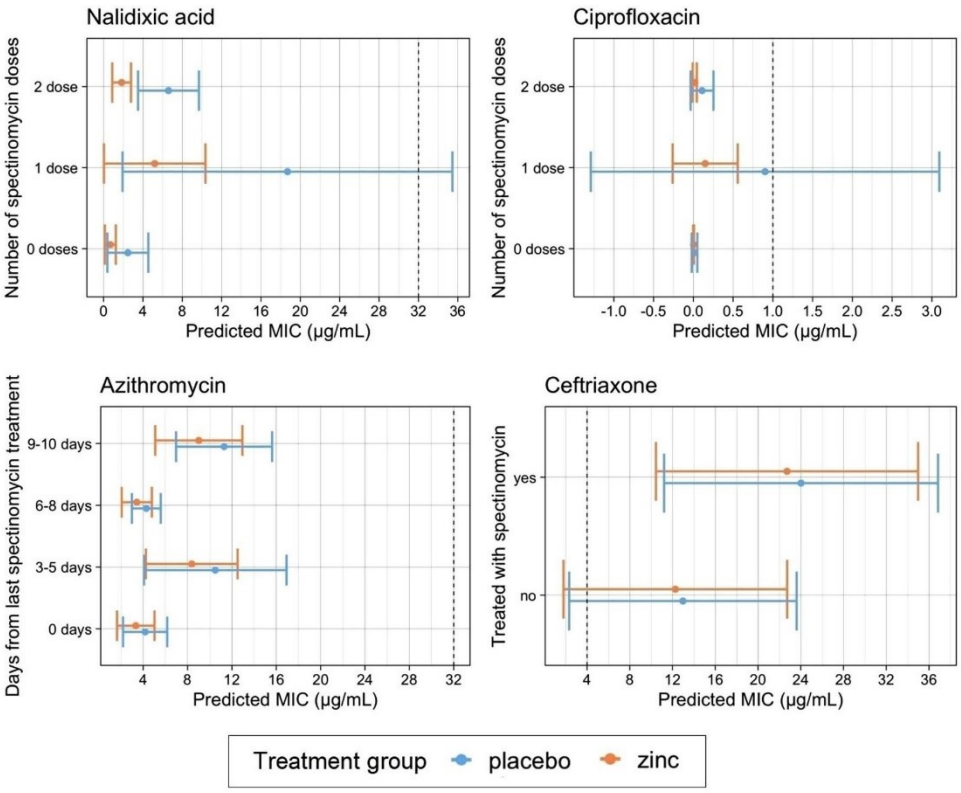

Figure 5. Predicted minimum inhibitory concentration (MIC, µg/mL) and corresponding 95% confidence intervals for *E. coli* accelerated failure time (AFT) models for each antimicrobial drug model, by therapeutic spectinomycin treatment status. Dashed lines mark resistant breakpoints based on CLSI criteria for each antimicrobial drug.

like that of swine. These GI physiological similarities and the same duration of zinc supplementation evaluated in this study (14 days) suggest that improved health outcomes of pre-weaned calves from zinc supplementation (80 mg elemental zinc) in this study likely occurred with minimal excess zinc excretion.

The age-dependent acquisition and loss of AMR in pre-weaned dairy calves has been well documented to show transient increases in AMR during early calf life[3,5,7,46–49], which corroborate findings from this study. Higher levels of multiple resistance in fecal *E. coli* isolates were observed in calves 2 weeks of age compared to day-old calves (OR = 53.6)[3]. Additionally, a case–control study evaluating the occurrence of AMR in fecal *E. coli* from diarrheic and healthy dairy calves found higher prevalence of resistance to ampicillin, tetracycline, or sulfonamide ($p \leq 0.05$) in diarrheic calves[50]. Similarly, this study found high phenotypic AMR (right-censoring of MIC data) in diarrhea-associated *E. coli* isolates collected from calves around 2 weeks of age, which limited our ability to model AMR dynamics of several antimicrobial drugs for *E. coli*. In enterococci isolates, age was significantly associated with a decrease in MIC for 5 of the 13 antimicrobials evaluated after accounting for zinc and spectinomycin exposure. The age-dependent trends in calf AMR has been thought to be driven by host immune status of increased susceptibility during early life that allows ease of colonization by resistant bacteria, exposure through the environment and dam (e.g. colostrum), and rapid establishment of the early gut microbiome[7,8]. A previous study demonstrated that the acquisition of the resistome throughout calf life occurs even in the absence of antimicrobial exposure[8]. Liu et al. also demonstrated that facultative anaerobes such as *Escherichia* and *Enterococcus*, which are frequent carriers of AMR, constitute greater portions of the fecal microbiota during early life but decrease in relative abundance over time[8]. Calf diarrhea has been shown to result in further perturbation of the gut microbiota, with multiple studies observing shifts or differences in gut microbial composition between healthy and diarrheic calves[51–54]. These findings collectively support the hypothesis that stabilization and maturation of the calf gut microbiota with age may impart differential fitness costs for resistance, providing susceptible bacteria a competitive advantage and ultimately leading to a decrease in the resistant enteric microbial population as observed in *Enterococcus* spp. in our study.

Although this study was unable to evaluate the effects of zinc supplementation in absence of antimicrobial exposures, these data provide practical insights on the combined effects of antimicrobial treatment, dietary heavy

metal exposure, and host-level factors on phenotypic AMR, interactions that remain undercharacterized. The AMR selective pressures in our study were from dietary milk (same concentration and duration of tetracycline and neomycin for all calves) and therapeutic spectinomycin (same dosage but varied in time and duration of treatment for each calf) which were reflected in the high right-censoring to tested drugs from the same antimicrobial class in our *E. coli* and *Enterococcus* spp. study isolates. These broad-spectrum antibiotics correspond to tetracycline and aminoglycoside/aminocyclitol classes and are commonly used for the treatment of diarrhea in calves; USDA reports from 2014 indicate that of pre-weaned heifers treated with antimicrobials for diarrhea and digestive disorders, aminoglycoside and tetracycline were the third and fourth most commonly administered primary antimicrobials, with 14.7% and 11.2% of calves with these diseases treated with drugs corresponding to these respective classes[12]. The other reported antimicrobials frequently used—third-generation cephalosporins (27.6%) and trimethoprim/sulfamethoxazole (18.7%)[12]—were not administered to calves in our study. Despite this, we also observed right-censored or elevated MICs to these drugs (trimethoprim/sulfamethoxazole and ceftriaxone) in our data, indicating the occurrence of AMR selection in absence of direct selective pressures. Regarding the differential exposure of therapeutic spectinomycin for each calf that was controlled for in all models, neither zinc supplementation nor spectinomycin modulated the predicted MIC of all tested antimicrobials to above or below CLSI resistant breakpoints. These predictions from AFT models allowed for the quantification of absolute changes in resistance to better understand AMR selection dynamics. For example, model predictions indicated that after receiving two doses of therapeutic spectinomycin, *E. coli* from placebo-treated calves had a significantly higher nalidixic acid MIC relative to those from zinc-treated calves. These predictions support aforementioned in vivo findings that the putative inhibitory effects of zinc on quinolone resistance may occur in combination with antimicrobial selective pressures[39]. Antimicrobial therapy has been shown to transiently modify the calf gut microbiota, which may favorably enrich resistant bacteria[55,56]. Indeed, model predictions from this study indicated that following spectinomycin treatment, MICs to certain antimicrobials increase in both *E. coli* (ceftriaxone) and *Enterococcus* spp. (ciprofloxacin, tigecycline, quinupristin/dalfopristin, streptomycin, and vancomycin). However, the decreasing trend in MIC to other antimicrobials—primarily in *Enterococcus* spp. to tylosin tartrate, gentamicin, chloramphenicol, erythromycin, daptomycin, nitrofurantoin, and penicillin—and the cyclical pattern in azithromycin MIC of *E. coli* following spectinomycin treatment underscores the complexity of AMR selection. With respect to the latter finding, previous studies have observed similar cyclical resistance trends in fecal *Enterobacteriaceae* from dairy cattle following antimicrobial treatment. This included one study that observed cyclical peaks in neomycin resistant *Enterobacteriaceae* counts following treatment with the drug[57], and another which observed cyclical re-emergence of ceftiofur-resistant *Enterobacteriaceae* counts following systemic antimicrobial drug treatment[58]. In some bacteria, antimicrobial exposure has been shown to induce mutagenesis and conjugation[59–61], which can be followed by emergence of additional compensatory mutations that alleviate fitness costs associated with resistance (e.g. positive epistasis)[62,63]. Such variation in fitness costs would support how modifications of the calf enteric microbiota from collective influences of antimicrobial therapy, disease (e.g. diarrhea), and other changes in host-factors culminate a microbial system that is conducive to the divergent and bidirectional selection of bacterial resistance.

In conclusion, the current study found a lack of evidence for the selection of phenotypic AMR to the majority of tested antimicrobial drugs in fecal commensal bacteria from dietary zinc supplementation in pre-weaned dairy calves. The primary limitations of this study include the cross-sectional assessment of *E. coli* at one time point which limited our ability to determine the temporality of association between dietary zinc treatment and AMR, and the small sample size of *E. coli* which may impact the reliability of study findings. Additionally, our study may have limited external validity due to samples originating from just one dairy. Future studies conducted across different dairies with varied environmental factors and management practices, with larger sample sizes, and evaluating multiple isolates per sample are needed to validate the effect sizes observed in our study, particularly for the significant findings of the antagonistic association between zinc supplementation and quinolone resistance in *E. coli*. As our main objective was to assess the impact of dietary zinc on AMR, AFT models specified in this study were not designed to obtain unbiased point estimates on the relationship of calf age and antimicrobial treatment with AMR. Additionally, the extreme model estimates observed in our study for two models (gentamicin and streptomycin for *Enterococcus* spp.) indicated that AFT models may not be optimal for data with a high frequency of right- and left- censoring; hence, alternative approaches such as truncated interval censored models should be explored for these types of data in future studies. Further research that evaluates a longer follow-up period (e.g. through post-weaning), the impact of other forms of dietary zinc (e.g. chelated zinc), and different resistance outcomes are needed. Additionally, genomic analysis of isolates in this study would provide additional insight on the AMR dynamics of dietary zinc supplementation, antimicrobial treatment, and disease status in pre-weaned dairy calves. Collectively, this work corroborates previous reports of host-level drivers (e.g. age and antimicrobial exposure) on AMR, and provides important insights into the largely unexplored dynamics of dietary zinc and AMR in pre-weaned dairy calves.

Data availability

Data from this study are presented in the Supplementary File. Additional source data are available from the corresponding author upon reasonable request.

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Author contributions

Conceptualization: K.Y.L., E.R.A., X.L., B.C.W., and S.S.A. Data collection and curation: K.Y.L., H.R.F., D.R.W., and S.S.A. Laboratory analysis: K.Y.L. Formal analysis: K.Y.L. and S.S.A. Investigation: K.Y.L. and S.S.A. Resources: X.L., E.R.A., and S.S.A. Writing—original draft preparation: K.Y.L. Writing—Review and editing: D.R.W., B.C.W., S.S.A. Visualization: K.Y.L. Project administration: K.Y.L. and S.S.A. Funding acquisition: K.Y.L. All authors have read the article and approved the submitted version.

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Materials

Supplementary Table 1. Primer sequences used for PCR confirmation of *E. coli* and *Enterococcus* spp.

Organism	Primer sequence	
E. coli	Forward	5'-CCG ATA CGC TGC CAA TCA GT-3'
E. coli	Reverse	5'-ACG CAG ACC GTA GGC CAG AT-3'
Enterococcus spp.	Forward	5'-AGA AAT TCC AAA CGA ACT TG-3'
Enterococcus spp.	Reverse	5'-CAG TGC TCT ACC TCC ATC ATT-3'

Supplementary Table 2. Drug panel and dilutions used for antimicrobial susceptibility testing of *E. coli* isolates

Drug Class	Abbreviation	Antimicrobial drug	Range (µg/mL)
beta-lactam	FOX	Cefoxitin	0.5 - 32
macrolide	AZI	Azithromycin	0.12 - 16
chloramphenicol	CHL	Chloramphenicol	2 - 32
tetracycline	TET	Tetracycline	4 - 32
beta-lactam	AXO	Ceftriaxone	0.25 - 64
beta-lactam	AUG2	Amoxicillin/clavulanic acid	1/0.5 - 32/16
quinolone	CIP	Ciprofloxacin	0.015 - 4
aminoglycoside	GEN	Gentamicin	0.25 - 16
quinolone	NAL	Nalidixic acid	0.5 - 32
beta-lactam	XNL	Ceftiofur	0.12 - 8
sulfonamide	FIS	Sulfisoxazole	16 - 256
sulfonamide	SXT	Trimethoprim-sulfamethoxazole	0.12/2.38 - 4/76
beta-lactam	AMP	Ampicillin	1-32
aminoglycoside	STR	Streptomycin	2.64

Supplementary Table 3. Drug panel and dilutions used for antimicrobial susceptibility testing of *Enterococcus* spp. isolates

Drug Class	Abbreviation	Antimicrobial drug	Range (µg/mL)
glycylcycline	TCG	Tigecycline	0.015 - 0.5
tetracycline	TET	Tetracycline	1-32
chloramphenicol	CHL	Chloramphenicol	2-32
lipopeptide	DAP	Daptomycin	0.25 - 16
aminoglycoside	STR	Streptomycin	512 - 2048
macrolide	TYLT	Tylosin tartrate	0.25 - 32
streptogramin	SYN	Quinupristin / dalfopristin	0.5 - 32
oxazolidinone	LZD	Linezolid	0.5 - 8
nitrofurans	NIT	Nitrofurantoin	2-64
beta-lactam	PEN	Penicillin	0.25 - 16
aminoglycoside	KAN	Kanamycin	128 - 1024
macrolide	ERY	Erythromycin	0.25 - 8
quinolone	CIP	Ciprofloxacin	0.12 - 4
glycopeptide	VAN	Vancomycin	0.25 - 32
lincosamide	LIN	Lincomycin	1-8
aminoglycoside	GEN	Gentamicin	128 - 1024

Supplementary Figure 1. Distribution of minimum inhibitory concentration from antimicrobial susceptibility testing for all *E. coli* isolates (n=44). Shaded areas indicate the range of tested antimicrobials. Numbers listed outside of the shaded range correspond to right/left censored counts.

	Distribution of MIC (µg/mL) - Number of *E. coli* isolates (n=44)															
Antimicrobial drug (Abbreviation)	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	
Cefoxitin (FOX)						0	0	0	2	0	1	12	29			
Azithromycin (AZI)				0	0	0	0	2	23	13	2	4				
Chloramphenicol (CHL)								0	0	9	2	0	33			
Tetracycline (TET)									0	0	0	0	44			
Ceftriaxone (AXO)				2	0	0	0	0	0	12	17	8	2	3		
Amoxicillin/clavulanic acid (AUG2)							0	0	0	3	0	37	4			
Ciprofloxacin (CIP)	16	0	21	1	0	0	2	0	0	0	4					
Gentamicin (GEN)						0	2	3	1	0	0	0	38			
Nalidixic acid (NAL)							0	0	30	9	0	1	0	4		
Ceftiofur (XNL)				0	0	2	0	0	1	17	24					
Sulfisoxazole (FIS)											0	0	0	0		44
Trimethoprim-sulfamethoxazole (SXT)				0	0	0	0	0	0	44						
Ampicillin (AMP)							0	0	0	0	0	0	44			
Streptomycin (STR)								0	0	0	1	0	0	43		

Supplementary Figure 2. Distribution of minimum inhibitory concentration from antimicrobial susceptibility testing for *E. coli* isolates from placebo calves (n=26). Shaded areas indicate the range of tested antimicrobials. Numbers listed outside of the shaded range correspond to right/left censored counts.

	Distribution of MIC (µg/mL) - Number of *E. coli* isolates (n=26)															
Antimicrobial drug (Abbreviation)	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	
Cefoxitin (FOX)						0	0	0	1	0	0	6	19			
Azithromycin (AZI)				0	0	0	0	2	13	8	0	3				
Chloramphenicol (CHL)								0	0	3	2	0	21			
Tetracycline (TET)									0	0	0	0	26			
Ceftriaxone (AXO)				1	0	0	0	0	0	7	10	5	2	1		
Amoxicillin/clavulanic acid (AUG2)							0	0	0	1	0	22	3			
Ciprofloxacin (CIP)	10	0	10	1	0	0	1	0	0	0	4					
Gentamicin (GEN)					0	1	3	1	0	0	0	21				
Nalidixic acid (NAL)						0	0	17	4	0	1	0	4			
Ceftiofur (XNL)				0	0	1	0	0	1	9	15					
Sulfisoxazole (FIS)											0	0	0	0	0	26
Trimethoprim-sulfamethoxazole (SXT)				0	0	0	0	0	0	26						
Ampicillin (AMP)							0	0	0	0	0	0	26			
Streptomycin (STR)								0	0	0	1	0	0	25		

Supplementary Figure 3. Distribution of minimum inhibitory concentration from antimicrobial susceptibility testing for *E. coli* isolates from zinc treatment calves (n=18). Shaded areas indicate the range of tested antimicrobials. Numbers listed outside of the shaded range correspond to right/left censored counts.

	Distribution of MIC (µg/mL) - Number of *E. coli* isolates (n=18)																
Antimicrobial drug (Abbreviation)		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	
Cefoxitin (FOX)							0	0	0	1	0	1	6	10			
Azithromycin (AZI)					0	0	0	0	0	10	5	2	1				
Chloramphenicol (CHL)									0	0	6	0	0	12			
Tetracycline (TET)										0	0	0	0	18			
Ceftriaxone (AXO)					1	0	0	0	0	0	5	7	3	0	2		
Amoxicillin/clavulanic acid (AUG2)								0	0	0	2	0	15	1			
Ciprofloxacin (CIP)	6	0	11	0	0	0	1	0	0	0							
Gentamicin (GEN)						0	1	0	0	0	0	0	17				
Nalidixic acid (NAL)							0	0	13	5	0	0	0				
Ceftiofur (XNL)					0	0	1	0	0	0	8	9					
Sulfisoxazole (FIS)												0	0	0	0	0	18
Trimethoprim-sulfamethoxazole (SXT)					0	0	0	0	0	0	18						
Ampicillin (AMP)								0	0	0	0	0	0	18			
Streptomycin (STR)									0	0	0	0	0	0	18		

Supplementary Table 4. Descriptive statistics for minimum inhibitory concentrations from antimicrobial susceptibility testing for *E. coli* isolates.

Antimicrobial drug	**All isolates**		**Placebo isolates**		**Zinc isolates**	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Cefoxitin (FOX)	>32	>32	>32	>32	>32	>32
Azithromycin (AZI)	4	16	4	>16	4	16
Chloramphenicol (CHL)	>32	>32	>32	>32	>32	>32
Tetracycline (TET)	>32	>32	>32	>32	>32	>32
Ceftriaxone (AXO)	16	64	16	64	16	>64
Amoxicillin/clavulanic acid (AUG2)	32	32	32	>32	32	32
Ciprofloxacin (CIP)	0.03	0.5	0.03	>4	0.03	0.03
Gentamicin (GEN)	>16	>16	>16	>16	>16	>16
Nalidixic acid (NAL)	2	16	2	>32	2	4
Ceftiofur (XNL)	>8	>8	>8	>8	8	>8
Sulfisoxazole (FIS)	>128	>128	>128	>128	>128	>128
Trimethoprim-sulfamethoxazole (SXT)	>4	>4	>4	>4	>4	>4
Ampicillin (AMP)	>32	>32	>32	>32	>32	>32
Streptomycin (STR)	>64	>64	>64	>64	>64	>64

Supplementary Figure 4. Distribution of minimum inhibitory concentration from antimicrobial susceptibility testing for all *Enterococcus* spp. isolates (n=167). Shaded areas indicate the range of tested antimicrobials. Numbers listed outside of the shaded range correspond to right/left censored counts.

Antimicrobial drug	Distribution of MIC (µg/mL) - Number of *Enterococcus* spp. isolates (n=167)																			
	0.012	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096
Tigecycline		0	0	7	39	75	46													
Tetracycline								0	0	0	0	0	1	166						
Chloramphenicol									0	0	53	91	3	20						
Daptomycin						0	12	45	22	80	7	1								
Streptomycin																23	0	8	4	132
Tylosin tartrate						0	1	9	17	12	0	1	0	127						
Quinupristin / dalfopristin						4	0	18	36	85	5	17	1	1						
Linezolid							0	0	75	89	3									
Nitrofurantoin									0	0	29	37	3	28	70					
Penicillin						0	0	10	63	17	44	13	20							
Kanamycin														2	0	0	0	0	165	
Erythromycin					4	0	25	0	5	4	1	128								
Ciprofloxacin					0	0	0	7	60	48	52									
Vancomycin						0	57	58	50	1	0	0	1							
Lincomycin							4	0	0	0	2	161								
Gentamicin														80	0	1	0	0	86	

Supplementary Figure 5. Distribution of minimum inhibitory concentration from antimicrobial susceptibility testing for *Enterococcus* spp. isolates from placebo calves (n=100). Shaded areas indicate the range of tested antimicrobials. Numbers listed outside of the shaded range correspond to right/left censored counts.

Antimicrobial drug	Distribution of MIC (µg/mL) - Number of *Enterococcus* spp. isolates (n=100)																		
	0.012	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096
Tigecycline		0	0	2	24	50	24													
Tetracycline								0	0	0	0	0	1	99						
Chloramphenicol									0	0	35	53	1	11						
Daptomycin						0	6	29	13	49	3	0								
Streptomycin																14	0	4	2	80
Tylosin tartrate						0	1	7	10	7	0	1	0	74						
Quinupristin / dalfopristin						3	0	12	22	50	3	9	0	1						
Linezolid							0	0	50	49	1									
Nitrofurantoin									0	0	20	20	3	16	41					
Penicillin						0	0	7	38	8	27	7	13							
Kanamycin														2	0	0	0	0	98	
Erythromycin					2	0	17	0	3	3	0	75								
Ciprofloxacin					0	0	0	4	38	28	30									
Vancomycin						0	33	34	33	0	0	0	0							
Lincomycin							4	0	0	0	1	95								
Gentamicin														52	0	1	0	0	47	

Supplementary Figure 6. Distribution of minimum inhibitory concentration from antimicrobial susceptibility testing for *Enterococcus* spp. isolates from zinc treatment calves (n=67). Shaded areas indicate the range of tested antimicrobials. Numbers listed outside of the shaded range correspond to right/left censored counts.

Antimicrobial drug	Distribution of MIC (µg/mL) - Number of *Enterococcus* spp. isolates (n=67)																		
	0.012	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	4096
Tigecycline		0	0	5	15	25	22													
Tetracycline								0	0	0	0	0	0	67						
Chloramphenicol									0	0	18	38	2	9						
Daptomycin						0	6	16	9	31	4	1								
Streptomycin																9	0	4	2	52
Tylosin tartrate						0	0	2	7	5	0	0	0	53						
Quinupristin / dalfopristin						1	0	6	14	35	2	8	1							
Linezolid							0	0	25	40	2									
Nitrofurantoin									0	0	9	17	0	12	29					
Penicillin						0	0	3	25	9	17	6	7							
Kanamycin															0	0	0	0	67	
Erythromycin					2	0	8	0	2	1	1	53								
Ciprofloxacin					0	0	0	3	22	20	22									
Vancomycin						0	24	24	17	1	0	0	1							
Lincomycin								0	0	0	1	67								
Gentamicin														28	0	0	0	0	39	

Supplementary Table 5. Descriptive statistics for minimum inhibitory concentrations from antimicrobial susceptibility testing for *Enterococcus* isolates.

Antimicrobial drug	**All isolates**		**Placebo isolates**		**Zinc isolates**	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Tigecycline (TGC)	0.25	0.5	0.25	0.5	0.25	0.5
Tetracycline (TET)	>32	>32	>32	>32	>32	>32
Chloramphenicol (CHL)	16	>32	16	>32	16	>32
Daptomycin (DAP)	4	4	4	4	4	4
Streptomycin (STR)	>2048	>2048	>2048	>2048	>2048	>2048
Tylosin tartrate (TYLT)	>32	>32	>32	>32	>32	>32
Quinupristin/dalfopristin (SYN)	4	16	4	8	4	16
Linezolid (LZD)	4	4	2	4	4	4
Nitrofurantoin (NIT)	64	>64	64	>64	64	>64
Penicillin (PEN)	4	>16	4	>16	4	>16
Kanamycin (KAN)	>1024	>1024	>1024	>1024	>1024	>1024
Erythromycin (ERY)	>8	>8	>8	>8	>8	>8
Ciprofloxacin (CIP)	4	>4	4	>4	4	>4
Vancomycin (VAN)	1	2	1	2	1	2
Lincomycin (LIN)	>8	>8	>8	>8	>8	>8
Gentamicin (GEN)	>1024	>1024	<=128	>1024	>1024	>1024

Supplementary Table 6. Final accelerated failure time (AFT) model for azithromycin minimum inhibitory concentrations for *E. coli* isolates (BIC 144.0987, Weibull distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	-0.23 (0.21)	0.80 (0.16)	0.273	0.53, 1.19
	Placebo	Referent	-	-	-
Days from last spectinomycin treatment	3-5 days	0.92 (0.35)	2.51 (0.89)	0.009	1.26, 5.02
	6-8 days	0.025 (0.27)	1.03 (0.28)	0.926	0.60, 1.74
	9-10 days	1.00 (0.29)	2.70 (0.79)	0.001	1.53, 4.78
	0 days	Referent	-	-	-
Intercept	-	1.43 (0.24)	4.18 (1.02)	0	2.59, 6.73

Supplementary Table 7. Final accelerated failure time (AFT) model for ciprofloxacin minimum inhibitory concentrations for *E. coli* isolates (BIC 189.5612, Weibull distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	-1.80 (0.90)	0.17 (0.15)	0.046	0.028, 0.97
	Placebo	Referent	-	-	-
Number of spectinomycin doses received	1	4.12 (1.67)	61.64 (102.96)	0.014	2.33, 1627.81
	2	2.01 (1.20)	7.45 (8.92)	0.094	0.71, 77.96
	0	Referent	-	-	-
Intercept	-	-4.22 (1.24)	0.015 (0.018)	0.001	0.0013, 0.17

Supplementary Table 8. Final accelerated failure time (AFT) model for nalidixic acid minimum inhibitory concentrations for *E. coli* isolates (BIC 181.116, Exponential distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	-1.28 (0.33)	0.28 (0.09)	0	0.15, 0.53
	Placebo	Referent	-	-	-
Number of spectinomycin doses received	1	2.02 (0.60)	7.57 (4.55)	0.001	2.33, 24.57
	2	0.98 (0.44)	2.67 (1.16)	0.024	1.14, 6.27
	0	Referent	-	-	-
Intercept	-	0.90 (0.43)	2.47 (1.06)	0.036	1.06, 5.73

Supplementary Table 9. Final accelerated failure time (AFT) model for ceftriaxone minimum inhibitory concentrations for *E. coli* isolates (BIC 172.8303, Exponential distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	-0.056 (0.34)	0.95 (0.32)	0.867	0.49, 1.83
	Placebo	Referent	-	-	-
Days on/from diarrhea		0.095 (0.049)	1.10 (0.054)	0.052	1.00, 1.21
Received spectinomycin treatment	Yes	0.62 (0.43)	1.85 (0.80)	0.152	0.80, 4.31
	No	Referent	-	-	-
Intercept	-	2.56 (0.42)	12.97 (5.43)	0	5.71, 29.47

Supplementary Table 10. Final accelerated failure time (AFT) model for tigecycline minimum inhibitory concentrations for enterococci isolates (BIC 422.7243, Weibull distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	0.055 (0.076)	1.06 (0.081)	0.468	0.91, 1.23
	Placebo	Referent	-	-	-
Age of calf (days)	-	-0.031 (0.0065)	0.97 (0.0063)	0	0.96, 0.98
Days from spectinomycin treatment	-	0.026 (0.0078)	1.03 (0.0080)	0.001	1.01, 1.04
Intercept	-	-1.21 (0.10)	0.30 (0.030)	0	0.24, 0.36

Supplementary Table 11. Final accelerated failure time (AFT) model for chloramphenicol minimum inhibitory concentrations for enterococci isolates (BIC 403.4433, Ggamma distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	0.072 (0.065)	1.07 (0.07)	0.270	0.95, 1.22
	Placebo	Referent	-	-	-
Age of calf (days)	-	-0.015 (0.0096)	0.98 (0.0094)	0.111	0.97, 1.00
Days from last antibiotic treatment	-	-0.0031 (0.011)	1.00 (0.011)	0.775	0.98, 1.02
Intercept	-	2.07 (0.11)	7.93 (0.91)	0	6.33, 9.92

Supplementary Table 12. Final accelerated failure time (AFT) model for daptomycin minimum inhibitory concentrations for enterococci isolates (BIC 522.3062, Weibull distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	0.15 (0.12)	1.16 (0.14)	0.216	0.92, 1.46
	Placebo	Referent	-	-	-
Age of calf (days)		-0.015 (0.0078)	0.98 (0.0077)	0.052	0.97, 1.00
Received spectinomycin treatment	Yes	-0.18 (0.12)	0.84 (0.10)	0.149	0.66, 1.07
	No	Referent	-	-	-
Intercept	-	1.10 (0.14)	2.99 (0.41)	0	2.30, 3.91

Supplementary Table 13. Final accelerated failure time (AFT) model for streptomycin minimum inhibitory concentrations for enterococci isolates (BIC 260.1047, Ggamma distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	0.20 (0.85)	1.22 (1.03)	0.816	0.23, 6.42
	Placebo	Referent	-	-	-
Age of calf (days)	-	-0.20 (0.14)	0.82 (0.11)	0.143	0.62, 1.07
Received spectinomycin treatment	Yes	0.67 (1.09)	1.96 (2.14)	0.537	0.23, 16.68
	No	Referent	-	-	-
Intercept	-	6.61 (1.70)	740.40 (1258.26)	0	26.48, 20702.87

Supplementary Table 14. Final accelerated failure time (AFT) model for tylosin tartrate minimum inhibitory concentrations for enterococci isolates (BIC 390.3383, Weibull distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	1.08 (1.01)	2.94 (3.00)	0.288	0.40, 21.50
	Placebo	Referent	-	-	-
Age of calf (days)	-	-0.0082 (0.12)	0.99 (0.12)	0.946	0.78, 1.26
Days from spectinomycin treatment	-	-0.30 (0.13)	0.74 (0.097)	0.023	0.57, 0.96
Intercept	-	7.93 (1.56)	2779.05	0	130.75, 59066.56

Supplementary Table 15. Final accelerated failure time (AFT) model for quinupristin/dalfopristin minimum inhibitory concentrations for enterococci isolates (BIC 551.3418, Ggamma distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	0.13 (0.13)	1.14 (0.15)	0.340	0.88, 1.47
	Placebo	Referent	-	-	-
Age of calf (days)	-	-0.018 (0.011)	0.98 (0.011)	0.099	0.96, 1.00
Received spectinomycin treatment	Yes	0.20 (0.14)	1.23 (0.17)	0.138	0.94, 1.61
	No	Referent	-	-	-
Intercept	-	0.80 (0.16)	2.24 (0.35)	0	1.64, 3.05

Supplementary Table 16. Final accelerated failure time (AFT) model for linezolid minimum inhibitory concentrations for enterococci isolates (BIC 303.3108, Weibull distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	0.13 (0.076)	1.14 (0.087)	0.079	0.98, 1.33
	Placebo	Referent	-	-	-
Age of calf (days)		0.0048 (0.0070)	1.00 (0.0070)	0.491	0.99, 1.02
Days from last spectinomycin treatment	1-3 days	0.24 (0.098)	1.27 (0.12)	0.016	1.04, 1.53
	4-7 days	0.017 (0.089)	1.02 (0.090)	0.844	0.86, 1.21
	8-23 days	0.044 (0.099)	1.04 (0.10)	0.660	0.86, 1.27
	0 days	Referent	-	-	-
Intercept	-	0.69 (0.11)	2.00 (0.22)	0	1.61, 2.49

Supplementary Table 17. Final accelerated failure time (AFT) model for nitrofurantoin minimum inhibitory concentrations for enterococci isolates (BIC 567.2407, Exponential distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	0.23 (0.25)	1.26 (0.32)	0.354	0.77, 2.06
	Placebo	Referent	-	-	-
Age of calf (days)	-	-0.055 (0.021)	0.95 (0.020)	0.009	0.91, 0.99
Received spectinomycin treatment	Yes	-0.73 (0.28)	0.48 (0.13)	0.008	0.28, 0.83
	No	Referent	-	-	-
Intercept	-	5.42 (0.42)	226.92 (94.47)	0	100.34, 513.15

Supplementary Table 18. Final accelerated failure time (AFT) model for penicillin minimum inhibitory concentrations for enterococci isolates (BIC 644.4164, Exponential distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	0.0061 (0.21)	1.01 (0.21)	0.977	0.67, 1.52
	Placebo	Referent	-	-	-
Age of calf (days)	-	-0.051 (0.013)	0.95 (0.013)	0	0.93, 0.98
Received spectinomycin	Yes	-0.012 (0.21)	0.89 (0.18)	0.569	0.59, 1.33
treatment	No	Referent	-	-	-
Intercept	-	2.57 (0.30)	13.06 (3.92)	0	7.25, 23.52

Supplementary Table 19. Final accelerated failure time (AFT) model for erythromycin minimum inhibitory concentrations for enterococci isolates (BIC 365.5822, Weibull distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	0.90 (1.02)	2.46 (2.52)	0.379	0.33, 18.29
	Placebo	Referent	-	-	-
Age of calf (days)	-	0.0065 (0.12)	1.01 (0.12)	0.955	0.80, 1.26
Days from spectinomycin					
treatment	-	-0.29 (0.14)	0.75 (0.10)	0.037	0.57, 0.98
Intercept	-	6.40 (1.45)	602.55 (875.78)	0	34.90, 10403.28

Supplementary Table 20. Final accelerated failure time (AFT) model for ciprofloxacin minimum inhibitory concentrations for enterococci isolates (BIC 435.9695, Ggamma distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	0.024 (0.10)	1.02 (0.10)	0.809	0.84, 1.25
	Placebo	Referent	-	-	-
Age of calf (days)	-	-0.019 (0.0082)	0.98 (0.0080)	0.019	0.97, 1.00
Days from spectinomycin					
treatment	-	0.017 (0.019)	1.02 (0.020)	0.370	0.98, 1.06
Intercept	-	0.74 (0.22)	2.09 (0.47)	0.001	1.35, 3.24

Supplementary Table 21. Final accelerated failure time (AFT) model for vancomycin minimum inhibitory concentrations for enterococci isolates (BIC 451.1961, Ggamma distribution).

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	-0.039 (0.099)	0.96 (0.095)	0.694	0.79, 1.17
	Placebo	Referent	-	-	-
Age of calf (days)	-	-0.0049 (0.0074)	1.00 (0.0073)	0.508	0.98. 1.01
Received spectinomycin treatment	Yes	0.089 (0.10)	1.09 (0.11)	0.384	0.89, 1.34
	No	Referent	-	-	-
Intercept	-	-0.56 (0.21)	0.57 (0.12)	0.007	0.38, 0.86

Supplementary Table 22. Final accelerated failure time (AFT) model for gentamicin minimum inhibitory concentrations for enterococci isolates (BIC 524.5724, Exponential distribution).|

Factor	Level	Coefficient (SE)	MIC Ratio (SE)	P-value	95% CI
Treatment group	Zinc	0.37 (0.35)	1.44 (0.51)	0.295	0.73, 2.88
	Placebo	Referent	-	-	-
Age of calf	8-14 days	-1.80 (0.71)	0.17 (0.12)	0.012	0.041, 0.67
	15-21 days	-1.31 (0.82)	0.27 (0.22)	0.111	0.054, 1.35
	23-28 days	-2.63 (0.92)	0.072 (0.066)	0.004	0.012, 0.44
	29-35 days	-0.44 (1.26)	0.64 (0.81)	0.725	0.054, 7.60
	5-7 days	Referent	-	-	-
Days from spectinomycin treatment	-	-0.097 (0.045)	0.91 (0.041)	0.030	0.83, 0.99
Intercept	-	8.75 (0.70)	6331.95 (4440.216)	0	1601.92, 25028.44

Supplementary Table 23. Prediction estimates from final accelerated failure time (AFT) model for azithromycin minimum inhibitory concentrations (MIC, μg/mL) for *E. coli* isolates.

		Predicted MIC (Zinc)				Predicted MIC (Placebo)				Predicted MIC (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Days from last spectinomycin treatment	0 days	3.33	0.86	0	1.65, 5.02	4.18	1.02	0	2.18, 6.17	-0.84	0.78	0.28	-2.37, 0.69
	3-5 days	8.38	2.11	0	4.24, 12.52	10.50	3.28	0.001	4.07, 16.92	-2.12	2.18	0.33	-6.39, 2.16
	6-8 days	3.42	0.69	0	2.06, 4.78	4.28	0.66	0	2.98, 5.58	-0.86	0.77	0.26	-2.36, 0.64
	9-10 days	9.02	2.01	0	5.09, 12.95	11.3	2.21	0	6.96, 15.63	-2.28	2.07	0.27	-6.33, 1.77

Supplementary Table 24. Prediction estimates from final accelerated failure time (AFT) model for ciprofloxacin minimum inhibitory concentrations (MIC, μg/mL) for *E. coli* isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Number of spectinomycin doses received	0 doses	0.0024	0.0027	0.37	-0.0029, 0.0077	0.015	0.018	0.42	-0.021, 0.050	-0.012	0.016	0.45	-0.044, 0.020
	1 dose	0.15	0.21	0.48	-0.26, 0.56	0.90	1.12	0.42	-1.29, 3.09	-0.75	0.97	0.44	-2.65, 1.14
	2 dose	0.018	0.013	0.18	-0.0081, 0.044	0.11	0.073	0.14	-0.035, 0.25	-0.091	0.072	0.21	-0.23, 0.05

Supplementary Table 25. Prediction estimates from final accelerated failure time (AFT) model for nalidixic acid minimum inhibitory concentrations (MIC, μg/mL) for *E. coli* isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Number of spectinomycin doses received	0 doses	0.69	0.28	0.016	0.13, 1.24	2.47	1.06	0.02	0.39, 4.55	-1.78	0.89	0.044	-3.52, -0.047
	1 dose	5.19	2.63	0.048	0.037, 10.35	18.69	8.55	0.029	1.92, 35.46	-13.50	6.72	0.045	-26.68, -0.32
	2 dose	1.83	0.49	0	0.88, 2.78	6.60	1.58	0	3.50, 9.69	-4.76	1.58	0.002	-7.85, -1.68

Supplementary Table 26. Prediction estimates from final accelerated failure time (AFT) model for ceftriaxone minimum inhibitory concentrations (MIC, μg/mL) for *E. coli* isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Received spectinomycin treatment	No	12.26	5.34	0.022	1.79, 22.73	12.97	5.43	0.017	2.32, 23.61	-0.71	4.23	0.87	-9.00, 7.58
	Yes	22.71	6.26	0	10.45, 34.98	24.03	6.54	0	11.21, 36.85	-1.31	7.85	0.87	-16.70, 14.07

Supplementary Table 27. Prediction estimates from final accelerated failure time (AFT) model for tigecycline minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Days from last spectinomycin treatment	0 days	0.31	0.03	0.00	0.25, 0.38	0.30	0.03	0.00	0.24, 0.36	0.02	0.02	0.47	-0.03, 0.06
	1 days	0.32	0.03	0.00	0.25, 0.39	0.31	0.03	0.00	0.24, 0.37	0.02	0.02	0.47	-0.03, 0.06
	5 days	0.36	0.04	0.00	0.28, 0.44	0.34	0.04	0.00	0.26, 0.42	0.02	0.03	0.47	-0.03, 0.07
	10 days	0.41	0.06	0.00	0.30, 0.52	0.39	0.05	0.00	0.28, 0.49	0.02	0.03	0.47	-0.04, 0.08

Supplementary Table 28. Prediction estimates from final accelerated failure time (AFT) model for chloramphenicol minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Days from last spectinomycin treatment	0 days	8.52	0.95	0	6.65, 10.38	7.93	0.91	0	6.14, 9.71	0.59	0.54	0.271	-0.46, 1.65
	1 days	8.49	0.99	0	6.56, 10.43	7.90	0.94	0	6.06, 9.75	0.59	0.54	0.271	-0.46, 1.64
	5 days	8.39	1.19	0	6.06, 10.71	7.80	1.12	0	5.61, 10.00	0.58	0.53	0.273	-0.46, 1.63
	10 days	8.26	1.51	0	5.31, 11.21	7.69	1.41	0	4.91, 10.46	0.57	0.53	0.277	-0.46, 1.61

Supplementary Table 29. Prediction estimates from final accelerated failure time (AFT) model for daptomycin minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Received spectinomycin treatment	No	3.47	0.63	0.00	2.25, 4.70	2.99	0.41	0.00	2.20, 3.79	0.48	0.42	0.26	-.34, 1.30
	Yes	2.90	0.39	0.00	2.13, 3.67	2.50	0.36	0.00	1.79, 3.22	0.40	0.32	0.22	-.23, 1.03

Supplementary Table 30. Prediction estimates from final accelerated failure time (AFT) model for streptomycin minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Received spectinomycin treatment	No	901.75	1718.45	0.60	-2466.34, 4269.85	740.40	1258.26	0.56	-1725.75, 3206.55	161.35	814.67	0.84	-1435.37, 1758.07
	Yes	1769.14	3045.05	0.56	-4199.05, 7737.33	1452.58	2355.66	0.54	-3164.43, 6069.59	316.56	1516.10	0.84	-2654.93, 3288.05

Supplementary Table 31. Prediction estimates from final accelerated failure time (AFT) model for tylosin tartrate minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC – Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Days from last spectinomycin treatment	0 days	8176.12	10455.33	0.43	-12315.95, 28668.19	2779.05	4333.91	0.52	-5715.27, 11273.36	5394.07	7686	0.48	-9667.21, 20461.36
	1 days	6081.64	8088.78	0.45	-9772.08, 21935.36	2067.14	3382.43	0.54	-4562.31, 8696.58	4014.5	5823.56	0.49	-7399.47, 15428.47
	5 days	1861.72	3027.34	0.54	-4071.76, 7795.19	632.79	1265.48	0.62	-1847.49, 3133.08	1228.92	2037.77	0.55	-2765.04, 5222.89
	10 days	423.92	899.63	0.64	-1339.33, 2187.16	144.09	365.00	0.69	-571.31, 859.48	279.83	581.72	0.63	-860.32, 1419.97

Supplementary Table 32. Prediction estimates from final accelerated failure time (AFT) model for quinupristin/dalfopristin minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC – Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Received spectinomycin treatment	No	2.54	0.52	0.00	1.52, 3.56	2.24	0.35	0.00	1.54, 2.93	0.30	0.34	0.37	-0.36, 0.97
	Yes	3.11	0.62	0.00	1.90, 4.33	2.74	0.51	0.00	1.75, 3.74	0.37	0.40	0.36	-0.42, 1.16

Supplementary Table 33. Prediction estimates from final accelerated failure time (AFT) model for linezolid minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC – Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Days from last spectinomycin treatment	0 days	2.29	0.24	0.00	1.82, 2.75	2.00	0.22	0.00	1.57, 2.44	0.29	0.16	0.08	-0.03, 0.61
	1-3 days	2.90	0.30	0.00	2.31, 3.48	2.53	0.32	0.00	1.90, 3.17	0.36	0.20	0.07	-0.02, 0.75
	4-7 days	2.33	0.30	0.00	1.75, 2.91	2.04	0.28	0.00	1.48, 2.59	0.29	0.16	0.08	-0.03, 0.62
	8-23 days	2.39	0.33	0.00	1.75, 3.04	2.09	0.36	0.00	1.38, 2.80	0.30	0.16	0.05	-0.0042, 0.60

Supplementary Table 34. Prediction estimates from final accelerated failure time (AFT) model for nitrofurantoin minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC – Zinc				Predicted MIC – Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Received spectinomycin treatment	No	286.38	136.52	0.04	18.80, 553.95	226.92	94.47	0.02	41.76, 412.08	59.46	74.99	0.43	-87.52, 206.44
	Yes	138.08	52.36	0.01	35.45, 240.70	109.41	36.06	0.002	38.74, 180.08	28.67	34.35	0.40	-38.66, 96.00

Supplementary Table 35. Prediction estimates from final accelerated failure time (AFT) model for penicillin minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Received spectinomycin treatment	No	13.14	4.22	0.002	4.87, 21.41	13.06	3.92	0.001	5.38, 20.74	0.08	2.74	0.98	-5.29, 5.45
	Yes	11.69	2.98	0.00	5.85, 17.53	11.62	3.06	0.00	5.62, 17.61	0.07	2.44	0.98	-4.70, 4.85

Supplementary Table 36. Prediction estimates from final accelerated failure time (AFT) model for erythromycin minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Days from last spectinomycin treatment	0 days	1481.63	1765.71	0.40	-1979.09, 4942.36	602.55	875.78	0.49	-1113.94, 2319.05	879.08	1290.93	0.50	-1651.09, 3409.24
	1 days	1111.13	1352.30	0.41	-1539.32, 3761.58	451.88	683.07	0.51	-886.92, 1790.67	659.25	964.63	0.49	-1231.38, 2549.89
	5 days	351.45	509.79	0.49	-647.73, 1350.63	142.93	261.76	0.59	-370.12, 655.98	208.52	326.92	0.52	-432.23, 849.28
	10 days	83.37	161.12	0.61	-232.43, 399.16	33.90	79.82	0.67	-122.55, 190.35	49.46	95.23	0.60	-137.19, 236.12

Supplementary Table 37. Prediction estimates from final accelerated failure time (AFT) model for ciprofloxacin minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Days from last spectinomycin treatment	0 days	2.15	0.51	0.00	1.15, 3.15	2.09	0.47	0.00	1.18, 3.01	0.05	0.21	0.81	-0.37, 0.47
	1 days	2.18	0.54	0.00	1.12, 3.24	2.13	0.50	0.00	1.15, 3.11	0.05	0.22	0.81	-0.37, 0.48
	5 days	2.34	0.69	0.001	0.98, 3.70	2.28	0.66	0.00	1.00, 3.57	0.06	0.23	0.81	-0.40, 0.51
	10 days	2.55	0.95	0.007	0.70, 4.40	2.49	0.91	0.006	0.70, 4.28	0.06	0.25	0.81	-0.44, 0.56

Supplementary Table 38. Prediction estimates from final accelerated failure time (AFT) model for vancomycin minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Received spectinomycin treatment	No	0.55	0.12	0.00	0.32, 0.78	0.57	0.12	0.00	0.34, 0.81	-0.02	0.06	0.69	-0.13, 0.09
	Yes	0.60	0.12	0.00	0.37, 0.83	0.63	0.12	0.00	0.40, 0.85	-0.02	0.06	0.69	-0.14, 0.09

Supplementary Table 39. Prediction estimates from final accelerated failure time (AFT) model for gentamicin minimum inhibitory concentrations (MIC) for enterococci isolates.

		Predicted MIC - Zinc				Predicted MIC - Placebo				Predicted MIC – (Difference, Zinc-Placebo)			
		MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI	MIC	SE	P-value	95% CI
Days from last spectinomycin treatment	0 days	9147.40	6884.36	0.18	-4345.70, 22640.50	6331.95	4440.22	0.15	-2370.71, 15034.62	2815.45	3602.90	0.44	-4246.11, 9877.00
	1 days	8300.11	6209.46	0.18	-3870.20, 20470.42	5745.45	4037.86	0.16	-2168.61, 13659.50	2554.66	3242.34	0.43	-3800.21, 8909.53
	5 days	5626.37	4258.12	0.19	-2719.40, 13972.14	3894.65	2869.00	0.18	-1728.48, 9517.78	1731.72	2151.63	0.42	-2485.39, 5948.83
	10 days	3460.66	2851.98	0.23	-2129.12, 9050.45	2395.52	1995.06	0.23	-1514.74, 6305.77	1065.15	1326.07	0.42	-1533.896, 3664.187

Chapter 4: Whole genome sequence analysis reveals high genomic diversity and potential host-driven adaptations among multidrug-resistant *Escherichia coli* from pre-weaned dairy calves

Under review in "Frontiers in Microbiology"

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Whole genome sequence analysis reveals high genomic diversity and potential host-driven adaptations among multidrug-resistant *Escherichia coli* from pre-weaned dairy calves

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Abstract

Food-producing animals such as dairy cattle are potential reservoirs of antimicrobial resistance (AMR), with multidrug-resistant (MDR) organisms such as *Escherichia coli* observed in higher frequency in young calves compared to older cattle. In this study, we characterized the genomes of enteric MDR *E. coli* from pre-weaned dairy calves with and without diarrhea and evaluated the influence of host-level factors on genomic composition. Whole genome sequence comparative analysis of *E. coli* (n=43) revealed substantial genomic diversity that primarily clustered by sequence type and was minimally driven by calf diarrheal disease status (healthy, diarrheic, or recovered), antimicrobial exposure, and dietary zinc supplementation. Diverse AMR genes (ARGs) – including extended-spectrum beta-lactamase genes and quinolone resistance determinants – were identified (n=40), with unique sets of ARGs co-occurring in gene clusters with large AMR plasmids IncA/C2 and IncFIB(AP001918). Zinc supplementation was not significantly associated with the selection of individual ARGs in *E. coli*, however analysis of ARG and metal resistance gene pairs identified positive associations between certain aminoglycoside, beta-lactam, sulfonamide, and trimethoprim ARGs with acid, tellurium and mercury resistance genes. Although *E. coli* in this study lacked the typical virulence factors of diarrheagenic

strains, virulence genes overlapping with those in major pathotypes were identified. Among the 103 virulence genes detected, the highest abundance and diversity of genes corresponded to iron acquisition (siderophores and heme uptake). Our findings indicate that the host-level factors evaluated in this study were not key drivers of genomic variability, but that certain accessory genes in enteric MDR *E. coli* may be enriched. Collectively, this work provides insight into the genomic diversity and host-microbe interface of MDR *E. coli* from pre-weaned dairy calves.

Introduction

Escherichia coli is a diverse and ubiquitous organism present in the healthy enteric microbiome of humans and animals and as a pathogen responsible for various diarrheagenic and extraintestinal diseases (Jackson et al., 2011; Braz et al., 2020). The occurrence of antimicrobial resistant (AMR) *E. coli* in food-producing animals, such as dairy cattle, has been identified across various cattle groups in farm environmental matrices, feces, food products (e.g. milk and cheese), and clinical samples (e.g. diarrhea and clinical mastitis) (Ombarak et al., 2018; Formenti et al., 2021; Jeamsripong et al., 2021; Majumder et al., 2021; Imre et al., 2022). The prevalence and persistence of drug-resistant *E. coli* is both a veterinary and human medicine concern, with pathogenic strains compromising animal health and safety of food products, and commensals serving as important reservoirs for the dissemination of AMR.

Multidrug-resistant (MDR) *E. coli* have been observed in higher frequency in younger cattle, particularly in calves around two weeks in age (Berge et al., 2005, 2010). This age-dependent and transient increase in AMR of dairy calves is thought to be driven by the early developing gut microbiome, in which initial exposure to the environment, antibiotic therapy, dietary changes, and other factors collectively contribute to the rapid establishment of the bovine resistome (Khachatryan et al., 2004; Noyes et al., 2016; Liu et al., 2019; Springer et al., 2019; Oh et al., 2020). Previous studies have demonstrated the dynamic nature of AMR selection and enrichment in calves, with the acquisition of AMR occurring beyond influences of antibiotic exposure (Liu et al., 2019; Haley and Van Kessel, 2022) and calves harboring greater diversity in AMR than the potential sources (e.g. dam) seeding their

resistome (Haley et al., 2020; Massé et al., 2021). Additionally, studies have suggested that biocides used as disinfectants and heavy metal additives in feed may contribute to the co-selection of AMR with biocide and metal resistance (Wales and Davies, 2015; Cheng et al., 2019).

In pre-weaned dairy calves, diarrhea is the leading cause of morbidity and mortality, which frequently results in antimicrobial treatment (Berchtold and Constable, 2009; Habing et al., 2017). To reduce AMR without compromising animal health, antimicrobial alternatives such as dietary zinc supplementation have been explored and shown to be effective in preventing diarrhea and expediting diarrheal recovery (Glover et al., 2013; Feldmann et al., 2019; Chang et al., 2020; Ma et al., 2020; Wo et al., 2022). In this work, we evaluated fecal MDR *E. coli* isolates from pre-weaned dairy calves in a zinc supplementation clinical trial using whole genome sequencing (WGS) comparative analysis. The objective of this study was to characterize AMR and virulence genes and to evaluate calf diarrheal disease status, dietary zinc supplementation, and antimicrobial treatment as potential drivers of genomic variability in MDR *E. coli*. We hypothesize that these host-level factors will contribute to differences in genomic AMR, virulence, and metal resistance profiles, and that the presence of certain genes will provide insight into the persistence of enteric MDR *E. coli* in calves.

Materials and Methods

Isolate source

Fecal *E. coli* isolates in this study were obtained from pre-weaned dairy calves enrolled in a double-blind, block-randomized, placebo-controlled zinc supplementation clinical trial assessing dietary zinc supplementation on diarrhea prevention and calf health. Details on the original trial procedures were previously described (Feldmann et al., 2019). Briefly, all calves were under the same management practices (e.g. housing and diet) and standard on-farm treatment protocols. The repository of 43 *E. coli* isolates correspond to pre-weaned dairy calves 2 weeks in age (range: 14-16 days). One representative fecal *E. coli* isolate per calf was used for analysis, with each isolate corresponding to a calf after 14 consecutive days of dietary zinc sulfate or placebo treatment. Treatments were administered during morning milk feeding with calves in the zinc group receiving 0.22g zinc sulfate monohydrate (80 mg of

elemental zinc) (Sigma-Aldrich Company, St. Louis, MO, USA) with 0.44g milk replacer powder, and calves in the placebo group receiving only 0.44g milk replacer powder (Feldmann et al., 2019). At the time of isolate collection, calves were in various stages of diarrheal disease (pre-diarrheic/healthy, diarrheic, or recovered) and exposure to antimicrobial treatment for diarrhea (0, 1, or 2 doses of 31.5 mL (1575 mg) spectinomycin administered once daily, SpectoGard, Bimeda, Inc., Le Sueur, MN, USA). Other antimicrobial exposures included tetracycline and neomycin administered through daily milk, which were consistent in dosage and duration over time for all calves throughout the study. Calf-level data corresponding to isolates were collected from daily assessment records for individual calves. All isolates were confirmed as *E. coli* using conventional PCR and underwent antimicrobial susceptibility testing (AST) using broth microdilution and the NARMS Gram Negative panel (YCMV3AGNF) as previously described (Lee et al., 2024).

DNA extraction and whole genome sequencing (WGS)

Genomic DNA was extracted from pure overnight *E. coli* cultures per manufacturer's protocol using the Qiagen's DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). WGS was conducted using methods from the 100K Pathogen Genome Project as previously described (Weis et al., 2017; Bandoy and Weimer, 2020; Aguilar-Zamora et al., 2022; Hurtado et al., 2022; Woerde et al., 2023; Hernández-Juárez et al., n.d.). Briefly, genomic DNA purity and integrity were assessed using the Nanodrop and the Agilent 2200 TapeStation with the Genomic DNA ScreenTape Assay (Agilent Technologies, Inc., Santa Clara, CA, USA), respectively. Sequencing libraries were constructed using the KAPA HyperPlus library preparation kit (Roche Sequencing Solutions, Pleasanton, CA, USA). Double-stranded genomic DNA was fragmented and indexed using Weimer 384 TS-LT DNA barcodes (Integrated DNA Technologies, Coralville, IA, USA), followed by dual-SPRI size selection and PCR amplification. Final library sizes were confirmed on the LabChip GX using the HT DNA 1K kit (PerkinElmer, Waltham, MA, USA). Library quantification was conducted using the KAPA Library Quantification Kit (Roche Sequencing Solutions, Pleasanton, CA, USA) to ensure normalized

concentrations for sequencing pooling. Final libraries were sequenced using the Illumina HiSeq X Ten with PE150.

Whole genome assembly and comparison

Genomic sequence data was processed as previously described (Bandoy and Weimer, 2020; Higdon et al., 2020; Flores-Valdez et al., 2021; Miller et al., 2021; Depenbrock et al., 2024). Briefly, Trimmomatic was used to remove low-quality sequence and adapters, and FastQC was used to review sequence quality. Paired-end reads from WGS were assembled using Shovill with the SPAdes assembler and a Kmer size of 31. Quality of assemblies was then evaluated using CheckM. Genome similarity was measured using Sourmash with Minhash signatures with a Kmer length of 31 and scaled sketch size of 100,000 per megabase (Brown and Irber, 2016). The matrix output from Sourmash was visualized in R using the pheatmap package (pheatmap function - RDocumentation, n.d.).

Multilocus sequence typing (MLST) and pangenome analysis

The sequence type (ST) for each genome was determined based on the Achtman seven-locus scheme (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) using the PubMLST database (Kaas et al., 2012; PubMLST - Public databases for molecular typing and microbial genome diversity, n.d.). Pangenome analysis was conducted using Roary as described previously (Page et al., 2015; Bandoy and Weimer, 2020; Miller et al., 2021). Pangenome composition and gene diversity estimation were then visualized using open source python script 'roary_plots.py' and native Rscript (create_pan_genome_plots.R), respectively (Higdon et al., 2020).

Identification of antimicrobial resistance genes (ARGs), virulence genes, metal resistance genes, and plasmid replicons

Genetic determinants for antimicrobial resistance (ARGs), virulence, metal resistance, and plasmid replicons were determined using Abricate and the ResFinder, VFDB, BacMet, and PlasmidFinder databases, respectively (Zankari et al., 2012; Carattoli et al., 2014; Pal et al., 2014; Chen et al., 2016; Seemann, 2024). Additionally, SNP based resistance for quinolones was identified using RGI with the CARD database (Alcock et al., 2020a, 2020b). Hits were determined if meeting the criteria of ≥90%

coverage and ≥95% identity. For metal resistance genes, only experimentally confirmed genes were included in the analysis.

Data analyses

Descriptive statistics on the distribution of ARGs, virulence factors, metal resistance genes, and plasmid replicons were conducted in SAS OnDemand for Academics. Differences in the mean number of ARGs and virulence genes by factors of treatment group, diarrhea status, and number of therapeutic antibiotic doses were evaluated using a t-test/ANOVA or Mann-Whitney U test.

Proportions of *E. coli* genomes with presence of ARGs and virulence factors were plotted as heatmaps in R using the pheatmap package. Rows of the heatmaps were clustered using the Euclidean distance metric and complete linkage method. Bar plots and violin plots of the distribution of ARGs and virulence factors, respectively, were visualized in R using ggplot2 (Wickham et al., 2023).

To investigate the differences in antimicrobial resistance, virulence, metal resistance, and pangenome composition amongst isolates, clustering based on the presence and absence matrices for each were assessed by grouping factors of treatment group, diarrhea status, sequence type, and antibiotic exposure as previously described (Lee et al., 2023). A PERMDISP2 procedure was conducted to evaluate if dispersions of groups for each grouping factor were homogenous (Anderson, 2006; Anderson et al., 2006). Permutational analysis of variance (PERMANOVA) and ANOSIM (analysis of similarity) were then performed to evaluate equivalence of centroids of groups and average of ranks of within-group to between-group distances, respectively (Anderson and Walsh, 2013). Additionally, non-metric multidimensional scaling was performed by grouping factor of sequence type for AMR and virulence genes. All tests were performed using 10,000 permutations and a Jaccard distance metric in R using the vegan package (Oksanen et al., 2022).

Logistic regression models were constructed to assess the association between the presence of ARGs with calf-level factors. Models were constructed with outcomes specified as the presence or absence of individual ARGs, quinolone resistance determinants (presence of any point mutations or plasmid-mediated quinolone resistance determinants), and extended spectrum beta-lactamase (ESBL)

resistance genes. Calf-level factors included in model building included treatment group (isolate from zinc- or placebo- treated calf), therapeutic spectinomycin exposure at the time of isolate collection, and diarrhea status of the calf at the time of isolate collection. Antibiotic exposure and calf diarrhea status were evaluated based on individual calf-level data collected through daily assessments. Specifically, spectinomycin treatment was coded as a binary variable (received treatment or not), number of doses received (0, 1, or 2 doses), or days from the last spectinomycin dose received, and diarrhea status was coded as days on or from diarrhea or a categorical variable (healthy/pre-diarrheic, diarrheic, or recovered). Final models were selected based on the lowest AIC after inclusion of confounders (antimicrobial exposure for all ARG models) and any other significant predictors. Given their public health significance, the association between the presence of extended spectrum beta-lactamase (ESBL) genes and other ARGs were also evaluated using Fisher's exact test.

Antimicrobial susceptibility testing data previously collected on study isolates (broth microdilution using the NARMS Gram Negative panel, YCMV3AGNF) were used to assess the concordance between genotypic and phenotypic resistance (Lee et al., 2022) for the following drugs: gentamicin, streptomycin, amoxicillin-clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, trimethoprim-sulfamethoxazole, azithromycin, ampicillin, chloramphenicol, nalidixic acid, ciprofloxacin, and tetracycline. Classification of isolates into susceptible, intermediate, and resistant categories were conducted using CLSI breakpoints, with the exception of streptomycin and azithromycin where NARMS breakpoints were used due to lack of CLSI breakpoints (Supplementary File). Multidrug-resistance (MDR) was defined as resistance to ≥1 drug in ≥3 antimicrobial classes (Magiorakos et al., 2012). Concordance included phenotypically resistant isolates with the corresponding ARG(s) (TP, true positive) and phenotypically susceptible isolates with absence of corresponding ARG(s) (TN, true negative). Discordance included phenotypically resistant isolates not having the corresponding ARG(s) (FN, false negative), and phenotypically susceptible isolates having the corresponding ARG(s) (FP, false positive). Sensitivity and specificity were evaluated as TP/(TP+FN) and TN/(TN+FP), respectively. For analysis, intermediate isolates were grouped with susceptible isolates.

To evaluate the co-occurrence of plasmid replicons and ARGs, a pairwise co-occurrence matrix was constructed and visualized as networks using Gephi (Bastian et al., 2009) as previously described (Lee et al., 2023). To assess the linkage patterns of ARGs and metal resistance genes, pairwise probabilistic co-occurrence analysis was conducted using default settings in the R package cooccur (Griffith et al., 2016).

Data availability

WGS data for isolates are available at the 100k Pathogen Genome Project BioProject (PRJNA186441) (Supplementary File).

Results

WGS of MDR E. coli isolates

E. coli genomes in this study had an average of 193 contigs, coverage of 112X, and quality score of 38. Additional quality metrics, AST data, and metadata of genomes in this study are available in the Supplementary File.

Concordance of AMR phenotypes with genotypes

E. coli isolates in this study were previously determined to be MDR through AST. To assess AMR concordance, predictions of AMR phenotype from genotype was evaluated for 13 drugs using previously collected AST data. Across all tested drugs, genotypic AMR predicted phenotypic AMR with an overall sensitivity of 100% and specificity of 98.58% (Table 1). Discordances in specificity included a streptomycin susceptible isolate with a streptomycin resistance gene (*aadA2*), and a ceftiofur intermediate isolate with carriage of an AmpC beta-lactamase gene (bla_{CMY2}).

E. coli genome population structure

Whole genome analysis of the isolates revealed a large genomic diversity of *E. coli* genomes. All-by-all comparison identified three main clusters that exhibited minimal to no relationship to calf disease status (healthy, diarrheic, or recovered calves), treatment group (placebo or zinc), or therapeutic antimicrobial treatment (0, 1, or 2 doses of spectinomycin). A total of 20 unique sequence types (STs) based on the 7-gene allelic profile were identified among 42 isolates, with one isolate unable to be

assigned to a ST. The most frequently occurring ST included ST362 (7/43, 16.28%), followed by ST10 (4/43, 9.30%), ST101 (4/43, 9.30%), and ST641 (4/43, 9.30%). STs correlated with group and individual clusters from whole genome comparisons, with distinctive variability in genome content observed within each ST (Figure 1), indicating that the genes used to define ST were stable, but the remainder of the genome contained large variations. Specifically, the most prevalent STs exhibited substantial heterogeneity in genome composition, particularly ST10, ST101, and ST641 which had variable accessory genes including those for AMR and virulence (Figure 1, Figure 2B, and Figure 4B). This observation indicated that WGS provided higher resolution characterization of strain variation than MLST, and prompted examining the pangenome for better understanding of the gene variation among isolates in this study.

Pangenome analysis of E. coli isolates

The pangenome of *E. coli* isolates in this study was open and comprised of 14,011 genes that included a core genome with 3,117 genes and a soft-core, shell, and cloud genomes of 219, 3,076, and 7,599 genes, respectively. Analysis of the cumulative gene curve representing the number of total homologous genes and conserved homologs indicated an open pangenome that was covered with approximately 10 genomes within this population (Supplementary Figure 1). While the core was represented within a smaller portion of the isolates, genes from the variable portion of the pangenome represented 77.75% variation in the isolate population.

Table 1. Genotypic prediction of phenotypic resistance in dairy calf *E. coli* isolates (n=43).

CLSI class	Antimicrobial agent	Phenotype: susceptible (No. of isolates)		Phenotype: resistant (No. of isolates)		Sensitivity[b] (%)	Specificity[c] (%)
		Genotype: resistant (FP)[a]	Genotype: susceptible (TN)[a]	Genotype: resistant (TP)[a]	Genotype: susceptible (FN)[a]		
Aminoglycosides	GEN	0	5	38	0	100%	100%
	STR	1	0	42	0	100%	0%
B-lactam combination agents	AUG2	0	2	41	0	100%	100%
Cephems	FOX	0	2	41	0	100%	100%
	XNL	1	1	41	0	100%	50%
	AXO	0	1	42	0	100%	100%
Folate pathway antagonists	SXT	N/A	N/A	43	0	100%	0%
Macrolides	AZI	0	40	N/A	N/A	N/A	100%
Penicillins	AMP	N/A	N/A	43	0	100%	N/A
Phenicols	CHL	0	10	33	0	100%	100%
Quinolones	NAL	0	39	4	0	100%	100%
	CIP	0	39	4	0	100%	100%
Tetracyclines	TET	N/A	N/A	43	0	100%	N/A
Overall	-	2	139	415	0	100%	98.58%

[a]FP false positive; TN true negative, TP true positive, FN false negative
[b]Sensitivity TP/(TP+FN)
[c]Specificity TN/(TN+FP)

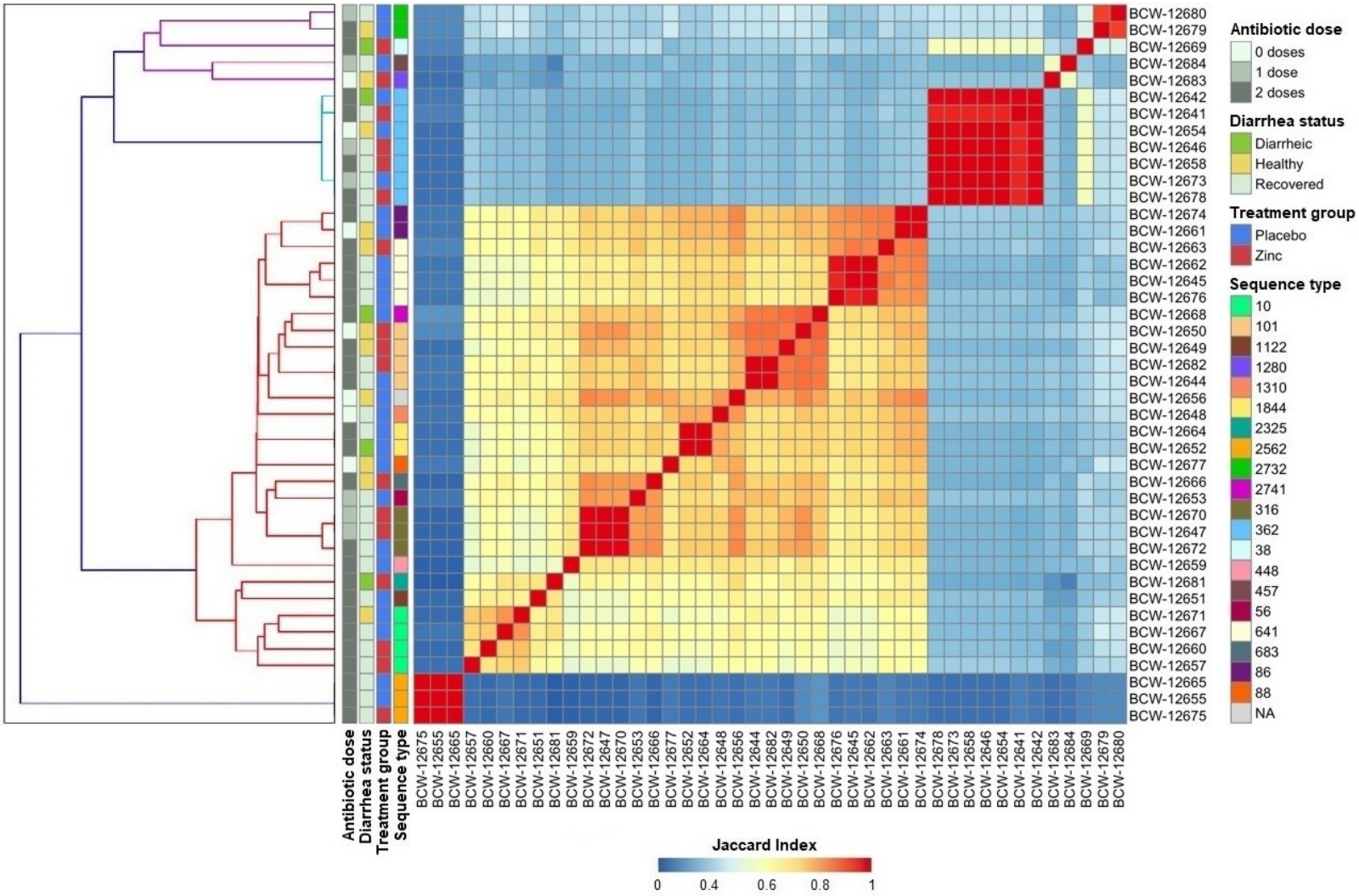

Figure 1. All-by-all comparison of genome similarity of *E. coli* isolates (n=43) from pre-weaned dairy calves, generated using MinHash sketches from draft whole-genome assemblies of k-mers with a length of 31 and sketch size of 100,000. The heatmap color gradient corresponds to the Jaccard Similarity Index (JSI) for each pairwise comparison, with values close to 0 and 1 corresponding to high genome dissimilarity and similarity, respectively.

AMR, virulence, metal resistance, and pangenome profiles & diversity

The collective ARGs, virulence genes, metal resistance genes, and pangenome elements of *E. coli* were evaluated using multivariate analysis to assess if variability in these genomic profiles were driven by host-level factors. Tests for differences in *E. coli* genomic content for AMR, virulence, metal resistance, and pangenome elements indicated that dispersion differences were not significantly different among isolates by treatment group, diarrhea status, and therapeutic antibiotic exposure (PERMDISP2 $p > 0.05$, Table 2). Additionally, grouping factors evaluated in this study accounted for a low proportion of variance in AMR, virulence, metal resistance, and pangenome composition in *E. coli* genomes (PERMANOVA R^2=9.39E-3-0.04), with equal or greater dissimilarities in average of ranks within group than those of between-groups across all factors (ANOSIM R = ~0 or R < 0) (Table 2). These analyses indicated that the host-level factors evaluated in this study – diarrheal disease status, dietary zinc supplementation, and antibiotic treatment – had minimal influence on the genomic composition of *E. coli*. These findings provided impetus to evaluate the distribution of genes individually with respect to host-level factors.

Antimicrobial resistance genetic determinants (ARGs)

Across the 43 *E. coli* genomes, a total of 40 ARGs among diverse antimicrobial classes were detected. The average and median number of ARGs per genome – including SNPs for quinolone resistance – was 13 ARGs with a range of 9 to 19. ARGs conferring resistance to antimicrobials of public health significance included seven SNPs in chromosomal genes – pS83L, pD87N, and pD87Y in *gyrA*, pS80I and pE84G in *parC*, and pI355T and pS458T in *parE* – and 2 plasmid-mediated quinolone resistance genes – qnrB19 and qnrS1 – associated with quinolone resistance, and those for AmpC (bla_{CMY-2}) and extended-spectrum ($bla_{CTX-M-15}$, $bla_{CTX-M-27}$, and $bla_{CTX-M-55}$) beta-lactamases (ESBL). The presence of ESBL gene(s) in *E. coli* was significantly associated with the presence of one or more quinolone resistance determinants ($p<0.05$, Fisher's exact test).

Table 2. Results of PERMDISP2, PERMANOVA, and ANOSIM tests

Group	PERMDISP2 P-value (F)	PERMANOVA P-value (R^2)	ANOSIM P-value (R)
AMR (ResFinder) genes (n=40)			
Treatment group	0.75 (0.10)	0.15 (0.04)	0.38 (4.23E-3)
Diarrhea status	0.74 (0.29)	0.87 (0.02)	0.49 (-5.89E-3)
Antibiotic doses	0.62 (0.48)	0.83 (9.39E-3)	0.85 (-0.09)
Virulence (VFDB) genes (n=103)			
Treatment group	0.86 (0.03)	0.73 (0.01)	0.75 (-0.035)
Diarrhea status	0.98 (0.02)	0.76 (0.03)	0.57 (-0.020)
Antibiotic doses	0.69 (0.38)	0.70 (0.02)	0.67 (-0.048)
BacMet genes (n=153)			
Treatment group	0.71 (0.15)	0.22 (0.03)	0.37 (7.93E-3)
Diarrhea status	0.96 (0.04)	0.93 (0.02)	0.49 (-4.04E-3)
Antibiotic doses	0.97 (0.03)	0.76 (0.01)	0.52 (-0.01)
Pangenome (Roary) elements (n=14011)			
Treatment group	0.92 (0.01)	0.34 (0.025)	0.42 (2.0E-3)
Diarrhea status	0.59 (0.54)	0.93 (0.03)	0.80 (-0.066)
Antibiotic doses	0.71 (0.35)	0.57 (0.02)	0.47 (2.63E-3)

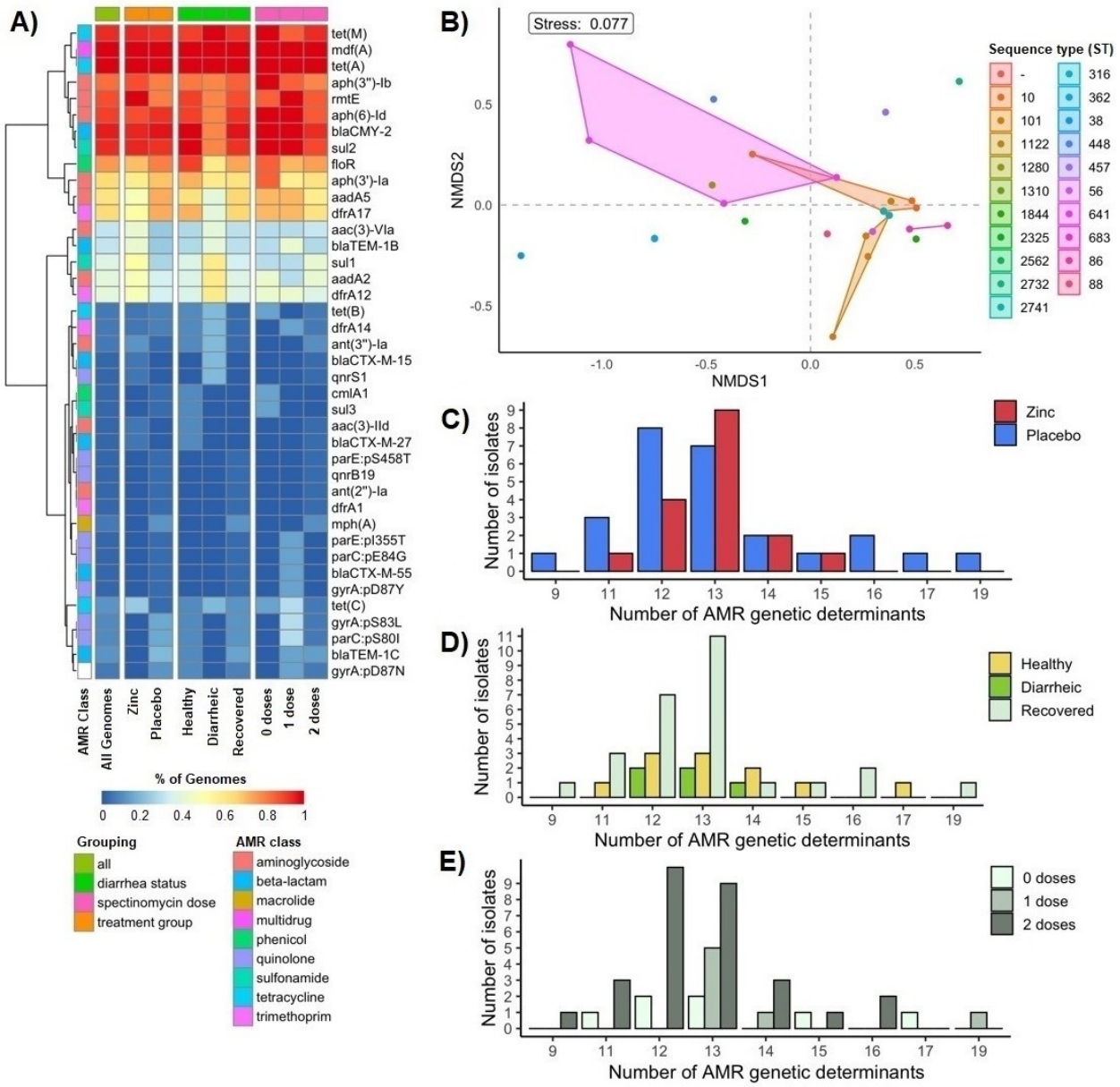

Figure 2. Antimicrobial resistance genetic determinants in fecal *E. coli* isolates from pre-weaned dairy calves (n=43). A) Heat map of ARG prevalence among isolates B) Non-metric multidimensional scaling of ARG composition of isolates by grouping factor of sequence type. Distribution of number of ARGs in *E. coli* isolates by C) treatment group D) diarrhea status and E) therapeutic antibiotic exposure.

ARGs present in more than half of the isolates included *mdf(A)* (43/43, 100%), *aph(6)-Id* (39/43, 90.7%), *rmtE* (38/43, 88.4%), *aph(3")-Ib* (36/43, 83.7%), and aadA5 (27/43, 62.8%) for aminoglycoside resistance, *blaCMY-2* (41/43, 95.3%) for beta-lactam resistance, *dfrA17* (28/43, 65.1%) for trimethoprim resistance, *floR* (33/43, 76.7%) for phenicol resistance, *sul2* (40/43, 93.0%) for sulfonamide resistance, and *tet(A)* (43/43, 100%) and *tet(M)* (40/43, 93.0%) for tetracycline resistance (Figure 2A). The average

number of ARGs across all genomes was 13 (SD = 1.73) and alongside collective AMR profiles, did not differ significantly by dietary zinc supplementation treatment group (zinc or placebo), diarrhea status (healthy, diarrheic, and recovered), and therapeutic antibiotic exposure (0, 1, or 2 doses) (Table 2, Figure 2C-E).

Mobile genetic elements associated with ARGs

As *E. coli* isolates in this study were MDR, it was of interest to investigate the mobile genetic elements associated with ARGs that may contribute to AMR co-transfer. Eighteen putative plasmids based on the presence of plasmid replicons were identified across all genomes, with a pairwise co-occurrence matrix indicating high frequency co-occurrence of AMR gene clusters with certain putative plasmids (Figure 5). The most frequently co-occurring gene network of *aph(6)-Id*, bla_{CMY-2}, *floR*, *mdf(A)*, *sul2*, and *tet(A)* was associated with the IncA/C2 plasmid replicon in 30 (69.8%) genomes. A second smaller network including *mdf(A)*, *rmtE*, and *tet(A)* co-occurred with the IncFIB (AP001918) plasmid replicon at a frequency of 20 (46.5%) genomes. At a minimum threshold co-occurrence of ≥10 genomes (about 25% of the genomes), a larger network of genes including *aac(3)-VIa*, *aadA2*, *dfrA12*, *mdf(A)*, *rmtE*, *sul1*, *tet(A)*, and *tet(M)* were detected with IncHI2/2A plasmid replicons. Screening for plasmid replicons among genomes in this study identified unique sets of ARGs in co-occurrence with primarily large AMR plasmids.

Association between dietary zinc supplementation and genotypic AMR

The relationship between genotypic AMR and calf zinc treatment group of isolates was examined to determine the association between dietary zinc supplementation in pre-weaned dairy calves and the selection of specific ARGs. From descriptive analysis, SNPs in genes for quinolone resistance were exclusively detected in isolates from placebo calves. Antibiotic exposure-adjusted logistic regression models identified higher odds of certain ARGs in *E. coli* isolates from zinc-treated compared to placebo calves (*dfrA12*, *aadA2*, *sul2*, *aac(3)-VIa*, *aph(3'')-Ib*, bla_{TEM-1B}, *sul1*, and alleles of bla_{CTX-M}), though none of these associations were significant (OR=1.60-2.92, $p>0.05$). Conversely, there were non-significant lower odds for other ARGs and point mutations associated with quinolone resistance for isolates from

zinc-treated to placebo calves (*aadA5*, *dfrA17*, *floR*, *aph(3')-Ia*, *bla*$_{CMY-2}$, *aph(6)-Id* (OR=0.23-0.82, p>0.05) (Figure 3, Supplementary Tables 1-15).

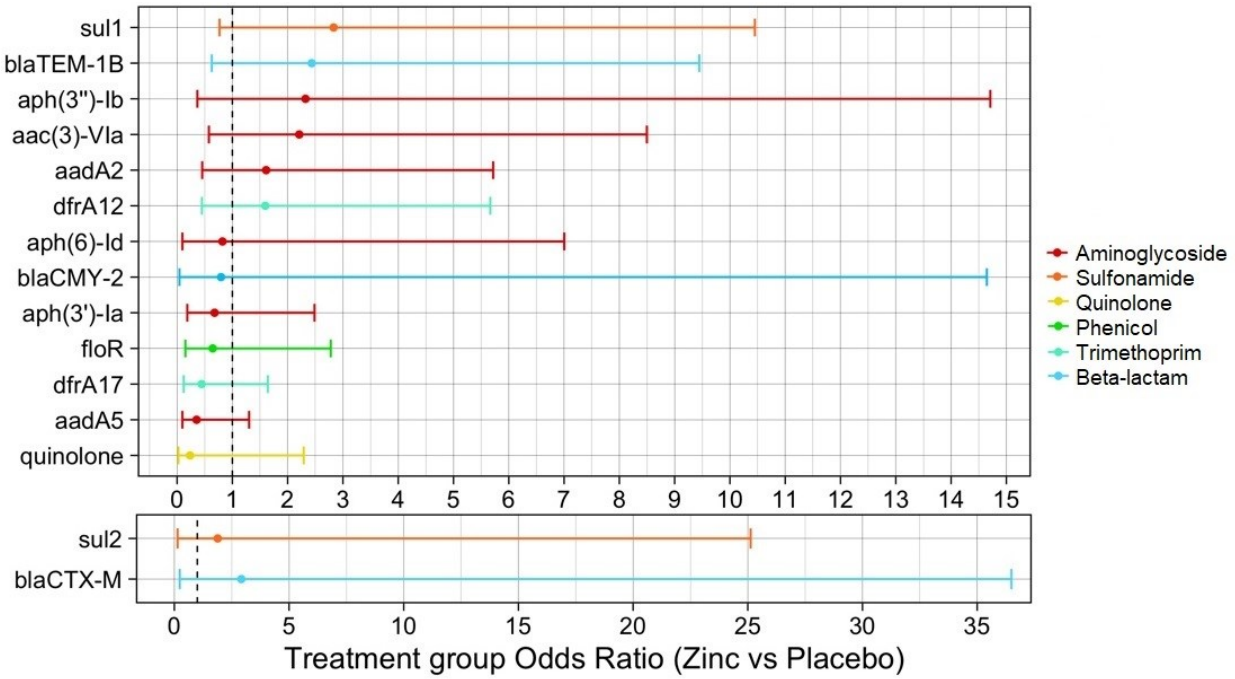

Figure 3. Antibiotic exposure-adjusted logistic regression models evaluating the association between presence of antimicrobial resistance genetic determinants (ARGs) and calf treatment group of *E. coli* isolates. Point estimates for each model are color-coded by antimicrobial class. Binary outcomes for quinolone and *bla*$_{CTX-M}$ models were specified as the presence/absence of any quinolone resistance mechanism (plasmid-mediated genes or point mutations) and the presence/absence of any *bla*$_{CTX-M}$ alleles, respectively.

Virulence genes

A total of 103 virulence genes corresponding to adherence/biofilm formation (n=36), iron/nutrient acquisition (n=40), secretion (n=21), toxin (n=4), and other functions (n=2) were detected across *E. coli* genomes. The average and median number of virulence genes were 40.58 and 38, respectively (range of 18 to 68). Four virulence genes related to enterobactin (*entB*, *entC*, *fepA*, *fepD* and *fes*) were detected across all isolates (Figure 4a). The number of virulence genes and collective virulence profiles across genomes did not differ significantly by dietary zinc supplementation treatment group, diarrhea status, and therapeutic antibiotic exposure (Table 2, Figure 4c-e).

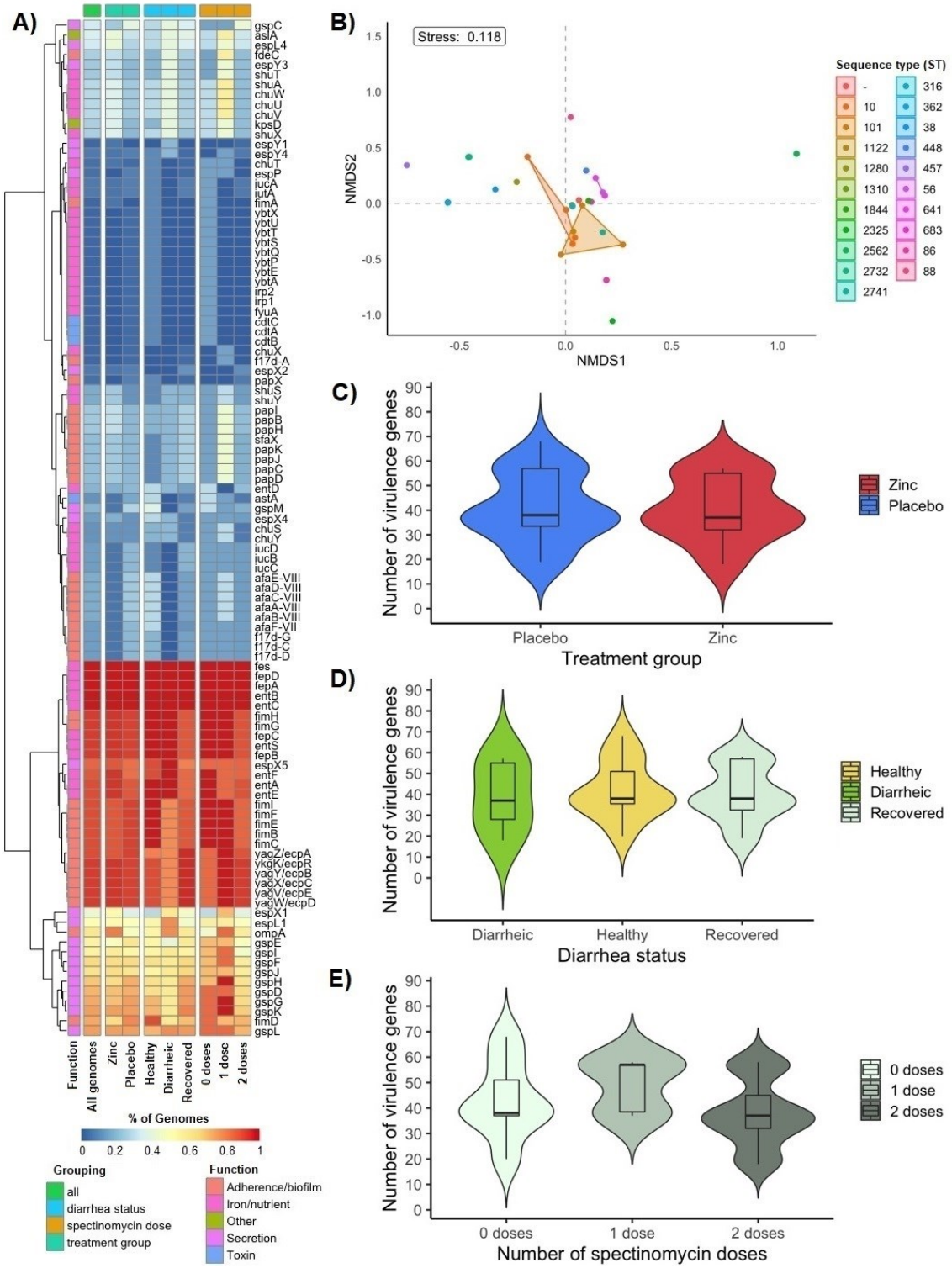

Figure 4. Virulence genes in fecal *E. coli* isolates from pre-weaned dairy calves (n=43). A) Heat map of virulence gene prevalence among isolates B) Non-metric multidimensional scaling of virulence gene composition in isolates by grouping factor of sequence type. Distribution of number of virulence genes in *E. coli* isolates by C) treatment group D) diarrhea status and E) therapeutic antibiotic exposure.

Virulence genes from the *afa-7* and *afa-8* clusters (*afaA-E*) encoding afimbrial adhesins were detected primarily in isolates from placebo calves (85.71%, 6/7), with the full gene set present in six isolates. Other virulence genes detected related to colonization included those encoding F17 fimbriae (*f17d-A*, *f17d-C*, *f17d-D*, and *f17d-G*) in six isolates, and P fimbriae in 12 isolates (*pap* genes) (Bertin et al., 2000; Bihannic et al., 2014; Ryu et al., 2020). Additionally, genes in the *fim* cluster (*fimA-I*) encoding type 1 fimbriae were present in the majority of isolates, though only one isolate harbored the *fimA* structural gene and three isolates the *fimH* adhesin gene. Major virulence genes related to secretion included those corresponding to Type II (*gsp*) and Type III (*esp*) secretion systems. Virulence genes for toxins, *astA* (enteroaggregative heat-stable enterotoxin) and/or *cdtABC* (cytolethal distending toxin), were identified in isolate(s) from pre- and post-diarrheic calves. Overall, virulence genes were interspersed in the population across calf zinc treatment group and diarrhea status. The largest number and diversity of virulence genes identified corresponded to iron/nutrient acquisition, including genes *chuSTUVWXY* (heme uptake), *entA-F* (enterobactin), *fepA-D* (enterobactin), *fyuA* (yersiniabactin receptor), *iucABCD-iutA* (aerobactin), and those in the *ybt* operon (yersiniabactin) (Figure 4a).

Association between AMR and metal resistance genes

A total of 153 metal resistance genes (MRGs) were identified across all *E. coli* genomes examined, with the average and median number of MRGs per genome being 128.42 and 127, respectively, with a range of 123 to 135. Co-occurrence analysis of ARGs and metal resistance genes included 16585 gene pairs and identified 96 positive and 77 negative co-occurrences. Positive associations including both ARGs and metal resistance were observed between aminoglycoside (*aac(3)-VIa*, *aadA2*), beta lactam (*blaTEM-1B*), sulfonamide (*sul1*), and trimethoprim (*dfrA12*) resistance and acid (*gadA* and *gadB*), tellurium (*terZ* and *terW*) and mercury (*merT*) resistance genes (Figure 6).

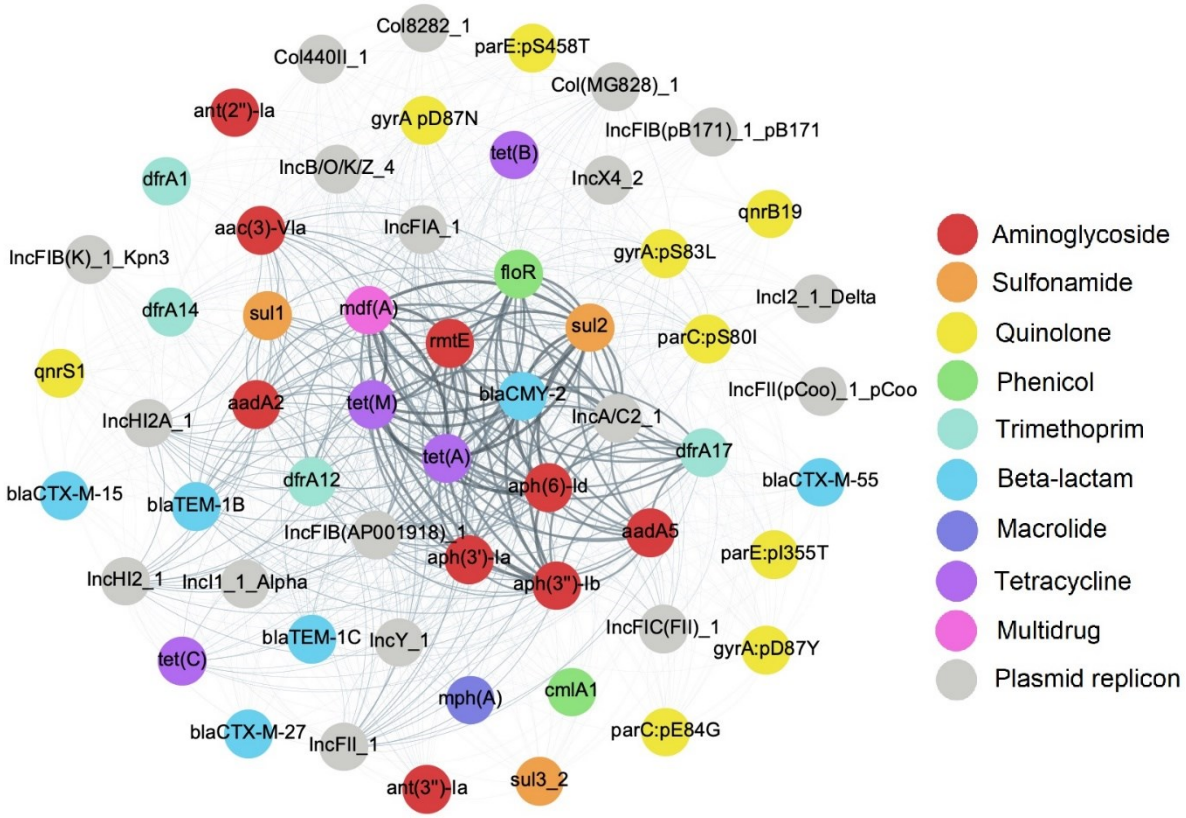

Figure 5. Co-occurrence network of plasmid replicons and antimicrobial resistance genetic determinants (ARGs) in *E. coli* isolates. Nodes representing ARGs are color coded by antimicrobial class and edges representing low to high frequency of co-occurrence are depicted in a light to dark color gradient.

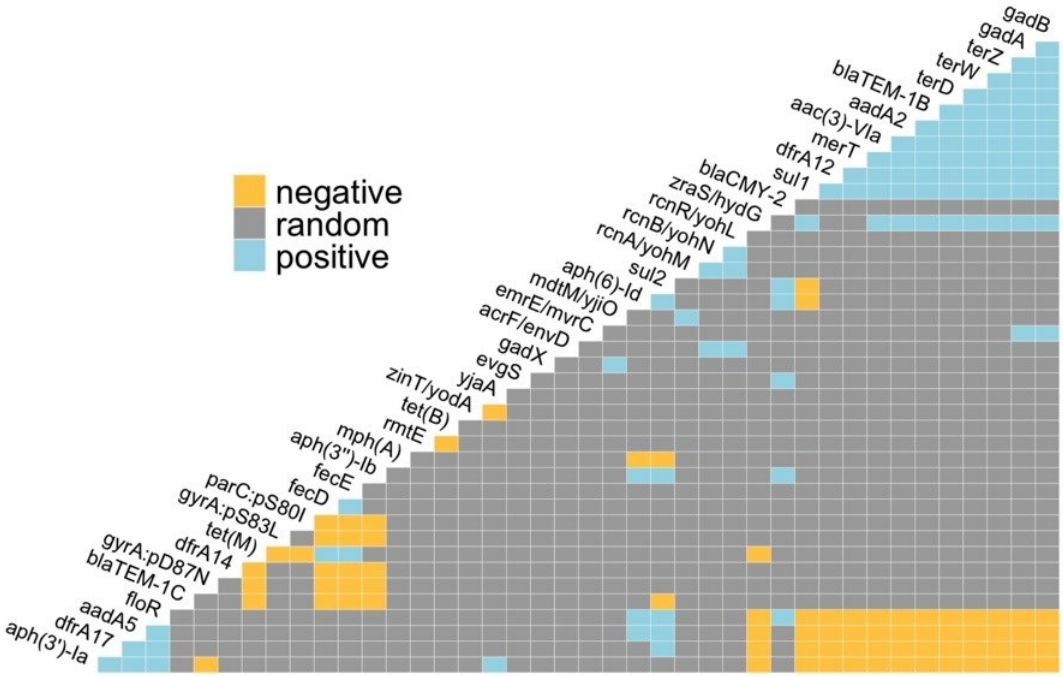

Figure 6. Pairwise probabilistic co-occurrence analysis for positive, negative, or random associations of antimicrobial resistance genetic determinants and metal resistance genes in *E. coli* isolates.

Discussion

The enteric microbiota serves as a symbiotic partner of the host, with crucial roles in intestinal health, metabolism, and host immune response (Casadevall and Pirofski, 2000; Kaiko and Stappenbeck, 2014; Jandhyala et al., 2015). The acquisition and loss of genes – such as those for AMR – in enteric microbes like *E. coli* can occur as adaptive responses to environmental (e.g. dysbiosis) and host changes (e.g. diet and disease). In this study, we investigated the host-microbe interface of enteric MDR *E. coli* from pre-weaned dairy calves to evaluate potential contributing factors to MDR persistence and better understand the relationship between genomic composition and host-level factors of antimicrobial exposure, dietary zinc supplementation, and calf diarrheal disease.

Whole genome sequence analysis revealed high genome variability and an open pangenome of multidrug-resistant (MDR) *E. coli* from dairy calves in this study. The diverse population structure of *E. coli* has been well-documented, with the frequent acquisition, loss, and modification of genes contributing to its large gene pool, fitness, and competitive ability to thrive in widespread geographical and host environments (Horesh et al., 2021). From all-by-all comparisons of the WGS, the isolates in this study clustered by sequence type (ST) but not host-level factors of disease status, dietary influences, or antimicrobial exposure. Common STs identified included ST362, a frequently occurring ST in calves that has been associated with extra intestinal infections (Falgenhauer et al., 2017; Vieille et al., 2019; Homeier-Bachmann et al., 2022). Other prevalent STs were those with zoonotic potential, such as ST641, were previously isolated from poultry and goat sources (Cortés et al., 2010; Zhuge et al., 2021; Treilles et al., 2023), ST10, a widespread lineage of pathogenic and commensal *E. coli* which are prominently MDR in animal populations (Haley et al., 2023; Silva et al., 2023; Wang et al., 2023), and ST101, another frequently occurring MDR clonal group frequently detected in food, water, food animal, and human matrices (Umpiérrez et al., 2017; Zhong et al., 2019; Sauget et al., 2023; Silva et al., 2023).

The accessory genome of *E. coli* encodes various characteristics for survival and reproduction, including those related to AMR (Hall et al., 2021). The early developing microbiota of calves has been observed to harbor high prevalence and diversity of ARGs (Liu et al., 2019; Haley et al., 2023), which is

corroborated by the large assortment of ARGs detected in *E. coli* genomes. In this study, the number of ARGs in *E. coli* did not correlate with antimicrobial use; however, the presence of ARGs corresponding to certain antimicrobial classes were consistent with the AMR selection pressures in our study; the high prevalence and diversity of tetracycline and aminoglycoside ARGs detected in *E. coli* genomes is reflective of the tetracycline and neomycin administered in dietary milk and spectinomycin for the therapeutic treatment of diarrhea.

A major mechanism of third-generation cephalosporin resistance in *Salmonella* and *E. coli* from food and food-producing animals is AmpC-type beta-lactamase bla_{CMY-2}, which was detected in almost every *E. coli* genome in this study, despite the lack of beta-lactam use in calves. The occurrence of bla_{CMY-2} in dairy cattle has been presumed to be from frequent use of ceftiofur for the intramammary treatment of mastitis and parenteral treatment of acute metritis and bacterial pneumonia (Durel et al., 2019). However, studies have found limited evidence for the direct dissemination of bla_{CMY-2} through ceftiofur use (Daniels et al., 2009; Schmidt et al., 2013) or associations between recent ceftiofur treatment and reduced-susceptible *E. coli* at the individual cow level (Tragesser et al., 2006). We found a high frequency of a co-occurrence networks with bla_{CMY-2}, ARGs corresponding to aminoglycoside, phenicol, sulfonamide, and tetracycline resistance, and the IncA/C2 plasmid replicon in our study isolates. These data support observations from other studies, in which the occurrence of bla_{CMY-2} in absence of cephalosporin use has been postulated to be from its acquisition on large MDR plasmids, followed by clonal expansion and/or the presence of indirect and co-selective AMR pressures that maintain these plasmids at the herd-level (Alcaine et al., 2005; Subbiah et al., 2011; Martin et al., 2012; Schmidt et al., 2013; Deng et al., 2015). Other beta-lactam ARGs conferring resistance to cephalosporins found in this study included ESBL genes from the bla_{CTX-M} family ($bla_{CTX-M-15}$, $bla_{CTX-M-27}$, and $bla_{CTM-M-55}$) from three *E. coli* genomes. In addition to being resistant to third-generation cephalosporins, ESBL- producing *E. coli* have important clinical consequences as they are frequently MDR to other critically important antimicrobials such as quinolones (Zurfluh et al., 2014; Azargun et al., 2018; Furmanek-Blaszk et al., 2023), a finding that is corroborated

through the significant association observed between the presence of ESBL and quinolone resistance determinants among *E. coli* in this study.

While antimicrobial use is perceived as a main driver of AMR, non-antimicrobial factors such as heavy metal exposure have also been recognized to influence AMR selection. Heavy metals such as zinc are frequently used as growth promoters or therapeutic agents in livestock species (Yazdankhah et al., 2014); for example, dietary zinc supplementation in pre-weaned calves may be used to reduce the burden of diarrheal disease and promote calf growth (Glover et al., 2013; Feldmann et al., 2019; Chang et al., 2020; Liu et al., 2023; Yu et al., n.d.). Little is known on the influence of dietary zinc on genomic AMR in cattle, however a previous study in swine found that high zinc in feed (2.5g/kg) significantly increased intestinal abundance of tetracycline and sulfonamide ARGs (Vahjen et al., 2015). As all *E. coli* genomes in our study had *tet* genes, we were unable to evaluate the selection of tetracycline ARGs. Adjusted logistic regression models found higher odds ratios for the presence of sulfonamide genes – *sul1* (OR=2.83, 95% CI 0.77-10.45) and *sul2* (OR=1.89, 95% CI 0.14-25.12) – in *E. coli* from zinc compared to those from placebo calves. Although these findings were not statistically significant, the direction of associations support the aforementioned findings of potential sulfonamide ARG selection from dietary zinc (Vahjen et al., 2015). We also found unique SNPs in the genes conferring quinolone resistance in isolates from placebo treated calves, suggesting an antagonistic effect of zinc on certain classes of ARGs. However, the lower odds ratio for the presence of quinolone ARGs from logistic regression in isolates from zinc compared to placebo treated calves (OR=0.23 95% CI 0.02-2.29) was also not statistically significant. These non-significant findings may be attributed to the small sample size of isolates in our study that may have resulted in inadequate power to detect differences in addition to other uncharacterized variables. Hence, future studies employing larger sample sizes are needed to ascertain the relationship between zinc exposure and ARG selection, particularly for those in our study (*sul2*, $bla_{CMY\text{-}2}$, *aph(3'')-Ib,* and $bla_{CTX\text{-}M}$ alleles) with large confidence intervals for point estimates.

Beyond the selection of individual ARGs, co-selection of both ARGs and metal resistance genes may occur through co-resistance, a phenomenon where dissimilar mechanisms for both resistances are

selected due to their genetic linkage (Wales and Davies, 2015). The linkage of ARGs and metal resistance genes has been well documented (Baker-Austin et al., 2006; Wales and Davies, 2015; Nguyen et al., 2019), and is supported by the several positive co-occurrences between diverse ARGs and mercury and tellurium resistance genes in *E. coli* from our study. Positive co-occurrence between ARGs for the same classes of antimicrobials and mercury and tellurium resistance genes were previously reported in fecal *E. coli f*rom dairy herds from Pennsylvania (Haley et al., 2023), suggesting that these specific genes are pervasive and selected for in dairy cattle and their farm environments irrespective of geographical location.

In addition to evaluating potential host-level drivers of AMR in calf *E. coli*, this study compared genotypic AMR – the presence of ARGs and point mutations conferring quinolone resistance – with phenotypic AMR data from antimicrobial susceptibility testing. Genotypic AMR exhibited a high degree of concordance with phenotypic AMR for genomically heterogeneous MDR isolates in this study. Despite the small sample size of isolates (n=43) from one host (dairy calves) and source (single dairy farm), our findings are consistent with previous work evaluating genotypic and phenotypic concordance in *E. coli* and *Salmonella* from cattle and/or food animal sources (Tyson et al., 2015; McDermott et al., 2016; Carroll et al., 2021; Lee et al., 2022, 2023). Discrepancies for streptomycin and ceftiofur as observed in two isolates in this study have been frequently reported (Tyson et al., 2015; McDermott et al., 2016; Lee et al., 2023), and may be a result of lack of CLSI breakpoints for these veterinary drugs, technical variability in AST/WGS processes (e.g. 2-fold variations in MIC from AST at intermediate and resistant cut-off thresholds), and choice of classifying intermediate isolates. As an example of the latter, grouping of intermediate and susceptible isolates for analysis resulted in discrepancy of a ceftiofur immediate isolate in this study; the genotypic and phenotypic AMR for this isolate would have been congruent if intermediate isolates were instead treated as resistant.

Diarrheal disease status of calves was not significantly associated with genomic variability in this study, including virulence profiles. *E. coli* can be categorized into various pathotypes depending on the presence of certain virulence attributes (Kaper et al., 2004), with common pathotypes associated with

neonatal calf diarrhea including enteropathogenic (EPEC), Shiga toxin-producing (STEC), enterotoxigenic (ETEC), and enteroaggregative (EAEC) *E. coli* (Awad et al., 2020). While MDR *E. coli* isolates in this study lacked the comprehensive virulence markers of these diarrheagenic pathotypes, they encoded a wide diversity of virulence genes that overlap with those in pathogenic strains. For instance, adhesin virulence genes observed in our study, *fim* and *pap* genes encoding Type I fimbriae and P fimbriae respectively, are associated with various pathotypes in both humans and animals (Bertin et al., 2000; Sarowska et al., 2019), and *f17* genes encoding F17 fimbriae and *afa-7* and *afa-8* gene clusters encoding afimbrial adhesion appear to be more host-specific and predominant in bovine *E. coli* associated with diarrhea and septicemia (Lalioui and Le Bouguénec, 2001; Bihannic et al., 2014; Shahrani et al., 2014). Additionally, detected in a few isolates were *cdtABC* and *astA* encoding cytolethal distending toxin (CDT) and enteroaggregative heat-stable enterotoxin (EAST1), which are typically present in EPEC and ETEC, respectively (Yamamoto and Echeverria, 1996; Osek, 2003; Gomes et al., 2016; Meza-Segura et al., 2017).

The most abundant virulence genes in MDR *E. coli* in this study were those involved in iron acquisition (e.g. sideophores and heme uptake). Iron plays a critical role in microbial metabolic processes and cell division, and its acquisition is an important host-microbe interaction that contributes to bacterial survival and pathogen infection (Caza and Kronstad, 2013; Nairz and Weiss, 2020). Previous studies identified several iron acquisition systems – some of which were also identified in our study (e.g. *iucABCD-iutA*) – to be significantly enriched in MDR bovine *E. coli* (Haley et al., 2023, 2024). Virulence factors and ARGs are essential for bacteria to overcome host immune responses and antimicrobial exposure, respectively. The simultaneous carriage of both in MDR *E. coli* may confer a fitness advantage in adverse conditions, promoting the co-selection and maintenance of these genes in MDR isolates as opposed to their susceptible counterparts (Beceiro et al., 2013). Moreover, the pre-weaned calf diet is primarily composed of milk, which is nutritionally negligible in iron and may contribute to a low-iron environment in the calf gut that has been hypothesized to favor the selection of MDR *E. coli* with more extensive repertoires of iron acquisition systems (Haley et al., 2023, 2024). During infection and disease,

host-driven iron sequestration occurs as an immune defense strategy to inhibit the growth of pathogens (Beceiro et al., 2013; Nairz and Weiss, 2020). As *E. coli* genomes in our study were from pre-weaned calves in various stages of diarrheal disease (pre-diarrheic, diarrheic, and recovered), we hypothesize that host-mediated iron withdrawal is another factor which may further favor the survival of MDR *E. coli* with high iron-scavenging capacity.

In conclusion, our analysis indicated that the genomes of MDR *E. coli* from pre-weaned dairy calves were highly diverse and minimally driven by the host-level factors evaluated in this study (dietary zinc supplementation, therapeutic antimicrobial treatment, and diarrhea disease status). Key limitations include the relatively small sample size of isolates and the absence of a susceptible and/or non-MDR group of *E. coli* genomes for comparison. Future work that evaluates longitudinal effects would provide greater insight on the relationship between genomic diversity and factors such as disease – which may occur in progressive stages – and antimicrobial exposure, which can rapidly and transiently impact the gut microbiome. Our findings corroborate previous reports of MDR *E. coli* from calves harboring diverse ARGs conferring resistance to clinically important drugs, enriched abundance of virulence factors for iron acquisition systems, and linkage of certain metal resistance genes and ARGs. These data suggest that the selection and persistence of MDR *E. coli* in calves are adaptive and attributed to the presence of these and/or other unidentified genes that confer a fitness advantage in the calf gut.

Data Availability Statement

Data from this study are presented in the Supplementary File, which will be publicly available with the final publication of this manuscript. Additional source data are available from the corresponding author upon reasonable request. WGS data of *E. coli* isolates in this study can be found at the 100k Pathogen Genome Project BioProject (PRJNA186441).

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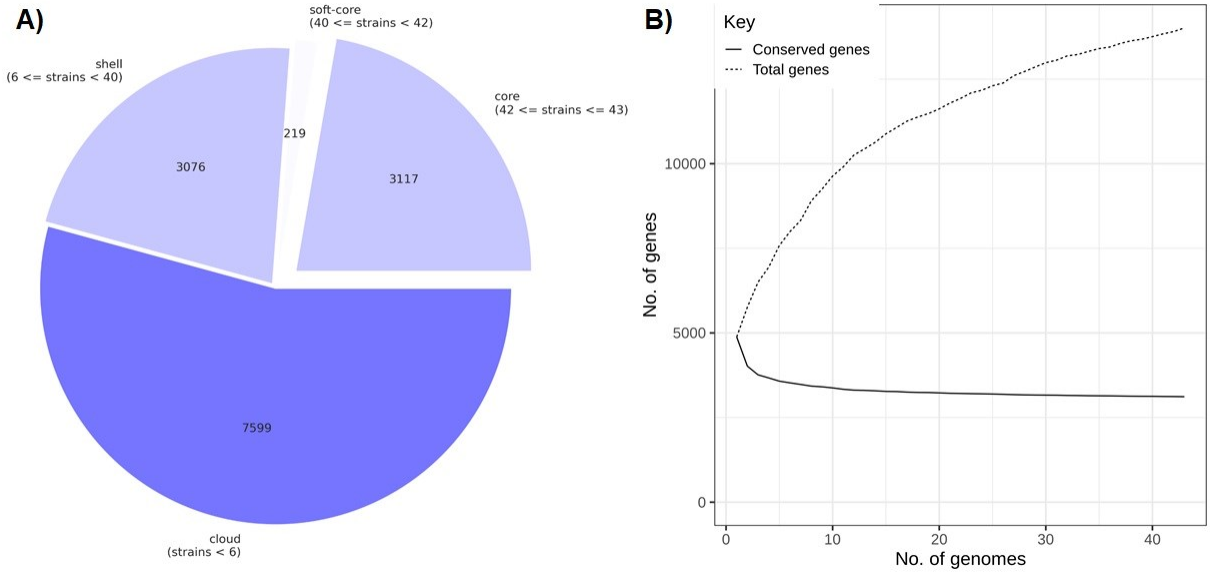
Supplementary Materials

Supplementary Figure 1. Pangenome analysis of *E. coli* genomes (n=43), conducted using Roary. (a) Summary of *E. coli* pangenome (14,011 genes) (b) Gene accumulation curve of the number of conserved homologous genes (homologs) and the total distinct homologs of the *E. coli* pangenome, depicted by solid and dashed lines, respectively.



Supplementary Table 1. Final logistic regression model for the association between the presence of *bla*$_{CTX\text{-}M}$ alleles in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	1.07 (1.29)	2.92 (0.23, 36.49)	0.41
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	0.14 (0.23)	1.15 (0.72, 1.81)	0.56
Intercept	-	-4.10 (1.93)	-	0.034

Supplementary Table 2. Final logistic regression model for the association between the presence of quinolone resistance determinants (point mutations and plasmid-mediated quinolone resistance determinants) in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	-1.45 (1.16)	0.23 (0.024, 2.29)	0.21
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	0.096 (0.15)	1.10 (0.81, 1.49)	0.53
Intercept	-	-2.03 (1.12)	-	0.070

Supplementary Table 3. Final logistic regression model for the association between the presence of *aac(3)-VIa* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	0.79 (0.69)	2.21 (0.58, 8.50)	0.25
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	0.054 (0.11)	1.06 (0.85, 1.31)	0.62
Intercept	-	-1.53 (0.82)	-	0.063

Supplementary Table 4. Final logistic regression model for the association between the presence of *aadA2* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	0.48 (0.65)	1.61 (0.45, 5.72)	0.46
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	0.04 (0.10)	1.04 (0.86, 1.26)	0.69
Intercept	-	-0.87 (0.73)	-	0.23

Supplementary Table 5. Final logistic regression model for the association between the presence of *aadA5* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	-1.04 (0.67)	0.35 (0.096, 1.30)	0.12
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	-0.084 (0.11)	0.92 (0.75, 1.13)	0.43
Intercept	-	1.51 (0.81)	-	0.061

Supplementary Table 6. Final logistic regression model for the association between the presence of *aph(3'')-Ib* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	0.84 (0.94)	2.32 (0.37, 14.71)	0.37
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	-0.23 (0.18)	0.80 (0.56, 1.13)	0.20
Intercept	-	2.95 (1.38)	-	0.033

Supplementary Table 7. Final logistic regression model for the association between the presence of *aph(3')-Ia* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	-0.39 (0.66)	0.68 (0.19, 2.49)	0.56
	Placebo	Referent	-	-
Spectinomycin treatment	Yes	-1.30 (1.14)	0.27 (0.029, 2.54)	0.25
	No	Referent	-	-
Intercept	-	1.91 (1.11)	-	0.084

Supplementary Table 8. Final logistic regression model for the association between the presence of *aph(6)-Id* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	-0.20 (1.09)	0.82 (0.096, 7.00)	0.86
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	-0.29	0.75 (0.45, 1.25)	0.27
Intercept	-	4.52 (2.17)	-	0.037

Supplementary Table 9. Final logistic regression model for the association between the presence of *blaCMY-2* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	-0.23 (1.49)	0.80 (0.043, 14.65)	0.88
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	-0.22 (0.33)	0.80 (0.42, 1.52)	0.50
Intercept	-	4.71 (2.65)	-	0.076

Supplementary Table 10. Final logistic regression model for the association between the presence of *blaTEM-1B* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	0.89 (0.69)	2.44 (0.63, 9.45)	0.20
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	-0.036 (0.10)	0.97 (0.79, 1.18)	0.73
Intercept	-	-1.00 (0.75)	-	0.18

Supplementary Table 11. Final logistic regression model for the association between the presence of *dfrA12* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	0.47 (0.65)	1.60 (0.45, 5.67)	0.47
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	0.049 (0.10)	1.05 (0.86, 1.28)	0.62
Intercept	-	-0.93 (0.73)	-	0.20

Supplementary Table 12. Final logistic regression model for the association between the presence of *dfrA17* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	-0.81 (0.67)	0.45 (0.12, 1.64)	0.22
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	-0.073 (0.11)	0.93 (0.76, 1.15)	0.49
Intercept	-	1.44 (0.80)	-	0.071

Supplementary Table 13. Final logistic regression model for the association between presence of *floR* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	-0.44 (0.75)	0.65 (0.15, 2.78)	0.56
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	-0.12 (0.13)	0.88 (0.69, 1.14)	0.34
Intercept	-	2.21 (1.00)	-	0.027

Supplementary Table 14. Final logistic regression model for the association between presence of *sul1* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	1.04 (0.67)	2.83 (0.77, 10.45)	0.12
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	0.084 (0.11)	1.09 (0.88, 1.34)	0.43
Intercept	-	-1.51 (0.81)	-	0.061

Supplementary Table 15. Final logistic regression model for the association between presence of *sul2* in *E. coli* genomes and treatment group of calves.

Factor	Level	Coefficient (SE)	Odds Ratio (95% CI)	P-value
Treatment group	Zinc	0.64 (1.32)	1.89 (0.14, 25.12)	0.63
	Placebo	Referent	-	-
Days from spectinomycin treatment	-	-0.35 (0.33)	0.70 (0.37, 1.34)	0.28
Intercept	-	4.98 (2.71)	-	0.066

Concluding Remarks

The dissertation addresses current gaps on the AMR of enteric bacteria of public health significance. The four presented chapters integrate statistical, microbiological, and genomic approaches to better understand the distribution and drivers of AMR in food and food-producing animals and their products, advancing knowledge that benefits both human and animal health.

In Chapter 1 and 2, data from expanded geographical sampling of the National Antimicrobial Resistance Monitoring System (NARMS) retail meat surveillance in California was assessed. Chapter 1 determined the prevalence and AMR profiles of *Salmonella* from retail meat in both northern and southern California. While the overall frequency of *Salmonella* in retail meats in California was low, diverse serotypes and AMR profiles were identified, including multidrug resistant (MDR) strains that were primarily *S.* Infantis. Whole genome sequencing detected antimicrobial resistance genes of public health significance and the IncFIB(pN55391) replicon among *S.* Infantis isolates, corroborating national NARMS data and worldwide reports of a disseminated *S.* Infantis clone with pESI-like mega plasmid carriage. Prior to data collection in southern California for Chapter 2, *E. coli* was not collected or evaluated from retail meats in California for NARMS. In Chapter 2, the first available data on *E. coli* from retail meats in southern California was evaluated. Using *E. coli* as an indicator bacteria for AMR, our results indicate that there was higher occurrence of AMR to certain antimicrobial drugs across different food animals, with a significantly higher odds of resistance observed for poultry counterparts (chicken or ground turkey) compared to non-poultry meats (beef and pork). WGS analysis also identified diverse AMR genes and heterogeneous gene networks. Findings from Chapters 1 and 2 advance understanding of the epidemiology of pathogenic and commensal enteric bacteria from retail meats in California and highlight the importance of routine monitoring and comprehensive characterization of foodborne bacteria across geographical locations and sample types. Work from these chapters also demonstrate the value of collaborative efforts involving academic partnerships, local public health departments, and federal agencies for addressing AMR, alongside the harmonized use of next-generation sequencing technologies for public health surveillance.

Substantial data is available on the prevalence and distribution of AMR in enteric bacteria. Comparably, less is known on the non-antimicrobial factors that may contribute to AMR and the extent to which they increase or decrease resistance. The standard approach to quantifying AMR involves microbiological testing to categorize bacterial isolates into susceptible, intermediate resistant, or resistant phenotypes. There remains a need for novel data analysis approaches that minimize data loss from dichotomization or categorization of MIC data (e.g. binary or multinomial logistic regression) and can also account for changes in resistance that may not cross breakpoint thresholds. Moreover, a common challenge with frequently used antimicrobial susceptibility testing (AST) methods – e.g. broth microdilution that tests twofold dilutions of antimicrobial drugs – are outcomes of censored MIC data. In Chapter 3, we evaluated the impact of zinc supplementation on the phenotypic AMR of fecal commensal bacteria. Accelerated failure time (AFT) models adapted for censored MIC data instead of time indicated that dietary zinc supplementation – which has previously been shown to have health and diarrheal prevention/recovery benefits in pre-weaned calves – was not associated with an increase in phenotypic AMR in fecal *Enterococcus* spp. and *E. coli*. In *E. coli*, our cross-sectional data suggest that zinc supplementation may be associated with a protective effect on AMR for quinolone antimicrobial drugs. Though further research is needed to validate these findings and investigate the influence of other factors (e.g. different management practices), these results suggest that the use of zinc supplementation as an antimicrobial alternative in dairy calves has simultaneous benefits of enhancing animal health and preventing selection of AMR. AFT models in Chapter 3 were shown to be well-suited for censored AST data with adequate distribution of MIC values and a robust alternative to models such as Cox models – which are heavily dependent on the proportional hazards assumption – and those resulting in data loss (e.g. logistic regression). Strengths of utilizing AFT models also include the ability to model censored MIC values on a continuous scale, whilst generating adjusted effect sizes (accounting for multiple confounders). AFT models however, may not be well-suited for handling high levels of right- and left-censored data; hence, alternative approaches such as truncated interval regression models should be explored for these types of data in future studies.

In Chapter 4, whole genome comparative analysis was conducted on a repository of MDR *E. coli* from Chapter 3. Although AMR interventions tend to be targeted towards animals and humans, it is important to recognize that it is the microorganisms – e.g. bacteria – rather than the hosts themselves that develop resistance. Such resistance can occur as adaptive processes to environmental and host-level influences that may be conducive to the selection of MDR populations. As opposed to the first two chapters of this dissertation, this chapter evaluated the entirety of bacterial genomes with the goals to identify potential genetic factors contributing to MDR persistence and to better understand the relationship between host-level factors and genomic composition. Pangenome analysis of MDR *E. coli* from pre-weaned dairy calves identified an open pangenome with high genomic diversity. The host-factors evaluated – zinc supplementation, antimicrobial exposure, and diarrheal disease status – did not account for considerable genomic variability of *E. coli* isolates. However, the high prevalence and diversity of virulence genes corresponding to iron acquisition – which corroborate findings from other studies on *E. coli* in cattle – suggest that certain accessory genes in MDR *E. coli* from calves may be enriched. Although a limited number of isolates were evaluated in this study, implications from this chapter highlight the need for future work on the population genomics of bacterial host adaptations. Specifically, high-resolution and comprehensive assessment of bacterial genomes in relationship to host-associated data will contribute to better understanding of how certain populations – such as MDR *E. coli* – establish themselves in host niches. Alongside comparative analysis of isolates from other host species, these directions for future research may aid the identification of novel approaches or targets to mitigate the emergence, persistence, and dissemination of AMR.

Taken together, this dissertation advances understanding of the distribution and risk factors for AMR, factors which may drive its development, and the underlying genomic profiles of these bacterial populations on the farm-to-fork interface. These findings highlight the importance of an integrated approach to addressing AMR and can be used to support future AMR monitoring and control strategies.