EXPLORING THE ECOLOGY OF *SALMONELLA* WITHIN COHORTS OF BEEF CATTLE

A Dissertation

by

GIZEM LEVENT

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

DOCTOR OF PHILOSOPHY

Chair of Committee,	H. Morgan Scott
Committee Members,	Keri N. Norman
	Sara D. Lawhon
	Robin C. Anderson
Head of Department,	Ramesh Vemulapalli

December 2019

Major Subject: Biomedical Sciences

ABSTRACT

Salmonella is a leading bacterial pathogen, causing a significant number of human infections and deaths every year in the United States. Recently, the increase in the prevalence of ceftriaxone and azithromycin resistance among human *Salmonella* isolates became a serious public health threat since both are used for the empirical treatment of salmonellosis. Analogs of these antibiotics (ceftiofur and tulathromycin, respectively) are widely used in beef cattle, which could be contributing to this problem, since beef products are one of several major sources of *Salmonella* outbreaks. A randomized controlled longitudinal field trial was designed to determine the effects of single-doses of ceftiofur and tulathromycin metaphylactic treatment on *Salmonella* prevalence, quantity and serotype distribution among cattle feces, sub-iliac lymph nodes, and hide samples. Beef cattle (n = 134) were divided 4 blocks consisting of three pens each. One pen in each block received either ceftiofur, tulathromycin, or else no antibiotic (i.e., negative control group) on Day 0. Feces (during the feeding period and at slaughter), sub-iliac lymph nodes and hide swabs (at slaughter) were collected from each animal, during periods before and after the treatment. *Salmonella* was isolated, quantified and tested for phenotypic antibiotic resistance using standard methods. Serotypes, sequence types, antibiotic resistance genes, and plasmids of *Salmonella* isolates were determined from whole-genome sequencing data. Phylogenetic analyses were performed to measure evolutionary distances between *Salmonella* comparing pens, source, days, sample types across the study period.

Data analyses indicated no significant effects ($P > 0.05$) of metaphylactic antibiotic treatments on the prevalence and quantity of *Salmonella*; however, there was a significant

($P < 0.05$) day (period) effect observed in both measures of *Salmonella* occurrence, increasing significantly from early spring through mid-summer. *Salmonella* isolates were mostly pan-susceptible and this was not affected by the antibiotic treatment. Serotypes found in cattle samples strongly clustered within pens and dynamically shifted their dominance over time; importantly, suggesting a strong interaction of this pathogen with the local ambient cattle pen environment. Further analyses are needed to understand the environmentally related dynamics of *Salmonella* originating from cattle.

DEDICATION

Yaptığım bu doktora çalışmasını, sevgi dolu, bana her zaman güvenen ve destek olan hayatımındaki en önemli iki varlığa annem Sevcan Levent ve babam Kadir Levent'e armağan ediyorum. Ve tabi ki bir de küçük arkadaşım Shiva'ya.

I dedicate this degree to my loving, supportive, and beautiful family, to my mother Sevcan Levent and my father Kadir Levent. And of course, to my little friend Shiva.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Morgan Scott for sharing his expertise with me on understanding the art of epidemiology and developing ways to think out of the box. He was also an educator for me who significantly contributed to my scientific and personal development. I would also like to acknowledge Gurhan Ciftcioglu for his encouragement to pursue my degree in the United States. I am also grateful to Robin Anderson who trusted me from the beginning until the end of this journey. Special thanks to Roger. Harvey for his effort to help me to find my career path, Michael Hume for giving me his enlightening speeches during the times I needed the most. Keri Norman and Sara Lawhon significantly increased my motivation during this study and provided continuous guidance and help with their positive attitudes. I would like to thank to Javi for not only being an expert and training me in the laboratory but also being a memorable friend and very special thanks to Roberta for her bear hugs. Exclusive thanks to the people that I value in my life that never let me be alone during these years Nagehan, Puren, Hank, Tolga, Jack, Ben, and Safak (SU).

Finally, I acknowledge my luck to have such a beautiful mother and father; I always felt their love and trust from 10,695 km distance every single day of my life. None of these would be possible without their support and encouragement.

In closing, I would like to thank that one lone vulture, out riding the thermals somewhere over southern Utah, for putting it all in perspective for me.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supported by the dissertation committee consisting of the chair, Dr. H. Morgan Scott from the Department of Veterinary Pathobiology, and the committee members, Dr. Keri N. Norman from the Department of Veterinary Integrative Biosciences, Dr. Sara D. Lawhon from the Department of Veterinary Pathobiology, and Dr. Robin C. Anderson (United States Department of Agriculture, Agricultural Research Service, College Station, TX).

The grant proposal of this research, entitled "*Prevalence, quantity and antibiotic resistance profiles of Salmonella enterica at slaughter in response to antibiotic use early in the cattle feeding period*", was submitted by the principal investigator of the project, H. Morgan Scott with the help of other investigators as follows: Dr. Keri N. Norman, Dr. Sara D. Lawhon, Dr. Samuel E. Ives (Department of Agricultural Sciences, West Texas A&M University), and Dr. Guy H. Loneragan (School of Veterinary Medicine, Texas Tech University).

The field study of this research was conducted in the experimental feedlot of West Texas A&M University located in Canyon, TX. Dr. Samuel E. Ives and his Master of Science student Ashlynn Schlochtermeier, MS (Department of Agricultural Sciences, West Texas A&M University) both contributed to cattle management in the experimental feedlot, sampling individual animals and shipping samples from West Texas A&M University to our laboratory in College Station, TX. In addition, West Texas A&M University, Department of Agricultural Sciences students Samantha Foos, Rebecca

Perrish, Dexter Tomczak, Antonio Adame, and Matt Heckel also contributed to the sample collection process.

Dr. Javier Vinasco Torres contributed to my laboratory base training. Roberta Pugh, MS, contributed to this research by ordering the materials that were needed for the research. Along with them, the graduate and undergraduate students that are members of our laboratory contributed by offering their help for extensive sample processing.

Dr. Henk Den Bakker (Department of Food Science and Technology, University of Georgia, Athens, GA) also contributed to this study by sharing his expertise in SNP analysis conducted for individual serotypes.

The instructors of the computational molecular evolution training at the Wellcome Genome Campus of Sanger Institute located in Cambridge, UK contributed this research by sharing their knowledge with me during the training.

Dr. Jing Wu contributed to this research by providing her assistance to use the MALDI-TOF device in the Clinical Microbiology Laboratory of the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University. Dr. Roger Harvey contributed to this research by providing the Sensititre® reader located in his laboratory located at USDA/ARS College Station, TX. Dr. Tom Edrington (Diamond V, Cedar Rapids, IA) also contributed to this study by providing his scientific recommendations on lymph node and hide sample processing.

Michael Dickens from the HPRC, Texas A&M university contributed to this research by providing help to troubleshoot the bioinformatic-related problems and also uploading the requested software to ADA Intel x86-64 Linux cluster. University Writing

Center of Texas A&M University contributed by providing the assistance with their students for editing of this dissertation. Finally, Texas A&M University provided me a high-quality research and education environment during my studies and research.

All other work conducted for the dissertation was completed by the student independently.

Funding Sources

This study was funded by The National Cattlemen's Beef Association: a contractor to the Beef Checkoff (Contract # 22615).

I was supported by a Texas A&M University Graduate Research Assistantship funded from H. Morgan Scott's start-up package. Texas A&M University awarded me the Lechner Scholarship for the 2016 Spring semester.

Additionally, the Graduate and Professional Student Council and the Office of Graduate and Professional Studies, Texas A&M University awarded me the Aggies Commit Fellowship in 2019, and the College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, awarded me the Graduate Student Advanced Development Training Initiative Award in 2019. Both awards financially contributed me to attend the computational molecular evolution training at the Wellcome Genome Campus.

I would like to also acknowledge the Graduate Student Association of the College of Veterinary Medicine, Texas A&M University for providing me two travel awards in 2018 and 2019, to be able to present my research in Chiang Mai, Thailand in 2018 and Chicago, IL in 2019.

Finally, I would like to also thank the Association for Veterinary Epidemiology and Preventive Medicine for selecting my first Ph.D. manuscript as the best manuscript in the field in 2019 and awarding me the Mark Gearhart Memorial Graduate Student Award.

NOMENCLATURE

ACSSuT	Resistance to ampicillin, chloramphenicol, streptomycin, sulfisoxazole, tetracycline
ACSSuTAuCx	Resistance to ampicillin, chloramphenicol, streptomycin, sulfisoxazole, tetracycline, amoxicillin-clavulanic acid, ceftriaxone
AIC	Akaike's information criterion
APHIS	Animal and Plant Health Inspection Service (USDA)
AR	Antibiotic-resistant
ARG	Antibiotic resistance gene
ARG-ANNOT	Antibiotic Resistance Gene Annotation
ASC	Ascertainment bias correction
ASSuT	Resistance to ampicillin, streptomycin, sulfisoxazole, tetracycline
AST	Antimicrobial susceptibility testing
BGA	Brilliant green agar
BIC	Bayesian information criterion
BIFSCo	Beef Industry Food Safety Council
BLAST	Basic local alignment search tool
bp	Base-pair
BPW	Buffered peptone water
BRD	Bovine Respiratory Disease
BWA	Burrow-Wheeler aligner
CARD	Comprehensive Antibiotic Resistance Database
CCFA	Ceftiofur as ceftiofur crystalline-free acid
CDC	Centers for Disease Control and Prevention

CFU	Colony forming unit
CHURDLE	Cragg's hurdle
CI	Confidence interval
CLSI	Clinical Laboratory Standards Institute
Da	Dalton
df	Degrees of freedom
ERS	Economic Research Service
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FASTA	Assembled sequence file
FASTQ	Raw sequence reads
FDA	The Food and Drug Administration
FoodNet	Foodborne Diseases Active Surveillance Network (CDC)
FSIS	Food Safety and Inspection Service (USDA)
G	Gamma parameter
GTR	Generalized time reversible
HACCP	Hazard analysis and critical control point
HIV	Human immunodeficiency virus
HPRC	High-performance research computer
ICC	Intra-class correlation coefficient
LLQ	Lowest limit of quantification
MALDI	Matrix-assisted laser desorption ionization
MDR	Multidrug-resistant (resistant to ≥ 3 classes of antibiotics)
MIC	Minimum inhibitory concentration

MLST	Multi-locus sequence typing
MPN	Most probable number
MS	Mass spectrometry
NAHMS	National Animal Health Monitoring System (USDA)
NARMS	National Antimicrobial Resistance Monitoring System
NCBI	National Center for Biotechnology Information
NIH	National Institutes of Health
NORS	National Outbreak Reporting System
OTU	Operational taxonomic unit
PATRIC	Pathosystems Resource Integration Center
PCR	Polymerase-chain reaction
PFGE	Pulsed-field gel electrophoresis
PHASTER	Phage Search Tool Enhanced Release
pMLST	Plasmid multi-locus sequence typing
RV	Rappaport-Vassiliadis R10
SISTR	*Salmonella in silico* Typing Resource
SNP	Single-nucleotide polymorphism
ST	Sequence type
Std. Dev.	Standard deviation
Std. Err.	Standard error
TOF	Time of flight
TSB	Tryptic soy broth
TT	Tetrathionate

USDA	United States Department of Agriculture
VCF	Variant Call format
WGS	Whole-genome sequencing
WHO	World Health Organization
XMFA	Extended multi-fasta files
ZINB	Zero-inflated negative binomial
ZIP	Zero-inflated Poisson

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION

1.1. Overview of *Salmonella*

The genus *Salmonella* was first discovered by Karl Joseph Eberth in 1880, then identified by Theobald Smith and named by Daniel Elmer Salmon in 1886 [1, 2]. *Salmonella* is a gram-negative, generally motile through the flagella, non-lactose fermenting, gas-producing, 2-3 μm in length, and facultative anaerobic bacillus genera found in the Enterobacteriaceae family. *Salmonella* is a persistent microorganism that can grow in temperature ranges from 6 to 48°C with optimal growth observed between 32-37 °C. The optimal pH-level required for *Salmonella* is between 6.5-7.5 [2, 3]. The genus *Salmonella* is divided into two main species that consist of 6 subgenera (I-VI), these are based on DNA differences observed in 16S rRNA sequences. When these differences were further analyzed by a DNA-DNA hybridization method, those subgenera were subgrouped as follows: *Salmonella enterica* (*I, II, III, IV,* and *VI*) and *Salmonella bongori* (V) [4].

Furthermore, these subgenera are divided into serotypes that currently result in more than 2,600 identified serotypes of the genus *Salmonella*. Within the same DNA-DNA hybridization study, these serotypes reported showing more than 80% genetic similarity. A minor portion of these serotypes (n = 23) belong to the *Salmonella bongori* species, and the remaining serotypes are members of the *Salmonella enterica* species. *S. enterica* is further subdivided into six subspecies based on their DNA, biochemistry, and serologic reaction differences (Figure 1).

These subspecies (with given subgenera numbers in parentheses) are listed as follows: *S. enterica* subspecies *enterica* (I), *S. enterica* subspecies *salamae* (II), *S. enterica* subspecies *arizonae* (IIIa), *S. enterica* subspecies *diarizonae* (IIIb), S. *enterica* subspecies *houtenea* (IV), and *S. enterica* subspecies *indica* (VI) [5].

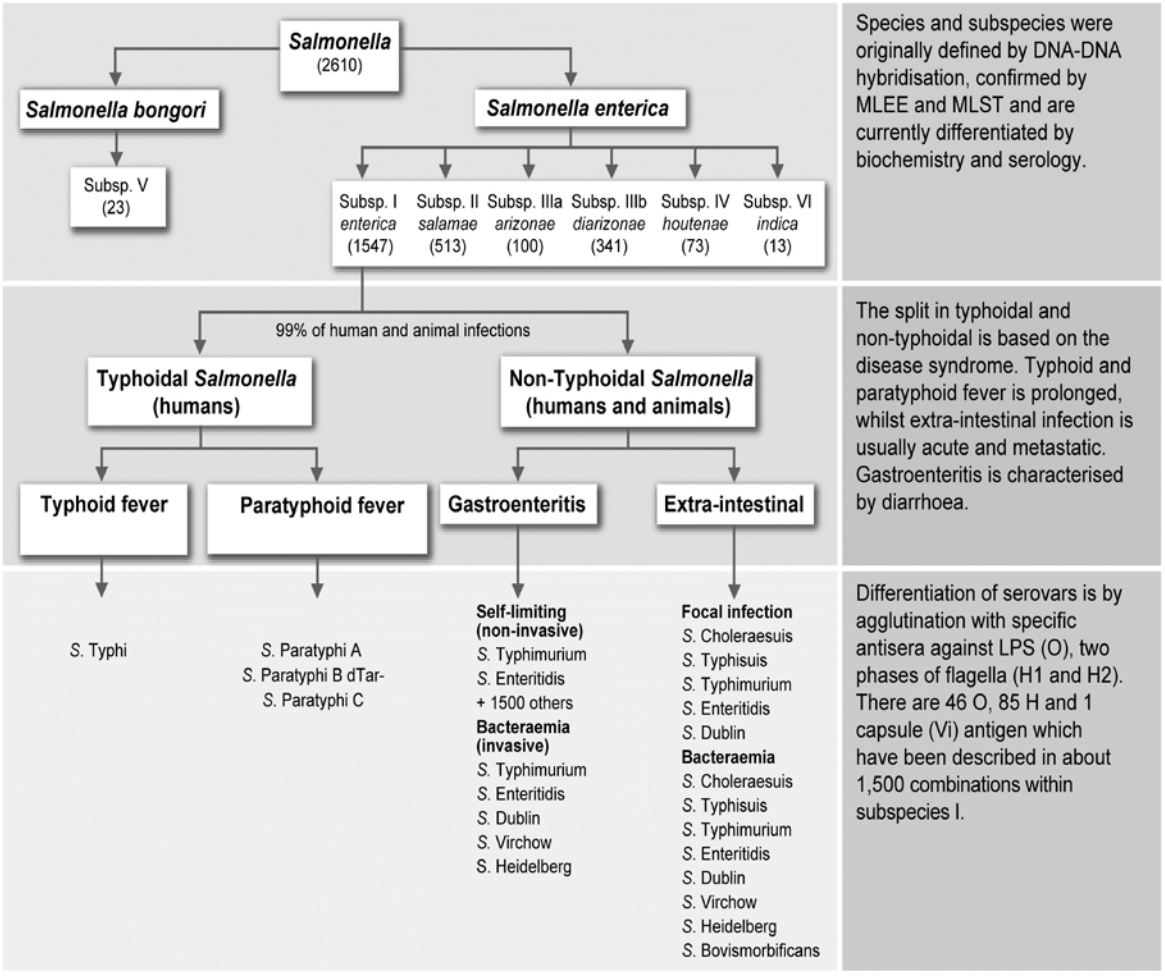


Figure 1. Overview of the *Salmonella* subspecies and serotypes

Adapted from Achtman et al. (2012) [5].

1.2. Clinical significance

S. enterica subsp. *enterica* is the largest subspecies within the *Salmonella* genus, consisting of 1,547 serotypes. The majority of these serotypes (99%) have the ability to infect or proliferate in warm-blooded animals, whereas the remainder of the subspecies usually cause infections and proliferate in cold-blooded animals [6]. *Salmonella* species are highly adapted microorganisms that can be also found in various niches of the ambient and built environment [6]. *Salmonella* serotypes that cause infections both in humans and animals are further divided into two groups according to their disease-specific syndromes.

Four *S. enterica* serotypes (*S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi B dTar-, and *S.* Paratyphi C) cause typhoid and paratyphoid fever only in humans; therefore, members of this group are also called "typhoidal *Salmonella*". The remaining serotypes belong to the "non-typhoidal *Salmonella*" group that may cause either symptoms of gastroenteritis that may be self-limiting, or can lead to invasive (bacteremia) or extra-intestinal symptoms (Figure 1). These symptoms can be followed by local infections and more diffuse bacteremia and septicemia. More than 1,500 serotypes belong to the non-invasive group that mostly cause self-limiting symptoms, such as diarrhea, abdominal cramps, and nausea in both humans and animals [5].

1.3. Research background

Each year in the United States, approximately 9.4 million foodborne illnesses occur due to exposure to foodborne pathogens [7]. *Salmonella enterica* subsp. *enterica* (hereafter referred to as simply *Salmonella* or non-typhoidal *Salmonella*) is one of the leading foodborne pathogens that is estimated to cause 1.2 million infections, 23,000

hospitalizations, and 450 deaths every year in the United States [7, 8]. According to the U.S. Centers for Disease Control and Prevention (CDC) National Outbreak Reporting System (NORS), a total of 1,833 *Salmonella* related outbreaks were calculated to have occurred in the United States between 2009 and 2017. Of these outbreaks, 1,186 (64.7%) were attributed to the consumption of contaminated food products while 35.3% of cases were attributed to direct animal contact, environmental sources, person-to-person contact, or various unknown reasons (publicly accessible national outbreak data are available at NORS dashboard [9]).

Most of the food-related clinical *Salmonella* infections are attributed – in order of importance – to ingestion of contaminated seeded vegetables, eggs, poultry, and beef products, respectively [10, 11]. Beef products are considered as one of the major sources of the *Salmonella* outbreaks, since the majority of *Salmonella* infections are attributed to a few important serotypes that mainly found in cattle [12, 13]. Equally important, non-meat sources of outbreaks are inevitably traced by to contamination by fecal matter from animals; very often, from food producing animals such as cattle, pigs, and poultry or else from wildlife.

Cattle are usually exposed to *Salmonella* in feedlots via the ingestion of contaminated feed and water [14]. *Salmonella* becomes a persistent problem in the feedlots because cattle that are exposed to *Salmonella* shed the microorganism through their fecal waste to the feedlot environment [2]. *Salmonella* also can be found in the digestive tract and the lymph nodes of healthy cattle [15, 16]. Hide and fecal origin *Salmonella* may be introduced to cattle carcasses at slaughter during the skinning process or via direct fecal contamination, respectively [17-19]. Carcass contamination may

subsequently lead to dissemination through the food chain and remain in or on the final beef products. In addition to fecal and hide origin *Salmonella*, cattle lymph nodes harboring *Salmonella* have also been reported as a potential contamination source for ground meat products. Cattle lymph nodes are embedded in fat tissue; later, this trim product contributes extensively to the fat content in ground beef products. During the grinding process, lymph nodes harboring *Salmonella* can be incorporated into the ground meat products [15, 19, 20].

Salmonella mostly causes self-limiting infections in adults, characterized by mild gastrointestinal symptoms, such as diarrhea, abdominal cramps, and nausea. These infections are often limited to the intestinal lumen and do not require antibiotic treatments. However, children 5 years of age or younger, adults 65 years of age or older, and adults with impaired immunity are high-risk groups to develop invasive *Salmonella* infections that can migrate from the intestinal lumen to the bloodstream, the lymphatic system, and other body sites. These infections – often requiring antibiotic treatment along with hospitalization – are characterized by positive blood culture and bacteremia, and include acute symptoms such as severe and bloody diarrhea, fever, and septicemic shock. The failure of the antibiotic treatment can result in patient death [21, 22]. Antibiotics used for the treatment of the human invasive (extra-intestinal) *Salmonella* infections include ceftriaxone (a 3rd generation cephalosporin), ciprofloxacin (a fluoroquinolone), and azithromycin (a macrolide) [22, 23]. Among these treatments, ceftriaxone and azithromycin are often the primary choices for empirical therapy of pediatric, obstetric, and recently also for adult cases of salmonellosis due to some adverse side effects of fluoroquinolone use [24-26].

Antimicrobial resistance among human *Salmonella* isolates became a global concern due to the observed increase of cephalosporin- and fluoroquinolone-resistance resulting in the failure of these antibiotics to treat human *Salmonella* infections [8, 27, 28]. In addition to clinically defined resistance, reduced susceptibility to fluoroquinolones (e.g., ciprofloxacin) – mainly induced by a plasmid-mediated resistance has also become an emerging problem [29, 30]. Based on the CDC Antibiotic Resistance Threat Report published in 2013, multidrug-resistant (MDR) *Salmonella* was classified as one of the serious-level public health threats causing 100,000 infections and 40 deaths annually in the United States [8]. According to this report, antibiotic-resistant (AR) *Salmonella* that show a pattern of resistance to at least one antibiotic is recovered from an estimates 100,000 patients annually. Of these recovered isolates, 66,000 are found to be resistant to at least five classes of antibiotics. Many of these AR *Salmonella* strain-related outbreaks were attributed to certain *Salmonella* serotypes such as *Salmonella* Enteritidis, followed by *Salmonella* Typhimurium, and *Salmonella* Newport serotypes [31].

In recent years, the CDC's concern about the increasing number of AR human *Salmonella* isolates was mainly focused on increasing ceftriaxone and ciprofloxacin resistance, both of which are the antibiotics that are used to treat human *Salmonella* infections (Figure 2). The CDC underlined the increases observed of the levels of ceftriaxone and ciprofloxacin resistance found in human *Salmonella* isolates between 1996 and 2015. According to this report, AR trends of *Salmonella* show levels of ceftriaxone resistance found in human *Salmonella* isolates gradually increased from 0.2% to 4.4% from 1996 to 2003. These levels have not dropped under the level of 2.4% from 2003 until 2017. Similarly, the number of isolates resistant to ciprofloxacin increased from below

0.5% to 3% by the year 2011 [8]. In addition to resistance, reduced susceptibility to these antibiotics was also increasingly observed in *Salmonella* isolates [32].

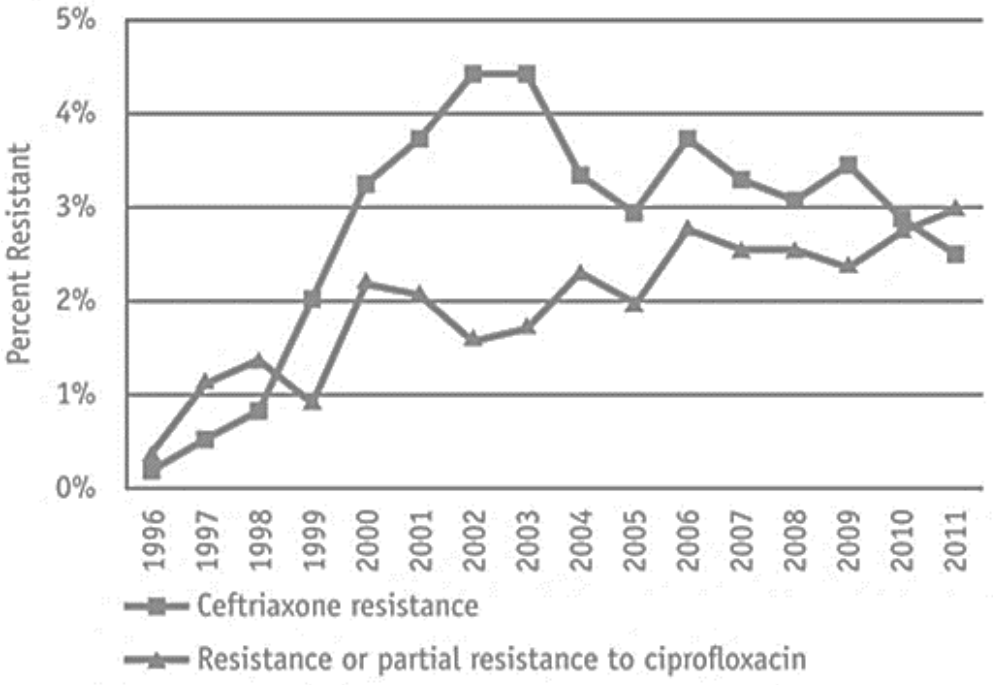


Figure 2. Ceftriaxone- and ciprofloxacin-resistant human *Salmonella* isolates from 1996-2011

Adapted from the CDC Antibiotic Resistance Threat Report [8].

The National Antimicrobial Resistance Monitoring System (NARMS) began monitor azithromycin resistance among human *Salmonella* isolates in 2011. One of the recent NARMS Human Isolates Surveillance Report published in 2018 also highlighted the increasing prevalence of azithromycin resistance in human *Salmonella* isolates (Figure 3). Although resistance to azithromycin is currently at low levels, the trends observed [32] for azithromycin resistance in human *Salmonella* isolates has increased from 0.0% to 0.2%

from 2012 to 2013 and then dropped to 0.0% by 2014. This level reached the highest resistance level (0.3%) in 2015 [32].

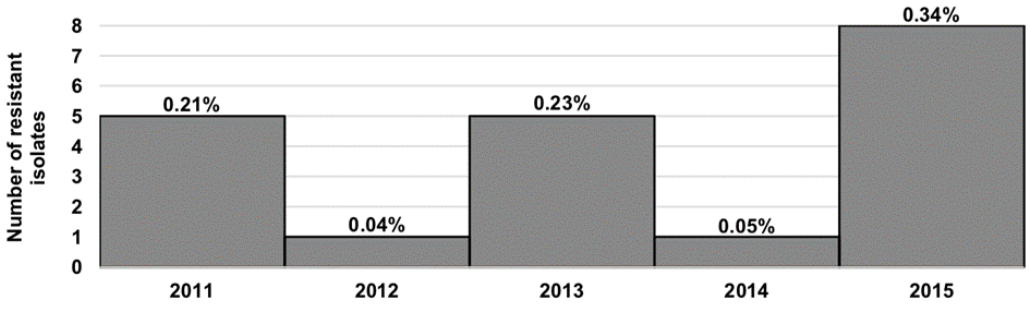


Figure 3. Azithromycin-resistant human *Salmonella* isolates from 2011-2015

Adapted from the NARMS Human Isolates Surveillance Report, 2018 [32].

Antibiotics are widely used in beef cattle for disease treatment, metaphylaxis (disease control), and prophylaxis (disease prevention) purposes. Metaphylaxis is commonly used to control infectious bacterial diseases in cattle herds during the onset and early stages of an outbreak. One of the major challenges of the cattle feedlot industry is bovine respiratory disease (BRD; also known as shipping fever) caused by pulmonary pathogens and stress factors that emerge upon arrival after transportation from the cow-calf/backgrounder/stocker industries or auction markets to the feedlots. BRD is the most common health problem of beef cattle causing both high morbidity and mortality[33]. BRD is estimated to affect 16.2% of cattle arriving to U.S. feedlots. More than half (60%) of the U.S. cattle population receive metaphylaxis to reduce the incidence of BRD in feedlots [34]. Following the initial metaphylactic treatments for BRD, cattle are not generally exposed to herd-level metaphylactic treatments during the subsequent feeding

period that may take three to ten months until the cattle reach the desired slaughter weight [35].

It has been also documented that antibiotics that are used for BRD in beef cattle for any therapeutic purpose (i.e. treatment, control, prevention) may significantly or transiently increase the number of AR bacteria among enteric populations recovered from cattle [36-45]. There are also other studies showing lesser effects of antibiotic use in enteric bacterial populations [46-51].

Ceftiofur crystalline-free acid (CCFA; also referred herein simply as ceftiofur) and tulathromycin are two antibiotics that are approved and commonly used to control BRD in cattle herds [34, 52, 53]. Ceftiofur (an analog of ceftriaxone) – as crystalline-free acid – is a long-acting 3rd generation cephalosporin formulation that was approved to treat BRD infections in both dairy and beef cattle in 2003; in addition, its label extends to the metaphylaxis of BRD in beef cattle. Tulathromycin (an azalide subclass of macrolide) is an analog of azithromycin that is also in the same subclass and was approved for use in beef cattle for BRD in 2005. Both ceftriaxone and azithromycin belong to two of five antibiotic classes that are listed as the highest priority critically important antimicrobials for human medicine by the World Health Organization (WHO) [54].

Beginning with the fact that BRD antibiotics can select for bacterial resistance, it is crucial to determine the selection effects of ceftiofur and tulathromycin on the resistant bacterial population in food-producing animals; that is, selection pressure that may increase the risk of human exposure to ceftriaxone- and azithromycin-resistant *Salmonella*.

1.4. Research significance

Salmonella is one of the most important bacterial foodborne pathogens, causing a significant number of human infections and deaths every year in the United States. Cases of salmonellosis attributed to MDR *Salmonella* infections may not be responsive to antibiotic treatment. Macrolides and 3rd generation cephalosporins play a critical role in the treatment of human *Salmonella* infections. Trends show increasing numbers of ceftriaxone- and azithromycin-resistant *Salmonella* recovered from human isolates. Ceftiofur and tulathromycin are analogs of these antibiotics, both of which are widely used in cattle herds to control BRD infections. Since antibiotic use in beef cattle may result in the selection of resistant bacterial populations in cattle, studies aiming to measure the direct effects of ceftiofur and tulathromycin use for BRD metaphylaxis in cattle are necessary.

Even though there are several epidemiologic observational and case-control studies existing in the literature aimed at understanding associations between antibiotic use and selection pressure for AR *Salmonella* in cattle, longitudinal randomized and controlled field trials are superior to these study designs; that is, they provide direct measurement of the effects of the antibiotic treatments on the bacterial population distributions of susceptible and resistant strains by controlling the environmental- and the host-related factors impacting the ecology – and consequently affecting the epidemiology – of antibiotic resistance. There are only a few longitudinal randomized controlled cattle trials that have measured the effects of these antibiotics on *E. coli* (an organism that is widely accepted as one of the indicator microorganism for AR profiles of gram-negative bacteria [37-40]), *Salmonella* [36], and metagenomic populations [41, 46, 47, 51] in beef cattle.

While a few of these studies reported a significant or transient effect of the antibiotic treatment on the resistance of enteric bacteria in cattle [36-41], the remaining studies tended to report little or no effects on these populations following antibiotic administration [46, 47, 51]. Among those, only Ohta et.al (2017) examined the direct effects of ceftiofur treatment in beef cattle on enteric *Salmonella* populations [36]. There are no existing studies in the literature measuring the effects of tulathromycin treatment on cattle *Salmonella* populations. The existing studies measuring the effects of tulathromycin on enteric bacterial populations in cattle were limited to metagenomic populations [46, 51]. These studies listed above explored the dynamics of the effects of these treatments for a maximum 28 days after the metaphylaxis; therefore, the potential public health risks of the treatments remained unknown until the slaughter age (90+ days). In addition, the focus of all these randomized controlled field trials was restricted to fecal samples. Therefore, no information was provided for the lymph node and hide origin *Salmonella* populations following the treatments.

The significance of this study was to utilize an epidemiological approach to fill two research gaps in the literature in order to understand the potential public health risks of ceftiofur and tulathromycin administration on the *Salmonella* populations in feedlot cattle. The first need was to determine the long (90+ days) term effects of these antibiotics at slaughter after they were administered early in the feeding period. The second need was to measure the effects of these antibiotics not only in fecal samples but also in the lymph nodes and on the hides, each of which can be a potential source of beef contamination at slaughter. For this study, sampling the feces throughout the feeding period is important to monitor time-related *Salmonella* population changes observed within the animals.

A main focus of this study was examining specifically the sub-iliac lymph nodes that are in the flank region of cattle. The extensive fat tissue mass surrounding the lymph node means these are more likely to be introduced into ground beef products than with other lymph nodes. To understand the pen/environment-related contributions to the *Salmonella* population, we also aimed to examine hide swabs collected from the brisket area of the cattle that were less likely to reflect animal-animal contamination. This region is most likely to reflect the environmental *Salmonella* carriage because of continuous contact of the brisket region with the pen floors during periods of rest.

Filling these gaps provides us an accurate and realistic approach to understanding the potential risk factors related to the consumption of AR *Salmonella* contaminated beef products obtained from the beef cattle that received BRD metaphylaxis upon arrival to the feedlot (at an early stage of feeding. In addition to filling the gaps listed above, this study also provides the serotype and phenotypic distribution of the *Salmonella* populations found in the cattle, since serotype and antibiotic resistance profiles tend to ne highly correlated in *Salmonella* populations.

1.5. Research objectives

The main objective of this study was to determine the effects of ceftiofur and tulathromycin treatments on the population dynamics of *Salmonella* in feedlot cattle from early in the feeding period until slaughter. The specific aims of the study were as follows:

1. To compare the prevalence, quantity, and phenotypic antibiotic resistance of *Salmonella* from feces collected before and after antibiotic treatments throughout the cattle feeding period, and to provide these comparisons among the feces, lymph nodes, and hides at slaughter age.
2. To evaluate the population structure and serotype distribution of *Salmonella* across different sample types, days, and treatments.

2. LITERATURE REVIEW

2.1. Cattle origin *Salmonella*

Salmonella in cattle are considered a serious food safety and animal health issue that cause significant negative impacts to the feedlot industry [55]. Cattle are usually exposed to *Salmonella* through ingestion of contaminated feed and water [14, 56]. *Salmonella*-infected cattle became carriers and shed *Salmonella* through their feces to the feedlot environment where *Salmonella* can became a persistent problem [2]. The risk factors for developing *Salmonella* infections in cattle include the number of cattle in the herd, feed and water quality, levels of pasture/pen contamination, fertilizer contamination, duration in pen, the density of *Salmonella* shedding of pen mates, and excessive numbers of rodents or wild animals on farms [2, 55].

The majority of *Salmonella* serotypes can reside in the intestinal tract of healthy cattle without causing disease in their host. However, a mostly cattle-specific serotype (*S.* Dublin, which also became associated with human infections recently) can result in acute (clinical) infections and other non-specific serotypes (e.g., *S.* Typhimurium, *S.* Enteritidis, *S.* Newport, and *S.* Cerro) can result in acute and sub-acute clinical infections in cattle [57]. Cattle infections caused by the *S.* Dublin serotype can be epidemic and highly contagious in dairy cattle herds resulting in high morbidity. On the other hand, *S.* Typhimurium outbreaks can be more sporadic but also with high mortality [58]. In recent years, *S.* Newport and *S.* Cerro serotypes also have been associated with *Salmonella* outbreaks in dairy cattle [59, 60].

The prognosis of salmonellosis in cattle depends on host immunity, the virulence factors of the serotype, and the dose *Salmonella* exposure [2]. Clinical symptoms of

Salmonella infections in cattle usually appear 5-7 days after the exposure and symptoms can vary from fever, depression, severe diarrhea (watery or bloody), abortion, respiratory disease (specifically, in calves) to septicemia and death [2, 55, 61]. On the other hand, sub-clinical infections usually progress without apparent clinical signs, but may cause local infections in cattle lymph nodes, spleen, or liver. These animals become latent carriers or intermittent shedders and consequently shed *Salmonella* through their feces after the initial infection and for a long-time period [2, 61].

Other *Salmonella* serotypes that are simply members of the commensal gastrointestinal microbiota of cattle are ubiquitous. These serotypes, that generally are not pathogenic to cattle, can also proliferate in the feedlot environment. However, these serotypes may be pathogenic for humans, causing gastrointestinal tract infections [62, 63]. Therefore, the ecology and epidemiology of *Salmonella* in cattle and their production environments needs to be better understood to assess the possible public health risks of *Salmonella* contamination in beef products and other cattle-related sources. In the literature, there are various detection, identification, and quantification methods available to evaluate the ecology and epidemiology of *Salmonella* in cattle feces, lymph nodes, and on hides, and in the cattle environment.

2.1.1. Detection of *Salmonella*

Salmonella isolation from various sample types, such as feces, tissues, soil, feedstuffs, food, and other environmental samples may take up to 5-7 days using various microbiological and molecular techniques and methods [3, 64]. The combination of both selective and non-selective enrichment media has been widely suggested to isolate

Salmonella from samples that harbor various bacterial species in the microbiota such as fecal, lymph node, and hide samples [3, 65]. Non-selective pre-enrichment broth, such as buffered peptone water (BPW) or tryptic soy broth (TSB), provide the main nutrients and chemicals – such as casein, peptone, sodium chloride, phosphate, glucose, and water – that are required for *Salmonella* growth, and are usually utilized as the initial step to increase the total number of target bacteria – including *Salmonella* – found in a sample. Following non-selective enrichment, a *Salmonella*-specific (selective) enrichment broth usually is utilized to increase the numbers of *Salmonella*. These specific enrichment broths, such as tetrathionate (TT), Rappaport-Vassiliadis R10 (RV), and selenite cystine, provide a selective media environment for *Salmonella* by inhibiting other microorganisms found in the sample. The majority of these selective features of *Salmonella* specific agars are combined with the compounds that *Salmonella* is naturally more resistant to than other members of Enterobacteriaceae. These compounds include – but are not limited to – selenite, tertigol, bile salts, iodine, brilliant green, and malachite green [2, 66]. The selectively enriched suspensions are further inoculated on a selective agar plate, such as xylose lysine deoxycholate agar, Hektoen enteric agar, bismuth sulfite agar, and brilliant green agar (BGA) to distinguish *Salmonella* from other microorganisms that can survive throughout these passages, and are further selected based on their standard colony morphologies [64, 67-69]. To increase the specificity of *Salmonella* isolation and eliminate false-positive *Salmonella* results, combining the pre-enrichment, specific enrichment broth and selective agar is highly recommended. Even though performing these methods can be laborious, and can take up to 5-7 days, using both non-specific and specific enrichment

media processed in the aforementioned sequence is accepted as the gold standard for highly sensitive and specific *Salmonella* detection and determination [3, 64].

Even though these combinations are generally enough to isolate *Salmonella*, they are often not successful in eliminating *Citrobacter spp.* and *Proteus spp*. that are commonly found in the environments that *Salmonella* inhabit; most often, this is due to similarities observed among the morphologies and/or indicative biochemical reactions. Essentially, selective enrichment media are often not enough to eliminate such microorganisms from samples for which the aim is to isolate *Salmonella.* For example, *Salmonella* is known to be naturally resistant to novobiocin up to a minimum inhibitory concentration (MIC) of 2,400 µg/ml; on the other hand, *Proteus spp*. are susceptible and inhibited at concentrations between 25-400 µg/ml [70]. Therefore, a selective medium combined with ≥ 25 µg/ml novobiocin (i.e., TT- novobiocin, RV- novobiocin, BGA-novobiocin) may be preferred due to its ability to suppresses the growth of *Proteus spp.* and to reduce *Citrobacter spp*. growth by up to 50% [16, 71, 72].

The conventional culturing methods listed above are standard methods to detect *Salmonella* in samples of animal origin. However, it should be noted that the sensitivity and specificity limits of non-selective and selective enrichment methods, and the effects of incubation parameters for detection or isolation of *Salmonella*, have been studied by few researchers [65, 73]. Significant ($P < 0.05$) differences in performance of five different culture methods used to isolate *Salmonella* in swine feces have previously been reported [65]. According to that study, initial selection by tetrathionate broth, followed by RV broth, was found to be superior to using only one single specific enrichment step. These methods later were also tested for the potential selection bias for different *Salmonella*

serotypes [74-76]. For example, adding novobiocin to the enrichment broth and then following this with inoculation of the suspension onto BGA plates improved the probability of selecting *S*. Dublin versus using an XLD plate [76], and when compared to the other methods. Gorski et al. (2012) [74] tested if certain media enrichments would favor certain serotypes or serogroups. They tested 10 serotypes that belong to 4 serogroups (B, C1, C2, and E) by using either TSB enrichment, TSB enrichment followed by RV, or else RV containing soy peptone broth. Among all methods tested, they found that mainly serogroup C2 and E isolates were selected compared to the remaining serogroups; of concern, serogroup B was detected at the lowest numbers.

One of the limitations of these microbiological methods is that they only provide the opportunity to detect *Salmonella* at the species or subspecies-level. Therefore, for the further discernment and identification of *Salmonella,* additional biochemical, serological, and molecular techniques are required.

2.1.2. Identification and characterization of *Salmonella*

Several traditional biochemical reaction tests are used for the initial identification of *Salmonella*. The principle of these biochemical tests is typically based on an active enzymatic reaction catalyzed for a biochemical compound that helps to differentiate *Salmonella* at the subspecies-level, except for a few serotypes. These biochemical reaction tests include, but are not limited to, glucose fermentation, negative urease reaction, hydrogen sulfide (H_2S) production, and negative indole tests [3]. For example, non-typhoidal *Salmonella* can ferment lactose, whereas the remaining subspecies cannot. On the other hand, because a large number of *Salmonella* serotypes require no growth factor

(thus, they can grow in a simple medium that contains glucose and ammonium ions), they are not easily distinguishable using biochemical reaction tests. However, several host-specific serotypes (e.g., *S.* Typhi, *S.* Gallinarum, and *S.* Abortusovis) require certain growth factors that are associated with host-specificity, and these can be distinguished by their biochemical reactions. For instance, *S.* Typhi A fails to produce H_2S; on the other hand, other non-typhoidal *Salmonella* serotypes can produce H_2S to decarboxylate the lysine [2].

In addition to these biochemical methods, whole-cell matrix-assisted laser desorption ionization (MALDI), time of flight (TOF) mass spectrometry (MS) has become an alternative novel cost-effective technology commonly used for rapid identification of ribosomal protein compositions (ribosomal fingerprints) of *Salmonella* species and subspecies [77, 78]. This method can successfully discriminate the soluble proteins at the mass range of 2000-20,000 Da (Dalton); that is, those usually belonging to the ribosomal proteins in a bacterial cell. The principle of this method is based on the desorption of bacterial cells on a matrix surface by nitrogen laser beams and then measuring the flight time of the ionized proteins by a spectrometer. The protein patterns of the molecules are recorded in a format calculated by the mass (m) amount divided by the electrical molecule charge (z). Later, the m/z result is compared with the main spectra protein database and presented as an output with confidence scores. This method produces highly accurate, rapid, cost-effective and reliable results and has been widely used in many clinical laboratories in the world since 2008 [79].

2.1.2.1. Identification of subtypes

The majority of *Salmonella* outbreaks have been found to be subtype dependent [12]. Therefore, identification of *Salmonella* at the subtype-level plays an important role in the investigation and understanding of the possible source and distribution of an outbreak. There are numerous subtyping methods that have been developed, both at culture- and molecular-level. The subtyping methods of *Salmonella* include, but are not limited to: serotyping, bacteriophage typing, pulsed-field gel electrophoresis (PFGE), and multi-locus sequence typing (MLST) [80].

Salmonella serotyping is one of the oldest subtyping methods and is based on serologic cell component characteristics of *Salmonella*. In recent years, *Salmonella* serotyping has gained importance due to fact that the majority of human *Salmonella* outbreak cases are usually traced back to several (<100) known human *Salmonella* serotypes [12, 62, 63]. There are several serological and molecular techniques available for *Salmonella* serotyping.

The serum slide-agglutination test is one of the traditional serological tests used for *Salmonella* identification at the serotype-level. The principle of the slide-agglutination test is based on agglutination reactions observed to occur between the polyvalent antisera and *Salmonella* specific antigenic components using commercial antiserum kits. These components consist of 1) bacterial surface (somatic O), 2) flagellar (H1 and H2), and 3) capsular (Vi) antigens. All members of *Salmonella* serotypes express somatic and at least one flagellar antigen. However, the capsular antigen is only expressed by *S.* Typhi, *S.* Paratyphi C, and *S.* Dublin serotypes. The antigenic formulae of *Salmonella* serotypes were initially defined by Kauffman-White and new antigenic formulae of recently

identified serotypes are updated by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France [81, 82]. This test (now named by some as: Kauffman-White-Le Minor) is widely used and accepted as a gold standard for preliminary identification of *Salmonella* species, subspecies, and serotypes [3]. However, the results of the test are heavily dependent on the decision of the observer, and thus can vary within and among laboratories. Furthermore, obtaining the specific antisera for more than 2,600 *Salmonella* serotypes and testing the isolate of interest against each antiserum is laborious, time-consuming, and costly for researchers.

There is also a molecular approach targeting the genes encoding the same specific antigenic cellular components in non-typhoidal *Salmonella* using whole-genome sequencing (WGS) and bioinformatic tools [83]. The serotypes of *Salmonella* are identified by the genomic variations observed in these components. Currently, there are two standardized, reliable, and generally accepted *in silico* tools available for *Salmonella* serotyping using high-throughput sequencing data: *Salmonella in silico* Typing Resource (SISTR) [84] and SeqSero [85]. Both tools determine *wzx* and *wzy* genes and *rfb* gene clusters, which are specific for somatic (O) antigens, as well as *fli*C and *flj*B gene alleles, which are specific for flagellar (H1 and H2, respectively) phases. These tools scan and determine the unique gene sequences in WGS data that are matched in certain *Salmonella* serotype classifications recognized in the Kauffmann-White-LeMinor Scheme [81]. A study comparing SeqSero with traditional serotyping found that the traditional serotyping method failed to identify 36 isolates where they were all identified using SeqSero. However, SeqSero was also reported to provide incorrect calling of antigenic determinants when serotypes were identical [83]. Another study showed that SeqSero is a superior tool

to SISTR due to providing an opportunity to determine the serotypes from the raw sequencing reads, rather than relying on assemblies [83]. Even though using *in silico* tools has several advantageous over traditional serotyping methods, one of the major deficiencies is the fact that these tools are fully dependent on the prior-defined sequences already existing in the serotype database. Therefore, it is highly important to identify serotypes using the most updated databases and using traditional methods for unidentified strains in order to be able to recognize newly emerging strains.

It has been shown that serotyping *Salmonella* is not enough when various homology or lineage differences can be observed within a single-serotype. For example, outbreak data collected from *S.* Enteritidis strains showed that traditional molecular serotyping methods were not enough to determine the lineages observed within these serotype-specific strains found in different locations and settings [86]. Therefore, other high-resolution subtyping methods have also gained importance to fully identify the lineages and homology characteristics of an outbreak strain.

One of the most common and traditional molecular identification methods is the PFGE. PFGE detects the genetic characteristics (DNA fingerprints) of bacterial isolates using restriction enzymes (endonucleases) to cut DNA into large fragments; later, these fragments are placed into a gel and exposed to an electrical field that is constantly changing voltage and direction. The electrical field separates the DNA fragments according to their sizes and creates strain-specific DNA patterns that can be compared. PFGE has highly standardized protocols that are widely accepted and applied in broad-spectrum pathogens; perhaps surprisingly, the results are comparable both domestically and internationally. Even though PFGE has good specificity for many pathogenic bacteria,

it is highly dependent on the choice of restriction enzymes (e.g., *xba*I, *Bln*I, *Spe*I) used, conditions of the electrophoresis gel, and the skills of the technician. It has been shown that PFGE also has low sensitivity for differentiating some *Salmonella* subtypes because of those varieties and lineages that have been observed within serotype-level that are not detectable with these restriction enzymes [80]. Up until 2014, this method was accepted as the gold standard by the U.S. national reference laboratory at the CDC (e.g., PulseNet) for bacterial molecular pathogen typing due to its high reliability, reproducibility, and accuracy.

Due to extensive labor and other limitations of the PFGE method, and the comparatively reduced costs of WGS methods (when factoring in other data that are provided), PulseNet and the national reference laboratories started to use WGS data for *Salmonella* typing since 2014. The WGS approach has not only replaced PFGE by providing higher accuracy and reproducibility, but also is preferred due to providing a wealth of genomic data about other genetic characteristics; these include – but are not limited to, antibiotic resistance genes (ARGs), genomic islands, virulence genes, non-synonymous and regulatory effectors (e.g., single-nucleotide polymorphic regions), and plasmids. WGS also provides an opportunity to perform genotypic comparisons to analyze the phylogenetic relationships across/among different species, subspecies, and subtypes. WGS data obtained from researchers are deposited in online databases, which gives scientists around the world the opportunity to obtain WGS data from various domestic and international laboratories and to perform comparisons [87]. The WGS method is proven to provide more robust phylogenetic inferences with a high epidemiological correlation for outbreaks when compared to PFGE and other subtyping methods [86]. Recently,

numerous WGS bioinformatics tools have been developed to perform *in silico* analyses of *Salmonella* using WGS data [5, 84, 85, 87].

MLST is one of the molecular subtyping methods using bacterial WGS data that was first proposed as a replacement for non-typhoidal *Salmonella* serotyping by Achtman et al. (2012) [5]. This method detects the SNP (single-nucleotide polymorphism) variations in the genomic sequences and gene clusters based on the similarities observed in DNA sequences; furthermore, these sequences are matched with previously identified MLST gene groups deposited in the MLST database to provide information on subtype groups [5]. The gene sequences that are utilized through MLST are based on the level of genes such as: ribosomal (rMLST), core genome (cgMLST), whole-genome (wgMLST), and legacy MLST. Legacy MLST is based on a concept that determines SNPs in the housekeeping genes, which are found to be specific for each sequence type (ST). For the *Salmonella* legacy MLST analysis, there are seven housekeeping gene fragments used: 1) *aro*C (chorismate synthase), 2) *dna*N (DNA polymerase III, β-subunit), 3) *hem*D (uroporphyrinogen III synthase), 4) *his*D (histidinal dehydrogenase), 5) *pur*E (phosphoribosylaminoimidazole carboxylase), 6) *suc*A (2-oxoglutarate dehydrogenase decarboxylase), and 7) *thr*A (aspartokinase I) [5]. The ST types are determined based on unique sequence variants found in the non-typhoidal *Salmonella* MLST database curated from public databases for molecular typing and microbial genome diversity platform (PubMLST; www.pubmlst.org).

Even though protein-level *Salmonella* typing at species- and subspecies- level has been explored by MALDI-TOF MS, this method was not successful in identifying *Salmonella* at the serotype-level without biomarkers due to protein similarities of serotypes

belonging to the same subspecies groups [77]. However, a study conducted by Dieckmann et al. (2011) showed that MALDI-TOF MS can successfully identify the five most common human *Salmonella* serotypes (*S.* Enteritidis, *S.* Typhimurium, *S.* Virchow, *S.* Hadar, and *S.* Infantis) observed in European countries by using the biomarkers for unique proteins prior to the MALDI-TOF MS process [78]. Serotyping using MALDI-TOF MS may well become a promising approach in the future by developing unique spectra databases, biomarkers, and different measurement parameters for the identification of an entire group of *Salmonella* serotypes.

2.1.2.2. Characterization of antibiotic resistance profiles

One of the most important aspects of *Salmonella* characterization for public health is determining the ARG traits at microbiological (phenotypic) and molecular (genotypic) levels. Phenotypic antimicrobial susceptibility testing (AST) can be determined based on colony growth observed under different concentrations of antibiotic-containing media. E-tests, disc-diffusion, agar dilution, and the broth (both macro- and micro-) dilution methods are traditional and widely used methods for the identification and characterization of phenotypic antibiotic resistance [88]. While disc diffusion method only can provide binary-coded data (resistant or susceptible), other methods can provide quantitative, semi-quantitative, or categorical data that are related to the MIC of an antibiotic; that is, by testing the growth of the target microorganisms under various concentrations of different antibiotics and antibiotic combinations.

Among those methods, the broth microdilution method is widely used in clinical microbiology laboratories due to time- and cost-effective features of the available

customized plates [89]. The results of phenotypic antibiotic resistance tests are mainly interpreted by the guidelines established by two major organizations: the Clinical Laboratory Standards Institute (CLSI) located in the United States [90] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) located in Europe [91]. One of the major differences among these organizations is the additional clinical interpretation classification of "intermediate resistance" that is defined in CLSI guidelines but not in EUCAST guidelines. This class refers to the MIC values that fall in the range between the MIC value of susceptible and resistant. Besides this difference, the way of each organization interpretations of MIC values for classification of resistance is also different. For example, the EUCAST uses the epidemiologic cut-off values, which are determined by the difference between the MICs of the wild-type strain (that are sensitive to the antibiotic tested) and those strains with acquired resistance. On the other hand, the CLSI uses clinically interpreted cut-off values for resistance that are based on the likely treatment success of the antibiotic for a given strain in the clinics. This incorporates both microbiological, pharmacological, and physiological parameters. These differences may result in slightly different interpretations of the antibiotic resistance for any tested microorganism and antibiotic [92].

In addition to these AST methods, using molecular techniques, the determination of ARGs in bacteria is also important to understand the molecular insights of antibiotic resistance mechanisms and their relation to phenotypic resistance [93].

Molecular methods to identify the ARGs are utilized either using standard PCR (polymerase-chain reaction) methods, which are usually performed with designed ARG specific primers [94], or using DNA-based microarray method with a known bacterial

species [95]. The ARGs can also be determined by using next-generation sequencing methods along with the bioinformatic tools applied on to WGS data that rely on comparing gene sequences against given ARG nucleotide or protein sequence databases [96]. Recently, WGS has become a cost-effective and robust method that is preferred to detect tall he ARGs from bacterial genomes. In addition to the ARGs, this method provides a large amount of other bacterial WGS related information [93, 97]. The most commonly used ARG databases include, but are not limited to, ResFinder [98], the Comprehensive Antibiotic Resistance Database (CARD) [99], and the Antibiotic Resistance Gene-Annotation (ARG-ANNOT) database [100].

Salmonella can acquire antibiotic resistance by chromosomal mutations that can cause rapid changes in bacterial populations to reduce the susceptibility to an antibiotic that originally either limits the growth of the bacterial cell or else destroy the bacterial cells. However, viable chromosomal mutations are considered as rare events in *Salmonella* [101, 102]. Another acquired resistance mechanism in *Salmonella* is through horizontal gene transfer that occurs via plasmids, bacteriophages, and transposons [103]. Conjugation events that are orchestrated by the plasmids are the most common horizontal transfer events in *Salmonella* and can result in the transfer of the up to seven or more antibiotic resistance elements at one time [104]. These transfers are often coordinated by a mobile DNA element that can be harbored in a plasmid located in the bacterial cell, or else or can be inserted into the bacterial chromosome as genomic islands, integrons, and insertion elements [105, 106]. Moreover, these resistance plasmids can be shared inter- and intra-species within and outside the family of Enterobacteriaceae [107].

Therefore, understanding the plasmidal relations of the ARGs in *Salmonella* provides insights into the epidemiology of AR *Salmonella* and their related sources [108]. Besides the ARG encoding plasmids, there are also different types of plasmids identified in *Salmonella*. These plasmids can either harbor virulence genes that are specific for *Salmonella* subspecies or serotypes, or else harbor the biological function genes to reduce the fitness cost of those genes for the bacterial DNA [109]. The plasmidal profiles of bacterial strains can be determined by a bioinformatic tool called PlasmidFinder. It matches the WGS data with a nucleotide-based plasmid database curated from PubMLST using plasmid MLST (pMLST) consisting of previously identified plasmidal DNA sequences [110].

Even though ARGs provide more accurate genetic information, from a public health perspective phenotypic resistance is still the most important outcome of the antibiotic resistance and is required to be determined along with the ARG information from the bacterial isolates. Currently, prediction of phenotypic resistance using WGS data became a popular and important research subject pursued by some utilizing machine learning [111]. The focus of such an approach is to develop phenotypic antibiotic resistance predictions accurately and to understand the patterns of antibiotic gene expressions using a large data set that contains both WGS data and isolate-specific MIC values [97, 112]. Even though this method provides an “idealistic” approach to identify the features of antibiotic resistance, the necessity of microbiological methods remains intact with respect to staying abreast of evolutionary aspects of bacteria.

2.1.3. Quantification of *Salmonella*

Quantification of *Salmonella* is important to determine for estimating the potential risks of *Salmonella* shedding in the feedlots and the potential risks of causing *Salmonella* infections in humans through carcass and environmental contamination. Therefore, *Salmonella* quantification in cattle feces, which is the primary source of shedding in the feedlots has been evaluated by researchers [69, 113]. Specifically, quantification of *Salmonella* in the feces, lymph nodes and on hide surfaces is important to better estimate the risks of human *Salmonella* infections that are proven to be related to the dose of exposure. The dose of human infection for *Salmonella* is reported to vary from $<10^2$ to 10^{10} [114], though it is ethically nearly impossible to update these risk estimates in the present day.

Determining the estimated quantity of *Salmonella* colonies in samples is often not simple and is inaccurate due to the detection or quantification (low- or high-) limits of the techniques, only a subset of a sample being tested, complex background microbiota, and uneven distribution of *Salmonella* in the samples. There are numerous cultural or molecular methods available for *Salmonella* enumeration from cattle sources [15, 67, 113, 115-118]. The most probable number (MPN, the results are expressed as MPN per weight or area of the sample) and viable plate count (the results are expressed as colony-forming units [CFU] per weight or area of the sample) are the two commonly used microbiological methods to quantify cattle origin *Salmonella* from feces [69, 113], lymph nodes [15, 118], hide [67, 69]. The direct plating method is one of the traditional culture-based *Salmonella* enumeration methods, which is based on serially diluting and plating the various *Salmonella* concentrations on selective agar plates to obtain *Salmonella* quantities per mass

or area by back-calculating the starting concentration, the direct plating method can be useful for the quantification of low concentrations of *Salmonella* in samples [119]. However, direct plating is a time- and material-consuming laborious method to obtain CFU counts from a high number of samples, and it is highly dependent on the pipetting accuracy of the technician. An automated spiral plating technique [119] provides the opportunity to spread the multiple dilutions of a bacterial inoculum on a single-agar plate. After the incubation of these plates, an automated plate reader also can be utilized to obtain the CFU counts of *Salmonella*. Using the spiral plater and plate reader together reduces time, cost and errors related to the human factors. This method is also commonly used to quantify CFUs of *Salmonella* from feces, lymph nodes and hides [117, 120, 121].

In addition to culture-based methods, a quantitative PCR based (qPCR) method can also be utilized by quantifying *Salmonella*-specific genes such as members of the *inv*/spa gene complex with specific primers [15, 67, 122]. Combining the PCR method with a selective pre-enrichment process can also decrease the lower limits of quantification (LLQ) of *Salmonella* when the density of *Salmonella* in the samples is expected to be low or variable in the samples. This additional pre-enrichment step also provides necessary adaptation time for cells that might be stressed or damaged during transport and storage prior to the PCR-based quantification methods [115].

However, the qPCR method is also laborious and can detect *Salmonella* at the gene-level, which also has limits of quantification. In addition, this method can determine the *Salmonella* quantity based on the quantity of total DNA, which fails to reflect the number and proportion of viable bacteria in the samples [115].

2.2. *Salmonella* in cattle and feedlots

The fecal *Salmonella* population in cattle depends on various environmental (i.e., season, farm management, and quantity of *Salmonella*) and cattle- related (i.e., behavior, source of cattle, *Salmonella* shedding density and frequency, and age) factors [123-125]. Cattle shed *Salmonella* to the pen environment through their fecal waste and consequently can contaminate other cattle housed in the same pen or feedlot [126]. This results in the contamination of cattle hide surfaces due to the direct contact with the pen floors. Cattle hides can also be contaminated with pen- or environment-origin *Salmonella* during dust events or due to aerosolization of pen-floor material by hoof action [127]. Moreover, cattle behaviors (e.g., grooming, licking the farm equipment) can also cause repeating contamination between the environment and the digestive tract of the cattle. Therefore, *Salmonella* in the feedlots builds and can become a persistent opportunistic pathogen over time.

Carcass-level contamination via hides can directly occur during hide skinning and the evisceration processes or indirectly occur via contact with the slaughterhouse environment or equipment at the harvesting stage [19, 128, 129]. In addition to fecal and hide origin *Salmonella*, lymph node origin *Salmonella* may also be incorporated into ground beef products during the fat/lean trimming events in food-processing facilities [20, 130, 131]. Cattle lymph nodes are embedded in fat tissue that is highly valued for improving the fat content of ground beef products. Since these lymph nodes are relatively small in size compared to the fat tissue that surrounds them, they can be difficult to identify and eliminate during the trimming events. Specifically, the massive fat tissues located in the chuck and flank regions of the cattle that are surrounding the pre-scapular

(superficial cervical) and prefemoral (sub-iliac) lymph nodes are considered as being of high-risk for incorporation into these beef products [20]. It has been shown that even healthy cattle can have *Salmonella* in their lymph nodes [132]. *Salmonella* in lymph nodes have also been found to be associated with host status, the route of transmission, inoculum amount, and serotype [121, 133-135]. In addition to cattle origin, the lairage environment was also reported as a possible origin of *Salmonella* contamination [122].

Clearly, the cattle feces, lymph nodes, and hides are commonly perceived as the primary contaminants of cattle origin *Salmonella* found on carcass surfaces and in beef products. Therefore, to estimate the public health risks of cattle origin *Salmonella*, there have been numerous studies conducted to determine the prevalence, quantity, serotype, and antibiotic resistance profile distribution of *Salmonella* in ready-to-harvest healthy beef cattle [15, 68, 69, 132]. These studies have been mainly observational studies conducted to analyze the possible risk factors (e.g., geographical, feedlot related, and seasonal) of fecal, lymph nodes and hide origin *Salmonella* populations in cattle presented for slaughter.

Vikram et al. (2017) collected fecal samples from cattle raised conventionally (n= 360) and without (n = 359) antibiotics from large slaughter plants located throughout the United States from February 2014 to January 2015 [136]. Overall, *Salmonella* prevalence was estimated at 13.8%, which did not statistically differ ($P = 0.42$) between cattle raised without antibiotics versus conventionally. However, the *Salmonella* prevalence in these cattle was significantly ($P < 0.01$) higher in summer (38.3%) when compared to fall (9.4%), winter (5.6%), and spring (1.7%) months. *Salmonella* prevalence in the feces of these cattle started to increase in May 2014 and peaked in July 2014. The researchers only

tested *Salmonella* isolates for phenotypic resistance to a 3rd generation cephalosporin (cefotaxime) and to nalidixic acid. Among all *Salmonella* isolates, only one isolate showed phenotypic cefotaxime resistance and no isolate was resistant to nalidixic acid, suggesting no reduced susceptibility was observed against ciprofloxacin. They also analyzed the abundance of ARGs among the conventionally and raised-without-antibiotic cattle samples. They found a significant increase ($P < 0.01$) in tetracycline, aminoglycoside, and macrolide resistance genes among conventionally raised cattle samples when compared to the samples from cattle raised without antibiotics. The ARGs encoding for 3rd generation cephalosporins were at low-abundance and did not differ between the two groups. However, in their study the antibiotic use information was not available for the cattle that were raised conventionally; this lack of differentiating information needs to be carefully considered before deriving any conclusions from this study about the selection effects of antibiotic use on antibiotic resistance observed in beef cattle feces.

A cross-sectional year-long observational study conducted by Kunze et al. (2008) involved collecting hide swabs (n = 1,081) from the perineal region of cattle at four slaughterhouses along with fecal samples (n = 600) from pen floors of six feedlots holding harvest-ready cattle in the southern United States [69]. Within this study, *Salmonella* prevalence and quantity, along with serotype and phenotypic antibiotic resistance profile of isolates, from hide and feces were determined. In their study, the *Salmonella* prevalence was reported higher in hide (69.6%) compared to fecal samples (30.3%). Furthermore, they also analyzed seasonal effects on the prevalence but did not report any significant difference ($P = 0.39$) among winter, spring, and summer. *Salmonella* CFUs were reported

as 1.73 $\log_{10}$/100 cm^2 for hide and 0.71 $\log_{10}$/g for feces. The serotype identification of 762 isolates recovered from this study showed that the most common serotypes isolated from hide and feces were *S.* Anatum (22.9% and 32.5%, respectively), *S.* Montevideo (22.9% and 19.6%, respectively), and *S.* Mbandaka (10.0% and 14.7%, respectively). *S.* Cerro (14.9%) was more prevalent on the hides than in the feces (4.3%), whereas *S.* Kentucky was more prevalent in the feces (16.0%) than in the hide samples (5.7%). The majority of *Salmonella* isolates were pan-susceptible (51.1%) or else singly resistant (33.1%) to either tetracycline or sulfisoxazole. The ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline) and the MDR-AmpC phenotypes were almost exclusively observed in 73.7% and 66.7% of *S.* Reading isolates, respectively. These isolates were recovered from 4.9% of the feces and 1.8% of the hide samples. In their research, a strong serotype and phenotypic resistance association were reported.

Gragg et al. (2013) also investigated the within animal diversity of *Salmonella* from feces, hides (over the foreshank region), and four different types of lymph nodes (mandibular, mesenteric, mediastinal, and sub-iliac) of 68 feedlot cattle at a slaughterhouse in Mexico [68]. Their study was conducted in early fall and the fecal and hide *Salmonella* prevalences were reported as 94.1% and 100%, respectively. The highest *Salmonella* prevalence found in lymph nodes was in mesenteric lymph nodes (91.2%) followed by sub-iliac lymph nodes (76.5%). Among 91 isolates, the most prevalent serotypes were *S.* Kentucky (15/91), *S.* Anatum (26/91), and *S.* Reading (15/91). In addition to these serotypes, *S.* Meleagridis, *S.* Cerro, *S.* Muenster, *S.* Give, and *S.* Mbandaka were also identified. They also reported four *Salmonella* isolates that remained as unidentified

serotypes. According to their results, certain serotypes may be better adapted to either lymph nodes or hides rather than feces illustrating that *S*. Meleagridis was more likely to be recovered from lymph nodes than hide and feces. In contrast, *S*. Kentucky was more likely to be recovered from feces and hides than from lymph nodes. Approximately 60.0% of isolates were susceptible to all antibiotics tested. Tetracycline resistance was observed in 22.0% of the isolates. MDR was observed for 13.2% of the isolates. The overall results are somewhat confounded by the fact that *Salmonella* isolation methods used for lymph nodes versus hides and feces were different, which may favor some serotypes over others (and as the authors stated in their conclusion). In contrast to Kunze et al. (2008), phenotypic antibiotic resistance was not fully associated with certain serotypes; rather, AMR phenotypes were associated with certain PFGE subtypes suggesting the feedlot origin of these cattle may have been in different regions and with different antibiotic use history. However, this information was not provided. It is also not known if there were any cluster effects of cattle in these data. Furthermore, the sampling frequency also was not provided to eliminate any effects of sampling period factors on the *Salmonella* population. Therefore, given the relatively small sample size (n = 68), conclusions from these data need to be carefully derived.

The same study group of Gragg et al. (2013) conducted another observational cross-sectional study to assess seasonal and regional effects on *Salmonella* prevalence, quantity, and phenotypic antibiotic resistance profiles in bovine sub-iliac lymph nodes at harvest [15]. The samples in this study (n = 3,327) were collected from slaughter plants in three different regions (i.e., southern, western, and northern United States) and during three different seasons (fall, winter/spring, and summer/fall) between 2010 and 2011. A

significantly higher (P = 0.0304) *Salmonella* prevalence was observed in summer/fall months compared to winter/spring. Also, *Salmonella* prevalence was significantly higher (P = 0.0198) among cattle in slaughterhouses located in the southern United States when compared to more northern locations. *Salmonella* enumeration from the subset (33.0%) of *Salmonella*-contaminated lymph nodes revealed CFU values ranging from 1.9 to 3.8 $\log_{10}$ CFU per gram of lymph node tissue. Serotyping results of the isolates showed that the major serotypes were *S*. Montevideo (44.0%) and *S*. Anatum (24.8%) among 24 serotypes recovered from the total of 266 tested isolates. Again, in their study, the majority of *Salmonella* isolates were found to be pan-susceptible (86.1%); on the other hand, 8.3% of *Salmonella* isolates were resistant to at least three classes of antibiotics. The MDR- AmpC resistance profile was mostly serotype dependent, and was observed in *S*. Reading, *S*. Newport, *S*. Dublin, and *S*. Typhimurium serotypes. Since this study had a much larger sample size and footprint compared to the previous study (10) and focused on different geographical plants in the United States with more extensive sampling (i.e., 76 lymph nodes per day with two days for each season), several potential confounding factors were likely to be eliminated. However, as the authors also stated in their conclusion, 11 of the 13 *S*. Reading serotypes in this study were from a single sample collection day (and thus, a single plant) in the summer months. This result shows a temporal and likely geographical clustering effect observed within their findings.

Another cross-sectional study was recently published by Webb et al. in 2017, evaluating *Salmonella* prevalence in the sub-iliac lymph nodes of healthy cattle at harvest collected during a one-year period from three different regions (i.e., western, southern, and midwestern) in the United States [132]. In their study, lymph nodes (n = 5,450) were

collected from 12 commercial slaughterhouses [132] and *Salmonella* were recovered from 5.3% of the total (289/5,450). The authors also reported an increase in prevalence in summer/fall (from June through October) months and in the southern when compared to the western and midwestern regions of the United States. Among positive samples, *Salmonella* CFUs ranged from 1.6 (limit of detection) to 4.9 $\log_{10}$ CFU per gram of lymph node. *S.* Montevideo (26.9%), *S.* Lille (14.9%), *S.* Cerro (13.0%), and *S.* Anatum (12.8%) were found to be the most common four serotypes out of 22 serotypes identified in 376 (i.e., with multiple isolates recovered from a subset of lymph nodes) *Salmonella*. Again, many of these isolates were pan-susceptible (80.6%) or else resistant to two or more antibiotics (10.7%). *S.* Dublin isolates recovered from this study were mostly (24/26) resistant to four or more classes of antibiotics. Tetracycline, sulfisoxazole, streptomycin, and chloramphenicol were the most commonly observed antibiotic resistance phenotypes among *Salmonella* isolates. In this study, the sample size may be sufficient to support a conclusion based on seasonal and regional differences, which were also forced into their statistical models as fixed effects, along with their interaction terms. However, the seasonal differences observed in such distant geographical locations and the unknown spatial distance within and between facilities also need to be further evaluated.

Estimating the nationwide *Salmonella* prevalence in feedlots is a challenging task due to a large number of feedlots and the wide distribution of these feedlots in various geographical locations throughout the United States. In addition, the point prevalences obtained from different sampling seasons show confounding effects on reported the *Salmonella* populations. Moreover, even though these three studies above (two in the U.S, one on Mexico) utilized standard *Salmonella* detection methods, the variations in these

methods can yield a bias when comparing results among laboratories [65]. Therefore, conducting a study, either a long-term study that can last for few years, or else collecting large nationwide data within a single-year, it is ideal to utilize a single-method to explore the actual locational and seasonal dynamics of *Salmonella* population among cattle and feedlots.

The only up-to-date nationwide *Salmonella* prevalence survey was conducted by USDA (United States Department of Agriculture) -APHIS (Animal and Plant Health Inspection Service) and published in 2014 [137]. This survey was conducted based on samples collected from 12 U.S. states, which accounted for over 95.0% of the cattle inventory in large feedlots during the year of the survey. From 68 large feedlots, three pens were randomly selected for *Salmonella* detection. In total, 5,050 individual samples were collected from 202 pen floors (25 samples from each pen). Their findings showed that 60.3% of the 68 feedlots had one or more samples that tested positive for *Salmonella.* The most common serotypes that were found in 50.4% of *Salmonella* recovered from those feedlots were *S.* Anatum (18.0%), *S.* Montevideo (17.2%), and *S.* Kentucky (15.2%). Phenotypic antibiotic resistance of these isolates was also tested against 14 antibiotics (amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim/sulfamethoxazole). Most *Salmonella* isolates were found to be pan-susceptible (74.6%) and the remaining resistance was observed as mostly singly resistance (15.9%). Of these singly resistant isolates, the majority of isolates showed single tetracycline resistance (21.4%), followed by sulfisoxazole resistance (13.1%).

These results showed similar prevalence patterns to the previously conducted USDA-APHIS survey conducted between 1999 and 2000 [138]. In that survey, samples 10,417 samples were collected from 73 feedlots from 11 States over the course of one year. *Salmonella* prevalence in different seasons and serotypes was determined. The highest prevalence was observed in summer (11.4%), followed by spring (6.8%), autumn (4.0%) and winter (2.5%) based on a total of 654 *Salmonella* positive isolates. The top five most common serotypes found in the earlier survey were: *S.* Anatum, *S.* Montevideo, *S.* Reading, *S.* Newport, and *S.* Kentucky.

Haneklaus et al. (2012) examined the effects of feedlot source on *Salmonella* prevalence in bovine lymph nodes recovered from cattle from seven different feedlots in the Southern United States [16]. In their study, two types of lymph nodes (279 superficial cervical and 28 iliofemoral) were collected from healthy cattle at harvest. The total *Salmonella* prevalence for both types of lymph nodes was found to be dramatically different among feedlots ranging from 0.0% to 88.2%, thereby, showing that feedlots are one of the key factors affecting the ecology and epidemiology of *Salmonella* found in the lymph nodes of cattle [16].

The observational studies and surveys listed above showed that *Salmonella* in cattle at slaughter age show a wide range of diversity depending on sampling from different feedlots, sample types and season. The key findings suggest that *Salmonella* prevalences during the summer were observed at higher levels when compared to other seasons. The prevalence on hides was generally higher than in the feces and lymph nodes. Most of the cattle origin *Salmonella* were pan-susceptible (74-80%) or else singly resistant to either tetracycline or sulfisoxazole. Major cattle origin *Salmonella* serotypes were *S.* Anatum, *S.*

Montevideo, *S*. Mbandaka, *S*. Cerro, and *S*. Kentucky. The MDR profile was often serotype-specific and typically found in *S*. Reading isolates, though also other serotypes across different regions. In addition to the cattle/feedlot origin *Salmonella*, it has been also reported that the hide surfaces can also be cross-contaminated with *Salmonella* during transportation to slaughter, further complicating our understanding of the original source of contamination at slaughter [122, 139].

2.3. *Salmonella* in slaughterhouses

Each year in the United States 30-33 million cattle are slaughtered in commercial slaughterhouses [140]. In order to reduce pathogen carriage in farm animals and carcasses at slaughterhouse and meat processing facilities, HACCP (Hazard Analysis and Critical Control Point) standards have been enforced in slaughterhouse and meat processing facilities by the USDA-Food Safety and Inspection Service (FSIS) since 1996 in the United States [141]. *Salmonella* has often been found at high prevalence on carcass surfaces and meat products by the Standard *Salmonella* surveys conducted by FSIS agency of the USDA [142]. To meet with the HACCP standards, slaughterhouse facilities often utilize carcass interventions such as physical and/or chemical applications at either post-hide-removal or pre-chill stages. The post-hide removal interventions include, but are not limited to: 1) carcass washing with cold, warm, or hot water, 2) chlorine wash, 3) caustic soda wash, 4) bacteriophage application, 5) de-hairing, and 6) lactic, acetic or peroxyacetic acid washes. The interventions applied to beef carcasses at the pre-chill stage include, but are not limited to: 1) trimming, 2) hot water wash, 3) steam water wash, 4) lactic acid wash and 5) peroxyacetic acid [143].

The latest Beef-Veal Carcass Baseline Survey published by FSIS in 2016 evaluated 2,736 post-hide removal and pre-chilled swabs of beef carcasses from August 2014 to December 2015. Post-hide removal swabs were collected when the carcass was de-hided and initial interventions were applied. Pre-chill swabs were collected after the last interventions applied (closer to the consumer) before the carcass was placed into the coolers. This survey, conducted by The Nationwide Microbiological Baseline Data Collection Program, showed that *Salmonella* prevalence was 27.1% (371/1,368) on the post-hide carcass and decreased to 3.3% (46/1,368) on the same carcasses after the pre-chill stage [143]. The same survey also identified 46 different *Salmonella* serotypes on the post-hide carcasses and this number decreased to 20 serotypes on pre-chilled carcasses. The predominant serotypes observed were as follows: *S.* Montevideo (21.5%), *S.* Anatum (15.9%), and *S.* Cerro (10.7%) were the three most commonly isolated serotypes from post-hide removal carcasses, while *S.* Montevideo (17.3%), *S.* Muenchen (10.8%), and monophasic variant of *Salmonella* Typhimurium and *S.* Agona (both at 8.7%) were more prevalent among *Salmonella* positive pre-chill carcasses (Table 1).

Studies conducted by Beach et al. (2002) [144] and Arthur et al. (2008) [122] also examined the potential contribution of cattle feces, hide and environment at slaughter. Both studies suggested environmental factors during transport and slaughterhouse contributed to the observed high-levels of *Salmonella* prevalence.

Beach et al. (2002) examined the *Salmonella* prevalence, serotype, and antibiotic resistance profiles recovered from rectal swabs, hide swabs of pre- and post-transit cattle, and carcass swabs, as well as environmental swabs collected from the transport vehicle [144]. In their study, a total of 100 feedlot cattle that were ready to transport to a

slaughterhouse were sampled before and after transportation (approximately 15 miles) from the same feedlot to the same slaughterhouse.

Table 1. Serotype frequency and diversity recovered from carcass surfaces at post-hide removal and pre-chill stages by FSIS in 2016

Serotypes	Post-hide removal	Pre-chill
Montevideo	80	8
Anatum	59	0
Cerro	40	0
Muenster	18	0
Muenchen	17	5
Agona	11	4
I 4,[5],12:i:	0	4
Give	0	3
Infantis	15	3
Newport	13	0
Meleagridis	8	0
Typhimurium	0	3
Derby	0	2
Kentucky	19	2
Uganda	0	2
Other	90	10

Source: The Nationwide Microbiological Baseline Data Collection Program: Beef-Veal Carcass Survey August 2014-December 2015 FSIS databases [143].

Their study reported a significant ($P < 0.05$) *Salmonella* prevalence increase in hide samples immediately before (19.8%) and after (52.2%) transportation, which they reported as related to the transport vehicle. Follow-up research conducted by the same authors examined the rectal, hide, carcass and environmental samples obtained during the previous study [145] and showed that *Salmonella* prevalence was highest in environmental samples (47.4%), followed by hide (37.5%), carcass (19.0%), and fecal (4.0%) samples with an overall prevalence of 26.7% (281/1,050). A subset (n = 120) of these isolates were further characterized for serotype identification and determination of antibiotic resistance profiles.

Among these, *S.* Anatum (18.3%), *S.* Kentucky (17.5%), *S.* Montevideo (9.2%), *S.* Senftenberg (8.3%), and *S.* Mbandaka (7.5%) were the most commonly identified *Salmonella* serotypes. In the study, 78.3% of the isolates recovered from feedlot cattle were recorded as pan-susceptible or else singly resistant to tetracycline (21.7%). The study showed only certain *Salmonella* serotypes (*S.* Anatum [16/26], *S.* Kentucky [5/26], *S.* Mbandaka [4/ 26], and *S.* Cerro [1/26]) had phenotypic tetracycline resistance profile. In contrast to the previous studies [15, 69], in the study of Beach et al. (2002, serotype and phenotypic AR profiles were not strongly associated.

Arthur et al. (2008) examined *Salmonella* carriage in cattle (n = 581) before leaving the feedlots and on arrival at one of three different slaughterhouses and after post-harvest stage to evaluate the potential origin of *Salmonella* contamination on carcass surfaces [122]. Cattle hide and fecal samples were samples at the feedlots a day before the transport to the slaughterhouse. The transport vehicles were surface sampled before and after cattle load. Lairage environment samples were collected from the lairage surface before cattle passing through each area. In addition, hide samples were collected at the slaughterhouse (post-harvest stage) before and after applications of the hide wash procedures in the cabinets, whereas the carcass samples were collected after hide removal but before the carcass interventions applied on the carcass surfaces. Results using PFGE analysis showed that 30.0% (15/50) of *Salmonella* found on carcasses, and 65.1% (656/1,007) of *Salmonella* recovered from hide samples at slaughter were attributed to the lairage environment. The only *Salmonella* contamination found on the carcasses was found in one slaughterhouse that did not utilize carcass wash procedures . The remaining two slaughterhouses that applied the carcass washes eliminated *Salmonella* contamination

on carcass surfaces. The authors concluded that carcass contaminations were mostly related with the lairage environment and that hide wash procedure significantly ($P < 0.05$) reduced *Salmonella* carriage in terms of prevalence and CFUs on the cattle hides.

Overall, these findings suggest that when the serotypes found on carcass surfaces are examined, they show similarities to the serotypes that are commonly found in cattle feces, lymph nodes, and on hides. These studies also suggest that when slaughterhouse interventions are regularly applied to hide and carcass surfaces, a reduction of *Salmonella* load and a shift in the population of dominant *Salmonella* serotypes can be observed [122, 143, 146]. However, these interventions are not enough to fully eliminate the public health risks of contamination resulting from cattle lymph nodes harboring *Salmonella*.

2.4. *Salmonella* in beef products

One of the main human exposures to *Salmonella* is via the consumption of contaminated beef products [13]. Each year in the United States an estimated 26- 28 billion pounds of beef products are produced and sold for human consumption [147]. *Salmonella* contamination in beef products usually occurs at slaughter or at meat processing stages. As described previously, contamination of carcass surfaces and inclusion of lymph nodes as fat trim in batches of ground meat may lead to the contamination of the final beef products. Contaminated beef products further pose a risk for human *Salmonella* infections when they are handled or cooked improperly prior to consumption. *Salmonella* is one of the leading concerns for the beef industry. Cattle at the age of slaughter are transported to slaughterhouses from various feedlots. After slaughter, valuable beef parts are separated from the carcasses. Other parts that are less desirable for

human consumption are often blended into ground meat along with fat tissue masses surrounding lymph nodes. Therefore, identification of a single animal or even a single feedlot as the point source of a beef-related *Salmonella* outbreak becomes exceedingly difficult. According to the USDA-FSIS, *Salmonella* is not considered an adulterant of raw beef products, since good food handling practices such as application of the adequate cooking temperatures (approximately 160 °F [71 °C]) to these raw meat products before consumption can eliminate *Salmonella* [148].

Zhao et al. (2006) examined 1,522 ground beef samples, along with the other meat types (chicken, turkey, and pork), collected from eight FoodNet (Foodborne Disease Active Surveillance Network) sites in 2002-2003. Among these ground beef samples, *Salmonella* prevalence was less than 2%; however, 6 of the 19 *Salmonella* ground beef isolates were resistant to ceftriaxone [149].

Another research paper evaluated beef products and their contribution to beef-related outbreaks [13]. That study showed that 22.9% of the outbreaks were related to ground beef consumption, 27.0% were related to roast beef, and a further 31.2% were related to other beef products (e.g., steak, brisket, jerky, barbecued beef, barbacoa, beef blood, ribs, and tripe). The remaining 18.7% was related to an unknown beef product among the total of 96 *Salmonella*-related outbreaks attributed to beef consumption between 1973 and 2011. Phenotypic antibiotic susceptibility profiles were examined only for 14 beef-related outbreaks. Their results showed that six of the 14 outbreak strains were pan-susceptible, the remaining eight outbreaks were MDR strains and all were recovered from ground beef products. These strains were mostly of the (5 of 8) *S*. Newport serotype. The MDR profiles of ACSSuTAuCx (ampicillin, chloramphenicol, streptomycin, sulfisoxazole,

tetracycline, amoxicillin-clavulanic acid, and ceftriaxone phenotype [n = 2]) and ACSSuT (n = 1) were observed in the three of the five *S.* Newport strains. The remaining three serotypes were *S.* Typhimurium and the other two had MDR profiles similar to the MDR *S.* Newport.

S. Montevideo isolates are known to be commonly isolated from ground beef samples but rarely cause human infections [32, 150, 151]. *S.* Newport and *S.* Typhimurium are two well-known serotypes that are most often associated with *Salmonella* outbreaks [31, 152, 153].

Among the 17,161 ground beef samples collected by USDA-FSIS in 2013, a *Salmonella* prevalence of 1.6% (277/17,161) was reported in the ground beef samples. Among the *Salmonella* positive samples, the most common serotype was *S.* Montevideo (31.0%), followed by *S.* Typhimurium (6.8%), *S.* Meleagridis (6.4%) *S.* Dublin (6.4%), *S.* Newport (4.6%), *S.* Muenchen (4.3%), *S.* Kentucky (4.3%), *S.* Cerro (3.9%), and *S.* Anatum (3.2%).

A recent *Salmonella* outbreak attributed to *S.* Newport serotype contaminated ground beef resulted in 403 reported cases in 30 states along with 117 hospitalizations but with no deaths reported in 2018 [154]. Even though *S.* Dublin serotype is highly adapted to cattle, human infections of *S.* Dublin are thankfully rare. Those few outbreaks caused by *S.* Dublin serotypes show the highest rate of hospitalization, invasive infections and deaths when compared to the other serotypes [142]. *S.* Meleagridis is not a commonly isolated serotype from beef products and is not often found associated with human *Salmonella* outbreaks [12].

Clearly, beef products containing *Salmonella* are a public health risk for humans and pose a potential financial burden to the beef industry. Certain *Salmonella* serotypes (e.g., *S*. Newport and *S*. Typhimurium) found in ground beef can cause severe human infections resulting in hospitalization [12]. Therefore, it is important to evaluate factors related to the serotype distribution in cattle, and specifically in lymph nodes, to fully address public health risks. To summarize, *Salmonella* contamination of beef products sourced from cattle (feces, lymph nodes and hides) needs to be better understood in order to decrease the public health impacts.

2.5. The public health burden of *Salmonella*

Foodborne pathogens in humans are one of the most important global problems due to their health and finance-related consequences. *Salmonella* is a leading foodborne pathogen resulting in 75.5 million illnesses and more than 28,000 deaths in the world annually [155]. The total number of people affected by *Salmonella* is difficult to estimate, since most of the people affected by *Salmonella* develop mild symptoms and recover without antibiotic treatment, and especially since infected individuals most often do not present to health care facilities or hospitals. Therefore, providing the exact numbers of people who are affected by *Salmonella* is difficult to estimate in both global and local settings. In addition, the long incubation period of *Salmonella* (12-72 h post-exposure), and possible recall or selection biases of food exposure information obtained from people during epidemiologic investigations, can result in an unattributable outbreak, especially when a small number of people are affected by the outbreak. This situation complicates

efforts to solve the epidemiological problems in order to detect the source of an outbreak [156].

Determining the source of an outbreak plays an important role in preventing the spread of a disease. It requires collaborative networking and exchange of the data that are collected from hospitals, retail markets, and agriculture settings. There are few countries that have established a collaborative surveillance agency to monitor the epidemiology of the foodborne pathogens. These countries are mainly developed countries such as Denmark, the United States, Canada, the United Kingdom, and Colombia. In the United States, the FoodNet of the CDC is the agency that routinely generates food-related outbreak reports through the collaboration of the United States Food and Drug Administration (FDA), USDA-FSIS, and outbreak reports collected from the health department of ten states. FoodNet reports the laboratory-confirmed bacterial infections and estimates population-level parameters.

Salmonella and *Campylobacter* are two of the leading foodborne zoonotic pathogens in the United States that are most often transmitted to humans via contaminated foods. The number of laboratory-confirmed bacterial pathogens that were reported to FoodNet was 18,375 in 2015; of these, 7,719 were confirmed as *Salmonella*, followed by 6,289 cases of *Campylobacter* [157]. In the same report, survey-based population-level incidence rate of salmonellosis was reported as 15.74 per 100,000 persons annually in the United States. The hospitalization percentage of these 7,719 laboratory-confirmed *Salmonella* cases was reported as 27.3%. The percentage of hospitalization rates increased by up to 58.3% among elderly individuals who in the age range of 60-85+. Based on FoodNet surveillance data, *Salmonella* infections were estimated to have caused

approximately 168,000 physician visits, 15,000 hospitalizations, and 400 deaths per year from 1996–1999 [158].

Besides the health-related consequences of *Salmonella*, salmonellosis is also a global financial problem all over the world due to expenses spent on medicine, loss of productivity, and related to deaths. USDA-Economic Research Service (ERS) report on the medical costs resulting from *Salmonella* estimated total costs at $312,738,453, productivity losses due to *Salmonella* at $81,380,620, the cost of deaths related to the *Salmonella* at $3,272,480,959 and with the total cost estimated at $3,666,600,031 in 2013 [159, 160]. In addition, there is a substantive cost resulting from the waste of food products when *Salmonella*-contaminated foods are recalled (these were not included in the ERS estimate). For example, the recent *Salmonella* outbreak reported in ground beef products resulted in the recall of 6.4 million pounds of ground beef products in October 2018, with an additional 5.2 million pounds of beef products recalled in December 2018 [154].

Symptoms of *Salmonella* usually occur 12-72 h after the digestion of contaminated food products. The incubation time of *Salmonella* in humans shows variability, which is mainly related to the intake dose of *Salmonella* and host immunity. *Salmonella* infections in humans usually cause mild symptoms and are self-limiting. These infections mainly result in gastroenteritis, which is often characterized by fever, acute watery diarrhea, nausea, vomiting and abdominal cramps [22]. The full recovery from salmonellosis in people may take from two to seven days and often does not require medical practitioner visits or medication. If these mild infections persist in the patients, they may be admitted to health care facilities to receive fluid and electrolyte replacements including sodium,

potassium, and chloride. However, immunosuppressed people, including children younger than 5 years old and adults older than 65 years old, as well as HIV (human immunodeficiency virus) patients with impaired immunity, people who receive cytotoxic therapy or have malnutrition, and people who are diagnosed with hemoglobinopathy, cirrhosis, or *P. falciparum* malaria are considered at highest risk for developing invasive (systematic) *Salmonella* infections [23, 161, 162].

Systemic *Salmonella* infections may occur following the migration of *Salmonella* from the intestinal lumen to the bloodstream and other body sites where the infection can cause life-threatening complications for patients. Symptoms of systemic *Salmonella* infections may consist of high fever, bloody diarrhea, and septicemia. These patients are admitted to hospitals and often require parental antibiotic treatments, which are known to have bactericide or bacteriostatic effects on *Salmonella* [163]. Historically, the recommended choice of antibiotics for the treatment of human *Salmonella* infections was ampicillin, chloramphenicol, or trimethoprim-sulfamethoxazole. Due to emerging resistance in *Salmonella* against these antibiotics, today the recommended empirical treatment for salmonellosis in humans includes newer generation cephalosporins (e.g., ceftriaxone), fluoroquinolones (e.g., ciprofloxacin) and macrolides (e.g., azithromycin) [164]. Fluoroquinolone use is limited in children and pregnant women due to adverse side effects on the developing cartilage of juvenile animals [24]. In addition, the FDA recently announced a black-box warning regarding the use of fluoroquinolone in adults due to potentially serious side-effects such as aortic vessel rupture, tendonitis, and neuropathy [26]. Therefore, ceftriaxone (100 mg/kg/day for two days) and azithromycin (20 mg/kg/day for 7 days) are the go-to choices available to practitioners for empirical therapy

of pediatric, obstetric, and most adult cases [22, 23]. Therefore, these antibiotics are critically important for human medicine, since the failure of the antibiotic treatment may cause death of these patients [54, 165].

2.5.1. Epidemiology of *Salmonella* in humans

Salmonella colonies can resist environmental changes such as temperature, pH, moisture, and solar radiation, which increases the survival rate of *Salmonella* outside of the usual host enteric niche. The ability of *Salmonella* to adapt to harsh conditions generates a persistent and complex problem for human exposure to *Salmonella*. *Salmonella* can be found in the gastrointestinal tract of animals, which can disseminate to other animals via soil, water and air [166]. Therefore, the transmission dynamics of *Salmonella* is a complex issue, since human exposure to *Salmonella* can be attributed to many different routes such as animal contact, water, food, person-to-person, and other sources, and ultimately can spread from agricultural to kitchen settings [125] (Figure 4).

Human exposure to *Salmonella* may occur via direct physical contact with *Salmonella*-colonized farm animals, handling contaminated farm equipment, and inhalation of contaminated aerosols at or near a farm environment. Farm irrigation systems can also carry these pathogens to nearby surface waters and rivers. Wastewater containing human feces, effluent from meat industries and wastewater from livestock can pose a potential risk factor for transmission of *Salmonella* despite treatments applied to reduce pathogen carriage [167]. Farm soil that is used as fertilizer for crop production can be contaminated by improperly amended animal manure. Therefore, both soil and water

contamination can lead to contamination of vegetables, crops, water, and be widely spread across multiple regions [166].

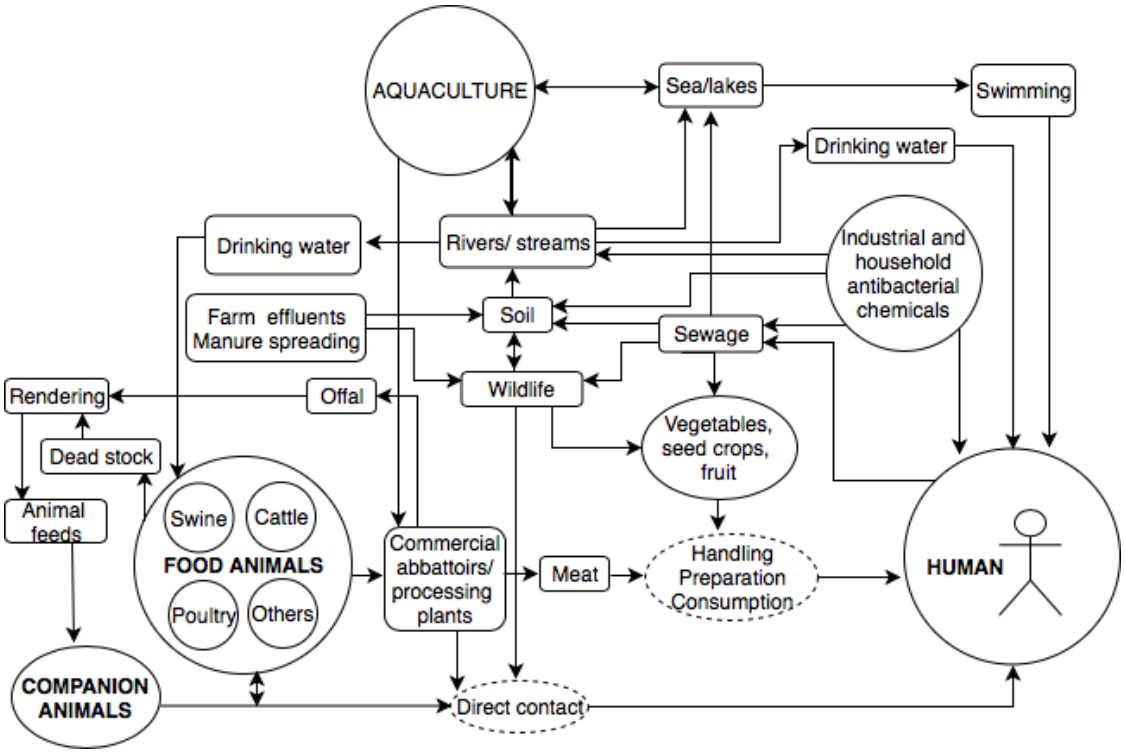


Figure 4. Epidemiology of zoonotic foodborne pathogens and associated antibiotic resistance

Modified from Health Canada, 2002 [168]; earlier adapted from Linton,1977[169].

Wild animals and rodents also play a potential role to carry and spread *Salmonella* to different locations [170]. Also, during animal transport, aerosolized fecal materials may also spread along the route taken by these vehicles [171]. At slaughterhouses and meat processing facilities, workers can be exposed to these microorganisms via direct contact or through the air. Offal products can pose an increased risk due to their reintroduction into various food animal production realms. For instance, rendered offal products are

commonly used as protein, fiber, calcium and fat sources in animal feed and pet foods, which can result in *Salmonella* exposure of farm animals and pets, respectively, and later to pet owners [172-174]. Handling exotic animals, especially reptiles has also been reported as a source of *Salmonella* infection in humans [170]. In addition to the routes described above, person-to-person *Salmonella* transmission may occur via fecal-oral transmission route due to a lack of hygiene practices in humans [175]. Animal origin food products may cause *Salmonella* outbreaks when these products are not entirely raised to the recommended temperatures to eliminate or reduce pathogen carriage of the products [148]. The consumption of uncooked vegetables, seed, and fruits that were contaminated with farm soil or water may also be sources of food-related *Salmonella* outbreaks [126]. Additionally, kitchen surfaces and equipment such as cutting boards, countertops, knives, and utensils that came into contact with a raw animal origin food product, can contaminate other food products that are consumed as raw or else cooked at lower temperatures [176].

Population-based data collected from 7,895 cases of laboratory-confirmed *Salmonella* infections from 1996 to 1999 by the FoodNet revealed an estimate of 40.5% of these infections were caused by *S.* Typhimurium, *S.* Enteritidis, or *S.* Heidelberg serotypes [177].

Based on the most up-to-date FoodNet 2015 Surveillance Report, involving 10 states and 20,098 laboratory-confirmed infections caused by foodborne pathogens, *Salmonella* was the most prevalent (15.7%) food-borne pathogen resulting approximately 2,100 hospitalization and 32 deaths. Among 7,220 *Salmonella* isolates that were serotyped, the most common serotypes were *S.* Enteritidis (19.2%), *S.* Newport (11.4%), and *S.* Typhimurium (10.4%). Interestingly, the incidence of infections recently caused by

S. Enteritidis and *S.* Newport was significantly higher ($P < 0.05$), while significantly lower ($P > 0.05$) for *S.* Typhimurium when compared to 1996-1998 data. *Salmonella* infections were highest in summer (from July through October) [157].

Approximately 80.3- 93.8 million human gastroenteric cases of salmonellosis that occur globally each year are attributed to the consumption of *Salmonella* contaminated food products [161]. Therefore, human consumption of animal food products is one of the most studied and most important routes of transmission of foodborne pathogens. Beef products play an important role in *Salmonella* related outbreaks [10, 11]. The salmonellosis cases attributed to beef products globally were caused by multiple *Salmonella* serotypes that include, but are not limited to: *S.* Newport, *S.* Enteritidis, *S.* Heidelberg, *S.* Montevideo, *S.* Saintpoul, *S.* Berta, *S.* Infantis, *S.* Thompson, *S.* Agona, *S.* Anatum, *S.* Chester, *S.* Hadar, *S.* Reading, *S.* Agama, *S.* Bovismorbificans, *S.* Braenderup, *S.* Cerro, *S.* Derby, *S.* Hartford, *S.* Kiambu, *S.* Ohio, *S.* Oranienburg, *S.* Singapore, *S.* Schwarzengrund, and *S.* Senftenberg serotypes [13].

2.5.2. Antibiotic resistance in *Salmonella*

Antibiotic resistance observed in food-borne pathogens is an important public health problem due to a persistent increase in AR clinical cases worldwide [165, 178, 179]. Antimicrobial therapies are mostly applied to patients diagnosed with *Salmonella* infections with clinical symptoms, especially to the patient group that is considered at highest risk to develop invasive *Salmonella* infections [180]. Even though antibiotic use can prolong *Salmonella* shedding in the patients, it is a lifesaving solution sometimes needed to eliminate the infection in patients [22]. As previously mentioned, the historical

empirical treatment of salmonellosis shifted from aminopenicillins, phenicols, and folate pathway antagonists to fluoroquinolones, 3rd generation (extended-spectrum) cephalosporins, and macrolides due to the isolation of the AR bacteria emerging in human infections caused by chloramphenicol and trimethoprim-sulfamethoxazole-resistant *Salmonella* serotypes [181]. Since then, AR *Salmonella* have become a persistent public health threat due to emerged antimicrobial resistance and reduced susceptibility (i.e., MICs of 0.12– 1 μg/ml) against the fluoroquinolones, the 3rd generation cephalosporins (Figure 2)**,** and azithromycin (Figure 3) in human *Salmonella* isolates [8, 28, 182].

NARMS has also been extensively tracking serotypes and phenotypical antibiotic resistance profiles of laboratory-confirmed human *Salmonella* isolates recovered from patients at the national-level since 1996. The NARMS monitoring program was first started with a human population of 13 states; today, NARMS represents 50 states and the District of Columbia, which is almost the entire US population [28]. Within introduction of low-cost WGS methods, NARMS began to also monitor and report molecular insights of antibiotic resistance since 2014 [183]. Therefore, complete ARG information along with phenotypic antibiotic resistance profiles obtained from human *Salmonella* isolates is somewhat limited.

The most up-to-date NARMS Annual Human Isolates Surveillance Report published in 2018 tested a subset of clinical human isolates (n = 2,364) for both phenotypic and genotypic antibiotic resistance [32]. In this report, *Salmonella* resistance against the antibiotics used for the treatment of salmonellosis in humans was extensively evaluated. According to this report, the majority (75.0% [1,775/2,364]) of the *Salmonella* isolates were pan-susceptible, and the remaining isolates (598/2,364) were resistant at least to one

antibiotic. Only 13 of the 1,775 phenotypic susceptible isolates demonstrated an identified an ARG and ARG mechanism corresponding to what should have been a resistance phenotype. Perhaps this could have been due to cryptic ARGs, which are not directly responsible for the expression of a resistance mechanism [184] or else to a low-quality base-call of the genomic sequence data. On the other hand, 64 of the phenotypic AR isolates did not harbor any resistance genes, which may be the result of the loss of a plasmid carrying ARGs during the storage or testing period or to unidentified ARGs, which are not yet available for searching in the ARG databases.

Bacterial resistance to nalidixic acid, which is the oldest quinolone, is usually characterized by a decreased susceptibility to higher-level fluoroquinolones such as ciprofloxacin. The observed decrease in susceptibility to fluoroquinolones is mainly observed in typhoidal *Salmonella* isolates, but also recently has become a concerning problem for non-typhoidal *Salmonella* [185]. The NARMS Human Isolate Reports used the MIC interpretation criteria for ciprofloxacin as: ≤ 1 µg/ml for susceptible, 2-4 µg/ml for intermediate, ≥ 4 µg/ml for resistant until 2012. Later, NARMS followed the new MIC interpretative criteria for ciprofloxacin as: ≤ 0.06 µg/ml for susceptible, 0.12-0.5 µg/ml for intermediate, and ≥ 1 µg/ml for resistant, which was also updated by CLSI in 2012 [32].

In the past, most of the quinolone resistance found in *Salmonella* was related to chromosomal mutations of the *gyr*A and *par*C genes [186]. However, during the last decade plasmid-mediated quinolone resistance (i.e., *qnr* gene-harboring plasmids) in *Salmonella* have become more prevalent [187, 188]. In the recently published NARMS Annual Human Isolates Surveillance Report [32], 5.8% [137/2364] of *Salmonella* isolates showed decreased susceptibility to ciprofloxacin, which was mostly (47.4%) found in the

S. Enteritidis serotype. Further, these isolates were examined for quinolone related ARGs. The majority of the plasmid-mediated quinolone-resistant isolates (n = 35) were found to be associated with a *qnr*B (n = 23) gene, followed by *qnr*S (n = 5), *qnr*A (n = 3), *oqx*AB (n = 3), and *aac*(6')*lb-cr* (n = 1) genes. In addition to the plasmid-mediated quinolone resistance, chromosomal mutations of the *gyr*A gene were observed in 64 isolates, whereas *par*C mutations were observed in only two isolates.

CLSI applied a resistance interpretive criteria for ceftriaxone at an MIC ≥ 64 μg/ml up until 2010; since then, the updated resistance interpretive criterion of ≥ 4 μg/ml has been used in the United States [189]. The most commonly observed ceftriaxone resistance mechanisms in *Salmonella* are the transferable plasmid-mediated AmpC β-lactamase gene (*bla*$_{\text{CMY}}$) and the extended-spectrum β-lactamases genes (*bla*$_{\text{CTX-M}}$ and *bla*$_{\text{SHV}}$) [190-192]. In the literature, there are a large number of *bla* genes resistance genes that have been identified in *Salmonella* isolates; these *bla* genes include, but are not limited to: CARB, CMY, CTX-M, DHA, KPC, NDM, OXA, and SHV [192]. In the NARMS Human Isolates Surveillance Report published in 2018 [32], phenotypic ceftriaxone resistance was found in 2.7% (n = 65/2,364) of the total isolates. Ceftriaxone resistance was mostly identified in the *S.* Dublin (66.7%) serotype, followed by *S.* Infantis (6.9%), *S.* 4,[5],12:i: (6.0%), *S.* Newport (4.7%), *S.* Heidelberg (4.4%), and *S.* Typhimurium (4.0%). Ceftriaxone resistance genes were observed in a total of 57 *Salmonella* isolates. Among those, the identified genes were *bla*$_{\text{CMY}}$ (n = 49), and in addition 8 isolates showed an extended-spectrum β-lactamase (ESBL) phenotype and were harboring either the *bla*$_{\text{SHV-12/30}}$ (n = 5) gene or *bla*$_{\text{CTX-M}}$ (n = 3) genes. Furthermore, a total of 242 isolates exhibited ampicillin

resistance due to the following genes: *bla*$_{TEM}$, *bla*$_{CMY}$, *bla*$_{CARB}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, and *bla*$_{OXA}$).

In addition, three of seven ceftriaxone-resistant *Salmonella* outbreaks that have recently occurred have been attributed to the consumption of beef products between 2011 and 2012 [190]. In those outbreaks, the ceftriaxone resistance gene (*bla*$_{CMY}$) encoded on IncA/C or IncI1 plasmids were found in two *S.* Typhimurium and one *S.* Newport strains that were phenotypically resistant to ceftriaxone. The full resistance phenotype observed in the *S.* Newport was MDR-AmpC (AmpC-ACSSuT) profile, which has commonly been associated with cattle and beef sourced outbreaks [150, 193].

In addition, there was an observed increased in the percentage of human *Salmonella* isolates resistant to ceftriaxone from 0.2% to 3.4% from 1996 to 2009 in the United States [28]. Among these resistant isolates, that were mostly MDR, the most common profile was the ACSSuT phenotype. Ceftriaxone resistance in human strains is mostly found associated with this MDR profile and has been observed in *S.* Typhimurium, *S.* Newport, *S.* Heidelberg, *S.* 4, [5],12:i:–, and *S.* Dublin serotypes.

At present, there are no clinical azithromycin breakpoints established for non-typhoidal *Salmonella* [189]; therefore *S.* Typhi breakpoints are used for the detection of phenotypic resistance in non-typhoidal serotypes. It has also been reported that non-typhoidal *Salmonella* and typhoidal *Salmonella* can show similar azithromycin MIC values [182]. NARMS included azithromycin in their phenotypic susceptibility testing panels as a replacement for amikacin in 2011 due to emerging concerns about azithromycin resistance in *Salmonella* [194]. Azithromycin is a relatively new choice of antibiotics to treat *Salmonella* infections in humans. Therefore, there are few historical phenotypic and

genotypic AR data or evidence of azithromycin resistance that have been reported. In *Salmonella,* there have been numerous ARGs of macrolide class antibiotics reported historically. These genes are listed as: *ere*, *erm*, *lnu*, *lsa*, *mef*, *mph*, *msr*, and *vga* genes [192]. In the NARMS Human Isolates Surveillance Report published in 2015 [32], phenotypical azithromycin resistance was observed among 0.3% (n = 8/2364) of the total isolates. Among these, 6 isolates were identified harboring the *mph*A (n = 5) gene and one isolate had co-existing *msc*E and *mph*E genes [32].

Sjolund-Karlsson et al. (2011), investigated phenotypic and genotypic azithromycin resistance in 575 *Salmonella* randomly selected isolates (232 human isolates submitted to the CDC, 227 animal [chicken, turkey, cattle, or swine] isolates submitted to the USDA, and 116 ground meat [chicken, turkey, or beef] isolates submitted to the FDA) from a total of 2,379 *Salmonella* isolates in 2008 [182]. Among these isolates, the majority were either *S.* Enteritidis or *S.* Typhimurium, whereas the animal origin serotypes were *S.* Kentucky, *S.* Heidelberg, and *S.* Montevideo serotypes, and the serotypes found in retail meat were either *S.* Heidelberg, *S.* Hadar or *S.* Typhimurium variant O:5—. The highest MIC range for azithromycin was observed in human isolates (with MICs of 1-32 µg/ml) followed by retail meat (with MICs of 4-16 µg/ml), and animal origin isolates (with MICs of 2-16 µg/ml) (Figure 5). Among these, two *Salmonella* isolates that were resistant to azithromycin (with an MIC ≥ 32) were screened for *ere*A, *ere*B, *erm*B, *mef*A, *mph*A, *mph*B, and *mph*D genes using PCR. The PCR results did not detect any of these macrolide resistance genes. This result may be related to the specificity and sensitivity of the PCR method they used or the phenotypic resistance may be related to other unidentified and different resistance mechanisms that are not in the ARG database by the time of the study.

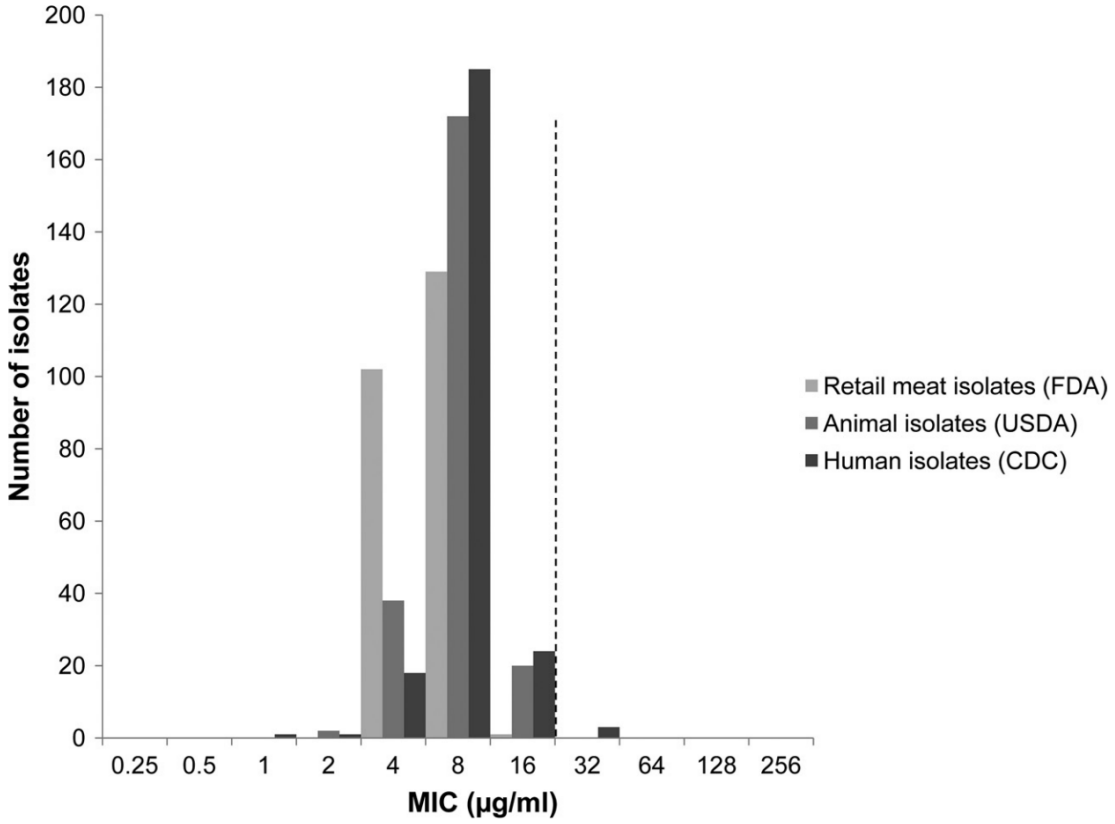


Figure 5. Azithromycin MICs observed from human-, animal-, and ground-meat-origin *Salmonella*

Adopted from Sjolund-Karlsson et al. (2011) [182]

Before 2013, the CLSI streptomycin resistance breakpoint was defined as ≥ 64 µg/ml, this was later updated to ≥ 32 µg/ml in 2014. In the NARMS Human Isolates Surveillance Report published in 2018 [32], a total of 251 isolates harbored a streptomycin resistance gene, among these isolates, the most common gene was the *str*A (n = 188), *str*B (n = 186), *aad*A (n = 84), *aph*(6)-*Ic* (n = 3), and *arm*A (n = 1) genes. In addition, gentamicin resistance genes (*aac*, *aad*B, and *arm*A) were also identified in 40 isolates. Tetracycline resistance genes were the most prevalent resistance genes found in this study and these were observed in 278 *Salmonella* isolates. Among those, the most common

tetracycline resistance gene was *tet*(A) (n = 129), followed by the *tet*(B) (n = 123), *tet*(G) (n = 25), *tet*(M) (n = 7), *tet*(C) (n = 4), and *tet*(D) (n = 3). The ARGs conferring resistance to either sulfisoxazole (*sul*1, *sul*2, and *sul*3) or trimethoprim-sulfamethoxazole (*sul*+*dfr*A.) were the second most prevalent (n = 264) ARGs. Chloramphenicol resistance genes (either floR, *cml*, *oqx*AB, *cat*A or *cat*B) were harbored by 75 isolates.

In the same NARMS Human Isolate Surveillance Report [32], 12.4% (293/2364) of *Salmonella* isolates that were MDR were mostly *S.* Dublin (91.7%), *S.* 4,[5],12:i:- I (68.8%), *S.* Typhimurium (18.3%), *S.* Infantis (15.3%), *S.* Newport (5.6%), or *S.* Enteritidis (4.2%) serotypes. The MDR *Salmonella* were mainly associated with the ACSSuT phenotype [192, 193, 195]. The ACSSuT profile corresponds to resistance against five CLSI classes of antibiotics as follows: aminopenicillins (ampicillin), phenicols (chloramphenicol), aminoglycosides (streptomycin), sulfonamides (sulfisoxazole), and tetracyclines (tetracycline). However, in this report, the ACSSuT phenotype was not identified. However, overall 5.0% of the isolates had the ASSuT phenotype (a phenotype similar to the ACSSuT but without chloramphenicol resistance) that was commonly observed though only in *S.* 4,[5],12:i:- I (59.1%) serotype. Chloramphenicol has not been used in U.S. food animal agriculture since 1985, though florfenicol has been available since the 1990s.

In recent years, a new MDR profile of *Salmonella* also has emerged by including amoxicillin-clavulanic acid and ceftriaxone resistance (the ACSSuTAuCx phenotype) to the ACSSuT phenotype, which shows phenotypic resistance to two additional classes of antibiotics. In the NARMS report [32], 1.3% (31/2,364) of the isolates showed an ACSSuTAuCx phenotype that was mostly found in the *S.* Dublin (58.3%) serotype,

followed by the *S.* Newport serotype (4.7%), *S.* monophasic Typhimurium I 4,[5],12:i:- I (2.7%), and S. Typhimurium serotypes (1.6%) [32]. Antibiotic resistance phenotypes that are commonly found in *Salmonella* are those against ampicillin, tetracycline, sulfisoxazole, streptomycin, and chloramphenicol. These phenotypes are mainly encoded by *bla*$_{CMY}$, *bla*$_{TEM-1}$, *tet*(A), *tet*(B), *sul*1, *sul*2, and *flo*R genes [192].

MDR *Salmonella* are a serious and threatening problem, since human cases related to these strains often result in a higher number of treatment failures, longer hospitalization periods, and higher mortality rates when compared to infections caused by antibiotic susceptible strains [165, 196, 197].

2.6. Public health risks of antibiotic use in food-producing animals

The use of antibiotics in food animals is commonly perceived as one of the key contributors to emerging antibiotic resistance problems in enteric commensal bacteria that might potentially pose a risk for public health [198-200]. Usually, antibiotic resistance found in foodborne pathogens is against tetracycline, streptomycin, and sulfamethoxazole, which are older antibiotics that were commonly used in the recent history of agriculture [31, 201].

Antibiotic use in food-producing animals can select for resistant bacterial populations and can cause potential direct or indirect effects to the public health. The risk of antibiotic use in food-producing animals is mainly dependent on dose, class, and duration of the antibiotics that are used [199, 202-205]. The direct effects of antibiotic use that can increase the risk of colonization of resistant bacterial populations in the human gastrointestinal tract may be caused by 1) physical contact with an animal treated with

antibiotics (e.g., direct contact) [206, 207], and 2) consumption of AR bacteria-contaminated food products [203, 207]. Human exposure to animal origin AR bacteria can be also caused indirectly as follows: 1) exposure to dust and air-particles carrying AR bacteria [171], 2) use of animal waste as fertilizer that can result in water contamination with AR bacteria [208, 209], 3) contact with contaminated domestic animal feed produced from by-products of food animals treated with antibiotics [174, 210, 211], and 4) horizontal gene transfer occurring in the human gastrointestinal tract via a mobile genetic element that harbor ARGs occurring between a non-pathogenic animal origin bacteria and a human pathogen [212-214].

There is abundance evidence suggesting that antibiotic use in animals contributes to the selection of antimicrobial resistance in *Salmonella* and other enteric populations, which potentially can transmit from animals to humans and pose a public health threat [203, 204, 206, 215, 216].

One of the oldest prospective field studies, conducted in 1976, showed evidence of transmission of antibiotic resistance from chickens to chickens and from chickens to humans [216]. When these chickens were fed tetracycline containing feeds, the authors showed an increased proportion of *E. coli* harboring tetracycline-resistant plasmids in the feces obtained from farm families, especially when compared to families living away from the farm location.,.

Among the older research projects on this subject published by Holmberg et al. in 1984, revealed a direct link of cattle origin AR *Salmonella* causing serious human infections via consumption of meat products [203]. Identification of the plasmidal profiles revealed that a plasmid that harbored tetracycline and ampicillin resistance genes in

Salmonella was transmitted to humans from beef cattle that were fed with chlortetracycline for growth promotion purposes in South Dakota.

A case-control study related to a massive MDR *S.* Typhimurium outbreak attributed to milk products was published in 1987 [215]. This strain carried a unique antibiotic resistance pattern and plasmid; therefore, the outbreak was readily traced back to its origin. Later, it was found that the contamination occurred in the milk plant and this strain repeatedly contaminated the milk products. Even though this study proved the transmission of antibiotic resistance carrying *Salmonella* between animal origin food-products to humans, the direct role of antibiotic use in dairy farms in this study remains unknown.

Resistant-*Salmonella* transmission was also reported to have occurred between a child and a cow via direct psychical contact [206]. A 12-year-old boy that was infected with an MDR *Salmonella* was traced back and revealed to have had direct contact with a cow on a family farm in 2000. The *Salmonella* that was isolated from the child was determined to be the identical strain of $bla_{\text{CMY-2}}$ gene harboring ceftriaxone-resistant *Salmonella* that was found in a cow treated with ceftiofur.

In parallel, the temporary withdrawal of antibiotics from animals has also shown a reduction in the AR *Salmonella* population [204]. Dutil et al. (2010) showed the relationship among ceftiofur resistant *S.* Heidelberg serotypes recovered from both poultry and human infections using the CIPARS (Canadian Integrated Program for Antimicrobial Resistance Surveillance) data during 2003–2008 [204]. Their finding showed a statistically significant ($P< 0.05$) and strong association among *S.* Heidelberg isolated from retail chicken and the incidence of ceftiofur-resistant *S.* Heidelberg infections in humans,

which were likely due to a large amount of *in ovo* ceftiofur use in hatcheries in Quebec, Canada. This study also showed that the withdrawal of ceftiofur injection from chicken eggs significantly decreased the prevalence and incidence of AR *Salmonella* recovered chicken products and humans, respectively; however, once ceftiofur began to be administered once again, the situation reversed to previous levels.

Due to existing evidence of antibiotic use in animals and their potential public health risk for selection of resistance, various countries and societies have enforced rules or given recommendations to limit/ban non-treatment purposes of antibiotic use in food animals. The first report (also known as Swann Report) recommending the banning of important human antibiotics as growth promoters in food-producing animals was published by the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine in the United Kingdom in 1969. This ban was proposed due to the observed increase of MDR *Salmonella* in human isolates that were found to be related to the large amount of antibiotic use as growth promoters in food-producing animals [217]. After this report, the growth promoter uses of medically important antibiotics were banned in the United Kingdom. After the United Kingdom, other European countries (e.g., Sweden in 1986 then Denmark in 1996) also banned the use of these antibiotics as growth promoters. In the United States, first regulation and prohibition were set for the extra (off)-label use of glycopeptides (vancomycin and avoparcin) and fluoroquinolones (danofloxacin and enrofloxacin) in 1997 and then cephalosporins (ceftiofur) in 2012 [218]. Additionally, the FDA proposed a voluntary withdrawal of all labels allowing medically important antibiotics in feed or water of food animals for production purposes and permitted the legal administration of these antibiotics via feed and water to registered veterinarians in 2013.

This voluntary withdrawal was fully implemented in 2017 [219]. This implementation ruled by the GFI (guidance for the industry) #213 was due to the increasing global threat of emerging antibiotic resistance against medically important antibiotics in pathogens found in food-animals [220].

Reducing the use of antibiotics in food-producing animals has been characterized by the reduction in observed antibiotic resistance in both animal and human isolates. For example, within the two years after the ban of avoparcin (a glycopeptide that is used as a feed additive in food animals in Europe) in 1997, a decrease in vancomycin-resistant enterococci isolated from both humans and animals was widely observed in many countries [221]. Similarly, a significant reduction of extended-spectrum cephalosporinase-producing *E. coli* in pigs and pork meat was observed after a voluntary ban of cephalosporin use in Denmark in 2010 [222].

Considering antibiotics are the most efficient treatment to combat bacterial infections in human, it is urgent and necessary to explore the consequences of antibiotic administrations, and prudently use these antibiotics in food-producing animals.

2.7. Antibiotic use in cattle

Antibiotics are widely used for the treatment of clinical infections, control (metaphylaxis), and prevention (prophylaxis) of common infectious diseases in feedlot cattle. Antibiotics are mainly administered to individual cattle to treat a clinically diagnosed infection. On the other hand, herd-level (i.e., groups of cattle) administrations are applied to prevent an expected disease or else to control the spread of infection in a herd when one or more individuals already show the clinical signs of an infection. To

accurately assess the associations between antibiotic resistance found in food-producing animals and antibiotic use in animal agriculture, it is essential to collect detailed information on the amount of medically important antibiotics used in food-producing animals.

In the United States, annual reports reporting the mass of antibiotics (kg of active ingredient/drug class) sold or distributed for use of food-producing have been routinely monitored and published by the FDA for approximately one decade. Based on the most up-to-date FDA data collected in 2016, an estimated 8,356,340 kg of medically important antibiotics were used in food-producing animals for the treatment, prophylaxis, metaphylaxis, and growth promotion purposes [223]. Since the beginning of 2017 (after the FDA's GFI #213), medically important antibiotics have only been used for animal health-related purposes, which yielded a complete cessation (5,770,655 kg [2016] to 0 kg [2017]) of the sale of these antibiotics for growth promotion purposes [223, 224]. Before the middle of 2016, these data included only the sales and distribution of antibiotics among the classes of food-producing and domestic animals. However, due to the variations of antibiotics used in different food-producing animal species, this approach was not found to be enough to determine the drivers of the antimicrobial resistance in certain animal species and humans in relation to antibiotic use. Therefore, starting from the mid-2016, FDA started to require sponsors to provide estimates of sales to four major food-producing animals: cattle, chickens, turkey, and swine [225].

Based on the latest FDA report published in 2018, domestic sales and distribution of antimicrobials approved for use in food-producing animals decreased by 33% from 2016 through 2017 [224]. In 2017, a total of 5,559,212 kg of medically important antibiotics

was used in food-producing animals. Approximately 2,349,271 kg (42%) of these antibiotics were used in cattle. The divided mass of major medically important antibiotics used for cattle was of tetracyclines (66%), followed by macrolides (11%), sulfonamides (8%), aminoglycosides (5%), penicillins (4%) and then cephalosporins and fluoroquinolones (less than 1%), respectively (Table 2).

In 2017, the major sales of antibiotics for cattle were observed in tetracyclines, followed by macrolides, sulfonamides, aminoglycosides, penicillins, amphenicols, cephalosporins, and fluoroquinolones. When these numbers are compared to the previous year (2016), the sales of the majority of the antibiotic classes largely decreased (between 16%-45%); this decrease was small for penicillin and cephalosporin class antibiotics (≤ 5%). However, the macrolides and fluoroquinolones sold for cattle use had increased by 41% and 24%, respectively (Table 2).

Table 2. Medically important antibiotics sold for cattle use between 2016-2017

Antibiotic class	Antibiotic	Estimated annual totals (kg)		Change (%)
		In 2016	In 2017	2016-17
Aminoglycosides	Dihydrostreptomycin, gentamicin, hygromycin B, neomycin, spectinomycin	161,646	124,675	-23%
Amphenicols	Florfenicol	-	49,321	N/A
Cephalosporins	Ceftiofur, cephapirin	24,677	23,512	-5%
Fluoroquinolones*	Danofloxacin, enrofloxacin	18,502	22,904	24%
Macrolides	Erythromycin gamithromycin, tildipirosin, tilmicosin, tulathromycin tylosin, tylvalosin	194,811	274,479	41%
Penicillins	Amoxicillin, ampicillin, cloxacillin, penicillin	99,935	96,936	-3%
Sulfonamides	Sulfadimethoxine, sulfamethazine	234,955	196,902	-16%
Tetracyclines	Chlortetracycline, oxytetracycline, tetracycline	2,840,519	1,560,542	-45%

*No animal species-level data present, the data include cattle, swine, and other species. Source: 2017 Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals, FDA [224].

2.8. Metaphylaxis of BRD

The two most common infectious diseases that were reported to occur in 3,214 cattle feedlots located in the 13 major cattle-on-feed states in the United States are BRD (97% of feedlots) followed by lameness (93% of feedlots) [34]. BRD is reported to occur in all of the feedlots in the United States [34]. BRD is a complex disease of cattle that is a co-infection of both viral and bacterial pathogens. The viral pathogens usually cause primary infections resulting in immune suppression in the host and with mild clinical signs such as decreased appetite, depression, nasal and ocular discharge, and coughing. Viral pathogens of BRD include bovine herpesvirus type 1 (BHV-1), parainfluenza-3 virus (PI3), bovine respiratory syncytial virus (BRSV), and bovine viral diarrhea virus (BVDV). Later, the bacterial pathogens of BRD become opportunistic pathogens of the cattle

respiratory tract and cause infection and disease when immunity is impaired [226, 227]. The bacterial pathogens associated with BRD are *Mannheimia haemolytica, Mycoplasma bovis, Pasteurella multocida,* and *Histophilus somni. M. haemolytica*, *P. multocida* and *H. somni* are normally present in the cattle nasopharyngeal microbiome. The immune status of cattle and exposure to pathogens are two drivers of BRD. The immunity of cattle is often related to environmental stress factors such as transportation (loading and unloading, duration of transport), dramatic climatic changes, and weaning [53, 228]. After the inclusion of both viral and bacterial pathogens in the disease complex, these cattle show severe clinical symptoms such as high fever, septicemia, and finally BRD can cause death [226, 227].

BRD is also known as shipping fever, since the clinical signs of BRD usually occur shortly after arrival to the feedlot from cow-calf, backgrounder, stocker industries or auction markets. Cattle are at a higher risk for BRD when they first arrive at feedlots from backgrounding/stockers or cow-calf sectors due to transport and environment-related stress factors. BRD is a major cause of morbidity, mortality, and economic loss in cattle industries affecting 16.2% of cattle arriving at feedlots. It is reported that metaphylactic antibiotic treatments significantly reduce the incidence of BRD [34, 53]. Therefore, an estimated 60% of cattle receive BRD metaphylaxis upon arrival to the feedlot [33, 34, 229].

The decision of BRD antibiotic administration at the feedlot is taken based on the health condition of the cattle upon arrival (e.g., weight, prior vaccination statue, history of exposure, source), shipping distance, or any other environmental stress factors such as dramatic temperature changes and precipitation (e.g., snow, sleet, rain) [228]. The

antibiotics that are used to control BRD in cattle are available in both injectable and oral forms. Injectable products are mostly preferred due to providing accurate dosing per animal weight, regardless of appetite or thirst. There were eight injectable antibiotics approved for metaphylactic use for BRD as of 2011: amoxicillin, ceftiofur, florfenicol, gamithromycin, tilmicosin, oxytetracycline, penicillin, and tulathromycin [34, 230], enrofloxacin was also approved for control use in 2012. Among these, ceftiofur is the only cephalosporin class antibiotic, whereas gamithromycin, tilmicosin and tulathromycin are the macrolide class antibiotics.

Based on the most up-to-date USDA-APHIS-NAHMS (National Animal Health Monitoring System) Feedlot 2011, Part IV: Health and Health Management on U.S. Feedlots with a Capacity of 1,000 or more Head Report published in 2011, tilmicosin was used by 57.6% of feedlots, followed by tulathromycin (45.3%), ceftiofur (39.7%), oxytetracycline (17.4%), florfenicol (9.2%), and gamithromycin (4.3%) [34]. Therefore, according to this report, tilmicosin, ceftiofur, and tulathromycin are the top three most commonly used antibiotics in the feedlots for BRD metaphylaxis. Among these antibiotics, tulathromycin (Draxxin®) and ceftiofur (Excede®) are two antibiotics that are preferred by farmers to treat or control BRD in feedlots because of their long-acting formulations and clinical effectiveness [34, 52].

Tulathromycin is a semi-synthetic long-acting azalide that was approved for use of beef cattle, but not dairy cattle, in 2005 [231]. It is mainly indicated to treat or control BRD of beef cattle and also to treat bovine foot rot disease and pinkeye. A single subcutaneous dose of 2.5 mg/kg tulathromycin by injection is recommended for metaphylaxis of BRD in cattle upon arrival to the feedlot and it has an 18-day residue

withdrawal period for the meat before the slaughter. Tulathromycin is the second most commonly used BRD metaphylaxis for cattle over 317 kg in the United States [34]. Tulathromycin was also reported as the most common BRD metaphylaxis used for cattle under 317 kg [34].

CCFA is a long-acting third-generation cephalosporin that was approved to treat BRD infections in both dairy and beef cattle in 2003; later, its name was changed to Excede® [232]. It is mainly indicated to treat or control BRD in cattle and to also treat bovine foot rot. A single subcutaneous dose of 6.6 mg/kg ceftiofur by injection is recommended for metaphylaxis of BRD in cattle upon arrival to the feedlots. Ceftiofur has a 13-day slaughter withdrawal period prior to slaughter. Ceftiofur is the third most commonly used antibiotic to control BRD in feedlots located in the United States [34].

2.9. Possible cross-selection risks of antibiotics for resistance

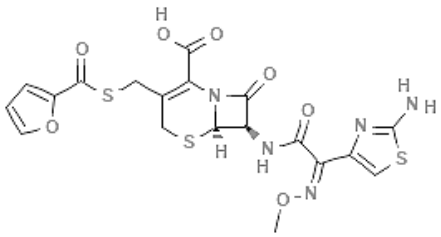
To address the relationship between the emerging cephalosporin and macrolide-resistant human *Salmonella* cases and antibiotic use in cattle, it is important to evaluate the cephalosporin and macrolide class antibiotics used in beef cattle and their potential for cross-selection for ceftriaxone and azithromycin resistance.

2.9.1. Cephalosporins

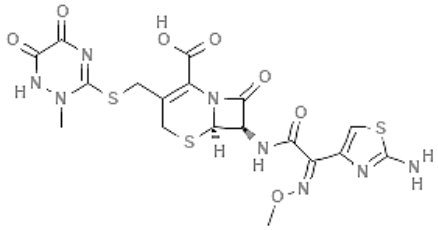
Ceftiofur and cephapirin are the only two approved cephalosporins that are available for cattle use [224]. Cephapirin is an older, 1st generation cephalosporin antibiotic and not used for metaphylaxis of BRD in cattle. However, ceftiofur, which like ceftriaxone is a 3rd generation cephalosporin that has been extensively used for metaphylaxis of BRD in

cattle. Ceftiofur has selection potential for ceftriaxone-resistant *Salmonella* largely because both antibiotics are in the same sub-class of cephalosporins and with very similar molecular structures [233] (Figure 6).

Even though it has been proposed that ceftiofur is degraded or inactivated in the bovine intestinal tract by facultative and obligate anaerobic bacterial species [234], in the literature, there have been numerous randomized controlled field trials that have reported significant, transient, or else no effects of metaphylactic ceftiofur treatments on phenotypic or genotypic ceftriaxone and/or ceftiofur resistance profiles of enteric bacteria in beef cattle populations, especially on *Salmonella* [36], *E. coli* [38, 39], or more broadly the fecal microbiome found in the cattle feces [37, 41, 47].



Ceftiofur ($C_{19}H_{17}N_5O_7S_3$)



Ceftriaxone ($C_{18}H_{18}N_8O_7S_3$)

Figure 6. Chemical structure of ceftiofur and ceftriaxone

Source: Open chemistry database (PubChem) at the National Institutes of Health (NIH) [233].

Studies measuring the effects of ceftiofur use on *Salmonella* populations in feedlot cattle are limited. Only Ohta et al. (2017) conducted a 26-day longitudinal randomized controlled cattle field trial to monitor the prevalence, phenotypic antibiotic resistance, and serotype distribution of *Salmonella* following a single-dose (6.6 mg/kg) of subcutaneous CCFA injection [36]. This study explored the effects of ceftiofur treatment (or no treatment) on fecal *Salmonella* populations in beef cattle. The trial was conducted in the research feedlot facility at West Texas A&M University, Canyon, TX. The antibiotic was administered to all animals in two pens (approximately 22 cattle) for two replicates (total of four pens, 44 cattle, 11 cattle in each pen) on Day 0 for the treatment group, the same number of animals were included in the control group with one of 11 cattle in each of 4 pens receiving the CCFA treatment, the fecal samples were collected before and after the antibiotic administration (on Days 0, 4, 8, 14, 20, and 26). According to this study, *Salmonella* prevalence significantly decreased ($P < 0.004$) by Days 4 and 8 in the ceftiofur treated group, but a recovery was observed after Day 8 until the end of the study. The ceftiofur treatment also selected for MDR *Salmonella* compared to the non-treated group, but this was not statistically significant ($P > 0.05$), since the *Salmonella* population that was found before the antibiotic treatment was pan-susceptible. However, after the treatment, 26.7% and 22.7% of the population exhibited the MDR phenotype in cattle that received ceftiofur on days 4 and 8, respectively. Interestingly, the MDR prevalence dropped to 3.2% and 3.6% by Days 14 and 20 and increased back to 12.9% by the end of the study (Day 26). In contrast, the control group, with only one ceftiofur treated steer, had the highest overall *Salmonella* prevalence. In this group, the MDR *Salmonella* was only 3.2% on Day 4, was not observed by Day 8 and then increased again to 5.7% on Day

14. By Day 20, 5.7% decreased to 3.8%. There were no MDR *Salmonella* observed in this group on Day 26 (Figure 7).

This study clearly showed that the ceftiofur treatment selected for MDR *Salmonella* starting 4 days after the treatment and continued selecting for MDR *Salmonella* in the feces by the end of the study. Ohta et al. (2017) also explored the serotype distribution of these *Salmonella* isolates using WGS data with SeqSero and MLST databases. The serotypes and matching ST types found in this study as listed from the most prevalent to least as: 1) *S.* Mbandaka (ST 413), 2) *S.* Kentucky (ST 198), 3) *S.* Montevideo (ST 138), 4) *S.* Give (ST 654), 5) *S.* Reading (ST 1628), and 6) *S.* Anatum (ST 64).

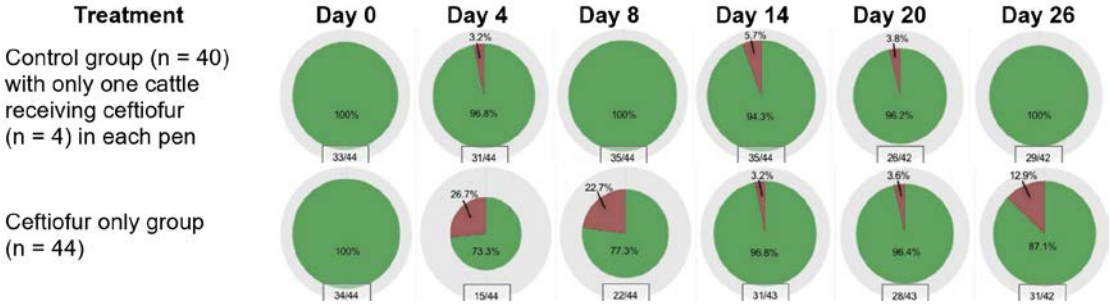


Figure 7. MDR *Salmonella* prevalence after single-dose ceftiofur treatment from Ohta et.al (2017)

Grey: the proportional size of the total samples tested, green: the proportional size of the pan-susceptible *Salmonella*, red: the proportional size of the MDR *Salmonella*. Adapted from Ohta et al.(2017) [36].

The molecular insights of this study also showed that the MDR profile of *Salmonella* was strongly associated ($P < 0.05$) with the serotype *S.* Reading. This serotype had the ACSSuT + Cef resistance profile and was first recovered after Day 4 in both ceftiofur treated and non-treated cattle; however, this serotype dramatically increased and

remained in the ceftiofur treated group until the end of the study, whereas in the control group it was always detected at low-numbers and was not detected on Day 26. Interestingly, this serotype was not identified on Day 0; however, *S*. Reading was likely present in the feces or environment but was not prevalent without the selection pressure of the antibiotic and therefore was not identified.

Other serotypes isolated from this study were mostly pan-susceptible. That study was the only published longitudinal controlled field study that measured the direct effects of ceftiofur on fecal *Salmonella* populations during 26 days following the antibiotic treatment. Due to the time limitation of this study, the dynamics of the MDR *Salmonella* population observed on Day 26 remained unknown at slaughter, which typically takes 90 or more days.

Within the same cattle study of Ohta et al. (2017), Kanwar et al. (2013) explored the effects of ceftiofur treatment on the *E. coli* population focusing on the phenotypic antibiotic resistance profiles along with the *bla*$_{\text{CMY-2}}$ gene, both before and after treatment on Days 0, 4, 12, and 24 [38]. The results of the studies published by Kanwar et al. (2013), showed that ceftiofur had a moderate selective pressure for MDR *E. coli* by reducing the proportion of ceftiofur or ceftriaxone susceptible strains against the resistant *E. coli* population especially on Day 4; however, this recovered back to the initial levels starting from Day 7, and a total recovery was observed by Day 26. This study also found that the ceftiofur treatment significantly increased the likelihood of an isolate being phenotypically resistant to five to ten antibiotics. The majority of the ceftiofur/ceftriaxone resistant isolates carried the bla$_{\text{CMY-2}}$ gene that was mainly found among isolates with phenotypic MDR profiles.

Within the same study, the effects of ceftiofur were also investigated focusing on the fecal microbiome population and *bla*$_{\text{CMY-2}}$, *bla*$_{\text{CTX-M,}}$ and 16S rRNA gene distribution using qPCR for only one replicate of cattle (two pens, 44 cattle) before and after treatment on Days 0, 4, 12, and 24 [37]. This study reported similar findings to the previous study. However, within this study, fecal samples from both replicates were explored (total of 4 pens with 44 cattle). Their results showed that ceftiofur statistically and significantly ($P <$ 0.05) increased the 16S standardized and non-standardized ceftiofur resistance genes (*bla*$_{\text{CMY-2}}$, and *bla*$_{\text{CTX-M}}$) copy numbers per gram feces on day 4 compared to Day 0; however, this trend was not significant after day 4 until Day 26. Interestingly, the total number of 16S genes were not affected by the treatment during this study.

Weinroth et al. (2018) further investigated these fecal samples collected from the study of Ohta et al. (2017), focusing on the effects of ceftiofur treatment on the resistomes of feces collected from only Day 0 and Day 26, and further focusing on the β-lactam genes using shotgun metagenomics [47]. They sequenced a total of 32 pooled metagenomic DNA samples. The β-lactam genes were found to be higher on Day 26 when compared to Day 0. However, this difference was not statistically significant ($P < 0.05$) and was not associated with ceftiofur treatment. Their results did not indicate any significant effects of treatment groups on the number of β-lactam genes after the treatment on Day 26.

Lowrance et al. (2007) investigated three different doses/regimens (single-dose of 6.6 mg/kg on Day 0, single-dose of 4.4 mg/kg on Day 0, and three doses of 6.6 mg/kg on Days 0, 6, and 13), of subcutaneously injected ceftiofur on fecal *E. coli* populations recovered from 30 cattle (each group had 10 cattle housed in two pens) [39]. Another 31 cattle remained as a control group without any ceftiofur treatment. Fecal samples from

these cattle were collected before treatment (on Day 0) and following the treatment on Days 0, 2, 6, 9, 13, 16, 20, and 28. The findings of this study clearly demonstrated that the ceftiofur treatment, regardless of dose and regimen, significantly increased the ceftiofur resistant and ceftriaxone resistant *E.coli* and reduced the susceptible *E. coli* population starting from Day 0 until Day 28; further, these levels were recovered back to Day 0 baseline values by Day 28. However, the exception was of the lowest dose (4.4 mg/kg; extra-label and illegal to use since 2012) regimen, which recovered back to baseline values after Day 26, and was not significantly different than the control group on that day. Following the multiple-dose administration (also illegal since 2012) of 6.6 mg/kg ceftiofur after Day 9, ceftiofur resistance was significantly higher than the remaining ceftiofur treatment groups on Days 13, 16, and 20. However, this group was not significantly different than the 6.6 mg/kg single-dose administered cattle group on Day 26. Their overall result shows that ceftiofur had a selective effect on the *E. coli* population, and repeated use of ceftiofur resulted in the persistence of resistance in cattle fecal *E. coli* population.

Alali et al. (2009) further investigated the study of Lowrance et al. (2007), to measure the effect of these regimens on the quantity the bla_{CMY-2} gene recovered from the feces on days 0, 3, 7, 10, 14, 18, 21, and 28 using qPCR [41]. This study showed that the administration of ceftiofur for all treatment groups increased the absolute and normalized (with 16*S*rRNA gene) number of bla_{CMY-2} genes detected in fecal samples compared to the control group throughout the 28-day period [41]. This study showed that administration of ceftiofur both at 4.4 and 6.6 mg/kg doses increased the selection pressure and the number

of both standardized and non-standardized *bla*$_{\text{CMY-2}}$ genes detected in fecal samples. This finding was similar to the findings of Kanwar et al. (2014).

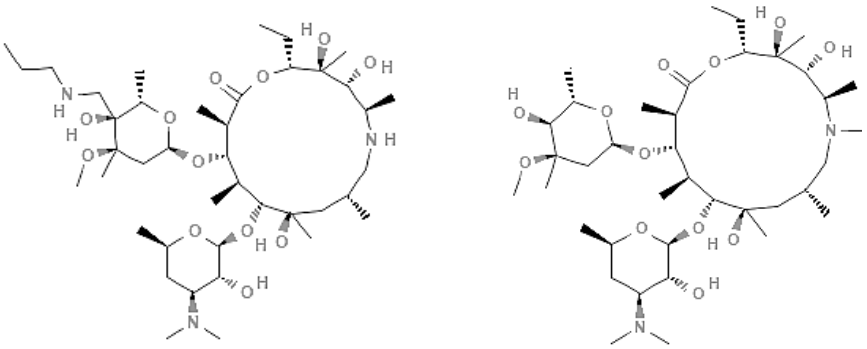
Overall findings of the studies presented above show that ceftiofur poses transient but significant selective pressures on both ceftiofur and ceftriaxone resistance profiles of *Salmonella* and *E. coli* recovered from the feces. Usually, after a single-dose of subcutaneous 6.6 mg/kg ceftiofur treatment on Day 0, the temporary selection pressure on these bacteria species or resistome subsides and the microbiome recovers close to baseline values by Days 26 or 28. However, the dynamics remain unknown until the slaughter age. In addition, none of these studies evaluated the populations observed in cattle lymph nodes or on the cattle hides, which are potent sources of carcass contamination by cattle origin *Salmonella* at slaughterhouses.

2.9.2. Macrolides

The macrolide class antibiotics that are used in cattle are erythromycin, gamithromycin, tildipirosin, tilmicosin, tulathromycin, tylosin, and tylvalosin (Table 2). To address the potential for these macrolides used in cattle to select for azithromycin resistance, it is important to understand the classification of macrolides and their chemical/molecular structures.

There are three major subclasses of macrolide antibiotics that are divided based on the number of carbons in their macrolactonic rings (C); these are listed as macrolides, azalides, and ketolides. These subclasses also are further divided into two groups as natural or semisynthetic. Azalides are 15-C semisynthetic antibiotics, which include the following: azithromycin, gamithromycin, and tulathromycin antibiotics. In the azalide

class, there is no natural group that exists [235]. The remaining antibiotics that are used for cattle are in the macrolide subclass, such as erythromycin (natural-14C), tylosin (natural-15C), and tildipirosin, tilmicosin, and tylvalosin (semisynthetic-16C) [235]. In cattle, gamithromycin and tulathromycin are the only azalide class macrolides used for BRD treatment and control [34]. Both antibiotics have the potential to select for azithromycin resistance in *Salmonella*. Among these two antibiotics, tulathromycin is the most commonly used macrolide for BRD metaphylaxis in cattle. Tulathromycin and azithromycin have very similar chemical structures [233] (Figure 8). Therefore, evaluating the potential effects of tulathromycin on *Salmonella* is important.



Tulathromycin ($C_{41}H_{79}N_3O_{12}$) Azithromycin ($C_{38}H_{72}N_2O_{12}$)

Figure 8. Chemical structure of tulathromycin and azithromycin

Source: Open chemistry database (PubChem) at the NIH [233].

The effects of tulathromycin metaphylaxis (single-dose 2.5 mg/kg) in relation to selection for azithromycin resistance has not been studied either for *Salmonella* or for *E. coli*, or for any other enteric bacterial species in cattle. There are only two recently published (Doster et al. [2018] and Holman et al. [2019]) randomized controlled cattle

field studies focused on the effects of tulathromycin and only on enteric microbiota and the resistome [46, 51].

Doster et al. (2018) provided a comparison of the fecal resistome and microbiome among tulathromycin-treated and non-treated beef cattle before treatment (day 1) and after treatment (day 11) early in the feeding period (35). Their study suggested that there was no significant difference ($P < 0.05$) in the cattle resistome and microbiome among tulathromycin treated and control groups; however, their results may be related to their small sample size (n = 15 per treatment group by day). The authors did, however, report a significant increase in the 16S rRNA normalized ARG abundance and the average relative abundance of microbial taxa between Days 1 and 11. Similar to the study of Weintroth et al. (2018) [47], Doster et al. (2018) ignored the likely peak day (close to Day 7) of antibiotic effect on enteric populations; importantly, their study did not measure the long-term effects of tulathromycin treatment out to slaughter eligibility.

Holman et al. (2019), evaluated the single-dose effects of metaphylaxis on the fecal microbiota of 24 beef cattle (12 each for tulathromycin treated and non-treated cattle groups) [51]. Cattle fecal swabs were collected three days before transportation (Day -5), before the tulathromycin treatment after two days of acclimation period (on Day 0), and following the treatment on Days 2, 5, 12, 19, and 34. The ARGs conferring macrolide resistance via *erm*A and *erm*X were quantified using qPCR and results were normalized using the 16SrRNA gene copy number. In their study, they only found *erm*X gene conferring macrolide resistance in the fecal microbiota of cattle, which did not differ among the treated and non-treated cattle when the results were normalized with the 16S gene copy numbers. These cattle had a relatively abundant *erm*X gene in their feces before

the treatment on Day 0. However, they reported a fecal microbial community shift observed before and after the transport, which was similar for all cattle prior to the cattle placement to the experimental feedlot. This observation was more likely related to the cattle group that was selected from a closed herd, and which was already commonly exposed to a similar environmental bacterial flora and other environmental factors that can affect microbial diversity. Specifically, the operational taxonomic unit (OTU) richness was reported to have decreased after the transportation, when cattle were housed in the same feedlot. These findings suggest that the feedlot environment may reduce the microbial diversity in cattle feces, following transport to the feedlot.

2.10. Summary

To summarize, the group-level administration of antibiotics to beef cattle represents a routine application by farmers to control BRD upon arrival to the feedlot. Because feedlots are the last production stage prior to slaughter, it is important to assess the contribution of the use of BRD control antibiotics – early, during the feeding period, and most importantly at slaughter – in affecting AR *Salmonella* populations in human. Macrolides and 3rd generation cephalosporins play a critical role in the treatment of human *Salmonella* infections. Ceftiofur and tulathromycin, which are the analogs of ceftriaxone and azithromycin are widely used in feedlot cattle for cohort-level control of BRD.

The study previously published by Ohta et al. (2017) showed an expansion of MDR *Salmonella* related to a single-dose of ceftiofur metaphylaxis on Day 4, which remained prevalent until the end of the study (Day 26) [36]. In their study, the MDR phenotype was strongly associated with *S.* Reading. Their study was the only published longitudinal

controlled field study that has measured the direct effects of ceftiofur on *Salmonella* populations for 26 days following antibiotic administration. However, in that study, the dynamics and persistence of MDR *Salmonella* between Day 26 and slaughter (typically, 90+ days) remains unknown. In parallel, studies conducted to measure ceftiofur treatment effects on the fecal *E. coli* populations [38, 39] and microbiome found significant or transient selective pressures on ceftiofur/ceftriaxone resistance [37, 41].

There is a lack of research measuring the effects of tulathromycin metaphylaxis on *Salmonella* and any other enteric bacterial species in cattle. Only Doster et al. (2018) and Holman et al. (2019) have conducted studies measuring the effects of tulathromycin on the cattle fecal microbiome and resistome, and only in limited ways. Both of these studies compared the enteric microbiome and resistome, between tulathromycin-treated and non-treated beef cattle population, before treatment and after treatment early (up to day 11 and 34, respectively) in the feeding period and showed no statistically significant ($P < 0.05$) effect [46].

None of the studies mentioned above was conducted until slaughter age and they also did not explore other contamination sources of beef products besides the feces. Clearly, there is a strong need for a longitudinal controlled study measuring longer-term effects of ceftiofur and tulathromycin on prevalence, quantity and antibiotic resistance patterns of *Salmonella* populations found not only in feces but also on hides and in lymph nodes of cattle, each of which represents potential contamination sources of *Salmonella* at slaughter.

Given the importance of the increasing ceftriaxone- and azithromycin-resistant *Salmonella* populations observed in human infections, the extensive use of these antibiotic

analogs in cattle feedlots, that beef is one of the important sources of *Salmonella* human outbreaks, and the lack of research on this subject, an immediate need for a randomized and controlled longitudinal study measuring effects of ceftiofur and tulathromycin on *Salmonella* populations is apparent. A study is especially needed to determine the antibiotic resistance profiles and the population dynamics of *Salmonella* monitored for a longer duration than might be affected by the antibiotic treatments. The focus of such a study should not only be on the fecal matter, but also the lymph nodes and the hide surfaces of cattle, which are known to be potential carcass and beef product contamination sources by *Salmonella* at slaughter in cattle.

3. MATERIALS AND METHODS*

A randomized and controlled cattle field trial was conducted with one hundred thirty-four crossbred yearling cattle (beef steers) to measure long term effects of single-dose ceftiofur and tulathromycin treatment on *Salmonella* populations in the West Texas A&M University Research Feedlot in Canyon, Texas, USA.

3.1. Study population

The animal field trial was approved by the West Texas A&M University/ Cooperative Research, Educational and Extension Team Institutional Animal Care and Use Committee (Protocol no. 05-09-15).

Previously backgrounded healthy cattle were purchased from two different sources; 99 cattle were purchased from Abilene, Texas (430 km southeast of the experimental feedlot) and 35 cattle were purchased from Hereford, Texas (48 km southwest of Canyon) (Figure 9). The prior antibiotic administration history of these cattle was unknown.

Cattle were transported to the experimental feedlot in Canyon, TX with an initial body weight of 310-370 kg. Upon arrival to the feedlot, individual steers were identified with a colored ear tag and an electronic ear tag in both ears. After a three-day acclimation period, these cattle were source- and weight-blocked and then randomly assigned to treatment groups to control for possible origin- and host-related confounders. The staff in the feedlot, slaughter plant, and in the microbiological laboratories were also blinded as to

*Part of this chapter is reprinted with permission from "Population Dynamics of *Salmonella enterica* within Beef Cattle Cohorts Followed from Single-Dose Metaphylactic Antibiotic Treatment until Slaughter" Levent G, Schlochtermeier A, Ives SE, Norman KN, Lawhon SD, Loneragan GH, Anderson RC, Vinasco J, Scott HM. 2019 Appl Environ Microbiol 85:e01386-19, Copyright © 2019 American Society for Microbiology.

treatment to avoid possible biases. This was possible since no differences existing in pen rations or other treatments after initial metaphylaxis was administered.

During the feeding period, the same ration was provided for all cattle, regardless of source, block, or treatment. Cattle were provided starter, grower, and finishing diets consisting of varying concentrations of wet corn gluten feed, chopped-corn stalks, steam-flaked corn, and mineral supplements (without antibiotics) progressively throughout the feeding period. Each pen was provided with two automated watering bowls.

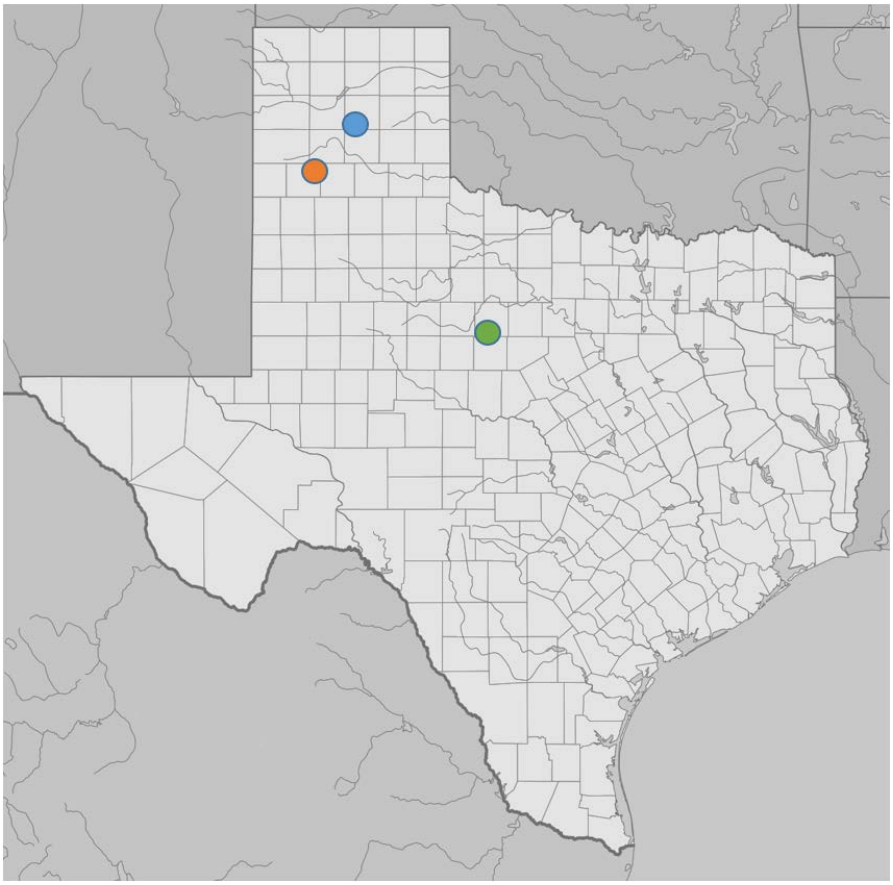


Figure 9. Map of Texas representing the sources of cattle and the experimental feedlot

The experimental feedlot in Canyon, TX (blue), Source 1: Hereford, TX (orange), and Source 2: Abilene, TX (green). Adapted from Wikimedia Commons, https://commons.wikimedia.org. The map has been made or improved in the German Kartenwerkstatt.

3.2. Experimental design

Beef cattle (n = 134) were allocated into four blocks (Blocks 1-4) by source and initial body weights. Steers in each block (33-35 cattle) were further randomly allocated into three pens. Each pen (11-12 cattle) in a given block received either ceftiofur, tulathromycin, or else remained as a control group. One or two cattle in the treatment pens remained as also a control (untreated) steer to measure the pen-level effects of the treatments. In the end, ten cattle received individual-level treatment (receiving either

ceftiofur or tulathromycin) in each antibiotic treatment pen (Table 3). Overall, 40 cattle received ceftiofur, 40 cattle received tulathromycin and 44 cattle remained as controls.

Table 3. Descriptive field trial data for cattle source, block, pen, treatment, and slaughter days

Source	Blocks	Pen identifier	Number of cattle	Pen level Treatment	Slaughter day
1	1	7	12	Tulathromycin	134
1	1	8	11	Control	134
1	1	9	12	Ceftiofur	134
2	2	51	11	Tulathromycin	141
2	2	52	11	Control	141
2	2	53	11	Ceftiofur	141
2	3	54	11	Control	120
2	3	55	11	Ceftiofur	120
2	3	56	11	Tulathromycin	120
2	4	57	11	Ceftiofur	99
2	4	58	11	Tulathromycin	99
2	4	59	11	Control	99

Reprinted with permission from Levent et al. (2019) [236].

After the pen allocations at the sorting pens, cattle were placed into their study pens (Figure 10). To eliminate possible source bias, cattle from Hereford (Pen 7-9) were placed at the far western end of the feeding area. The cattle from Abilene were allocated into the remaining nine pens (Pen 51-59).

After the placement, the trial began (Day 0) on March 14th, 2016. Before the antibiotic treatments were administered, fecal contents were collected *per rectum* from the individual cattle by feedlot staff eligible to perform these tasks under the AUP. These samples were considered as the study baseline samples for feces. After sampling, the cattle

in the treatment groups that were assigned to have individual treatments received a single subcutaneous injection of either tulathromycin or ceftiofur.

Figure 10. Satellite view of pens located in the experimental feedlot

34°58'02.2"N 101°48'04.8"W, retrieved on October 2, 2019. Source: https://goo.gl/maps/pzWU9f2LeG2EsMXCA.

Tulathromycin (Draxxin®, Zoetis, Kalamazoo, MI) was injected subcutaneously in the neck at a therapeutic dose of 2.5 mg/kg. Ceftiofur crystalline-free acid (Excede®, Zoetis Inc., Kalamazoo, MI) was injected subcutaneously in the posterior aspect of the ear at 6.6 mg/kg. Cattle remaining as control animals did not receive any antibiotic treatment.

3.3. Sampling frame

Fecal samples were the only type of sample that was collected before the treatment and after the treatment during the feeding period in order to evaluate pre- and post-treatment effects of antibiotics on *Salmonella* from Day 0 until Day 99. In addition to the feeding period, fecal samples, lymph nodes and hide swabs were collected at slaughter age starting after Day 98 (on Days 99, 120, 134, and 141), in order to measure the dynamics of the *Salmonella* population in ready-to slaughter cattle (Figure 11). This trial was completed on August 2nd, 2016.

Each block of cattle was sent to slaughter on a single day after at least 98 days of feeding. The decision as to timing of slaughter was made based on cattle approaching their desired body weights (varying from 450-635 kg) for slaughter. Each block of cattle was sent to slaughter on different days: Block 4 (Day 99), Block 3 (Day 120), Block 1(Day 134), and Block 2 (Day 141) (Table 4).

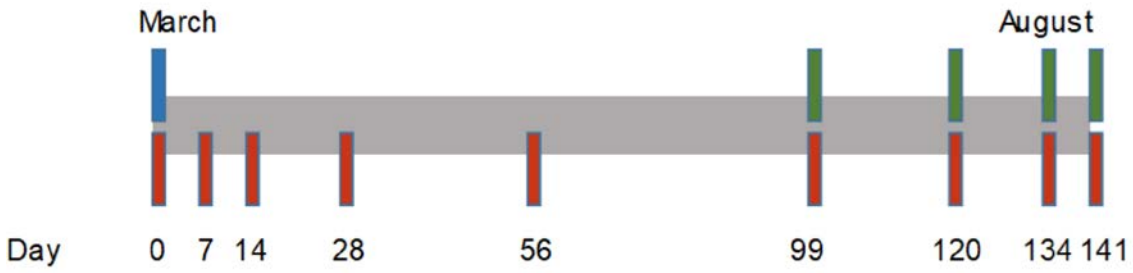


Figure 11. Timeline of the study

Antibiotic injection (blue) and fecal (red), and lymph node and hide collection (green) days. Fecal and hide samples were collected one day prior to the day indicated at slaughter. Reprinted with permission from Levent et al. (2019) [236].

Table 4. Planned sampling day, date, and sample type collection scheme

Sampling day	Sampling date	Sample type	Number of cattle	Pen
0	14-Mar-2016	Feces	134	All
7	21-Mar-2016	Feces	134	All
14	28-Mar-2016	Feces	134	All
28	11-Apr-2016	Feces	134	All
56	09-May-2016	Feces	134	All
99	21-Jun-2016	Feces, LN, Hide	33 (Block 4)	57-58-59
120	12-Jul-2016	Feces, LN, Hide	33 (Block 3)	54-55-56
134	26-Jul-2016	Feces, LN, Hide	35 (Block 1)	7-8-9
141	02-Aug-2016	Feces, LN, Hide	33 (Block 2)	51-52-53

LN (lymph node).

Feces were collected before treatment (Day 0) and then one week after the treatment (Day 7) to measure the short-term effects of antibiotics. Individual cattle were also sampled two weeks (Day 14), four weeks (Day 28) and eight weeks (Day 56) later in order to determine longer-term feeding period effects. Effects at slaughter of the early period antibiotics on *Salmonella* were measured in feces collected the day before slaughter (after day 98 for all cattle in all blocks).

On each fecal sampling day, individual cattle were restrained in a squeeze chute and weighed in the animal handling facility at 5 a.m. in the morning. Sterile full-length obstetric sleeves were used to collect approximately 25 g of feces from each cattle *per rectum*. After collection, each glove was inverted, knotted, and labeled with four-digit individual animal identifiers.

Two bilateral sub-iliac lymph nodes from the flank region also were collected during the slaughter process from each carcass that had passed both ante- and post-mortem inspections conducted by federal USDA-FSIS inspectors. Fatty tissues containing lymph

nodes from the flank region of individual steer carcasses were excised before the fabrication of the carcass and placed into 2.5-gallon Zip-lock plastic bags. Each bag was marked with a unique four-digit animal identifier.

Protocols from previously published studies were adapted to determine *Salmonella* carriage on cattle hides at slaughter [69, 117, 237]. Briefly, a one m^2 area of the brisket (cranial ventral) hide area of individual steers was rubbed with a sterile sponge (Whirl-Pak™ Speci-Sponge Environmental Sampling Bag, Nasco), which was pre-moistened with 25 ml Butterfield's phosphate buffer solution (REMEL®, Lenexa, KS) using sterile gloves. Hide swab collection was performed one day prior to slaughter in order the measure the likely *Salmonella* carriage on hide surfaces when cattle entered the plant. These samples were collected at the same time as the terminal day fecal samples.

Feces, lymph node and hide samples were immediately placed into the cooler with ice packs to maintain the cold chain at 4°C and shipped to the laboratory at the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University after each sampling day.

3.4. Sample processing

Microbiological methods, including those performed on animal tissues, were performed under a Texas A&M University Institutional Biosafety Committee permit (IBC2017-049). Sample processing and microbiological methods were performed in biosafety level-2 biological safety cabinets.

Upon the arrival to the laboratory, fecal samples were placed into sterile glycerol (at a ratio of 1:1) and also stored without glycerol in 5 ml polypropylene tubes,

homogenized via vortexing, and then preserved at −80 °C until the time of microbiological and molecular processing. In contrast, the lymph nodes and hide swabs were first pre-enriched with TSB (Bacto, Becton Dickinson, Sparks, MD) and then placed into glycerol and non-glycerol tubes, homogenized and stored at −80 °C. Prior to starting the microbiological processing of feces, samples were taken out of the freezer and thawed on ice at room temperature for about 30 min. A 0.5 g aliquot of each fecal sample was weighed and placed into 10 ml sterile plastic tubes with 5 ml of TSB. The suspension was homogenized via vortex for 1 min.

Two large chunks of fat tissue were received from each individual animal. The embedded lymph nodes were found by dissection and carefully cleaned from the excess fat and fascial tissue without causing rupture to the lymph node capsule by using a sterile knife, scalpel, and scissors (Figure 12).

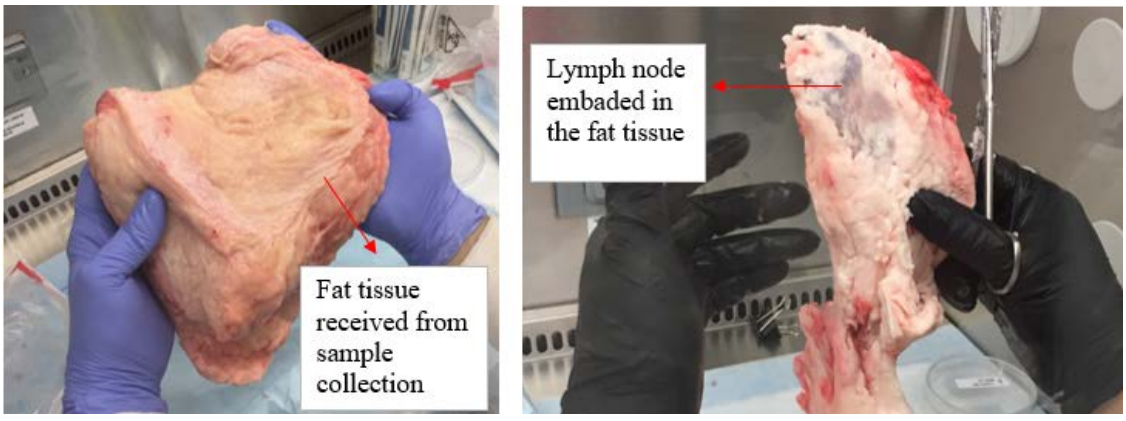


Figure 12. Photo of a lymph node embedded in fat tissue

Afterward, each lymph node was placed onto a sterile petri dish and weighed. The trimmed and cleaned lymph nodes were exposed to boiling water, depending on size, for 3 to 6 seconds to achieve surface sterilization via parboiling. Right after the sterilization, each lymph node was placed inside the filter bag of a sterile plastic stomacher filter bag (Seward, Norfolk, UK) and then pulverized with a rubber mallet. After the pulverization step, 80 ml of TSB was added into the bag. Later, the suspension was homogenized using a stomacher (Seward Circulator 400, Norfolk, UK) at 230 revolutions per min (rpm) for 2 min.

The pre-moisturized hide swab sponges in the plastic Whirl-pack bags were suspended with 75 ml of TSB. These bags were placed into the stomacher and homogenized at 230 rpm for 2 min.

3.5. Quantification of *Salmonella*

After homogenization, each of the three sample type TSB suspensions was subjected to a short incubation at 42°C for 3 h. After the 3 h incubation, a 50 µl aliquot of the suspension was spiral-plated onto BGA (Difco, Becton Dickinson, Sparks, MD) containing 25 g/L of novobiocin sodium salt (Sigma Aldrich, N1628-5G) – in order to reduce the false-positive counts [70] – using the Eddy Jet 2 Spiral Plater (Neutec Group Inc, Farmingdale, NY). The BGA plus novobiocin plates were incubated at 37 °C for 18 h. After incubation, colonies typical (pink and rounded) of *Salmonella* were counted to obtain CFU numbers using the Flash & Go Automatic Colony Counter (IUL Instruments S. A.). The CFUs were later back-calculated – either per gram (feces and lymph node) or per m2 (hide) basis – accounting for the appropriate dilutions.

3.6. Isolation of *Salmonella*

After the initial 3 h incubation of the three sample types, 1 ml of TSB suspension was transferred into 9 ml tetrathionate broth (Difco, Becton Dickinson, Sparks, MD) containing 180 µl iodine solution (REMEL®, Lenexa, KS) and further incubated at 37 ºC for 24 h. After incubation, 100 µl of the bacterial suspension of tetrathionate broth was transferred into 10 ml RV (Difco, Becton Dickinson, Sparks, MD) and further incubated at 42 ºC for 18 h. Upon completion of RV incubation, a 50-µL aliquot was spiral-plated onto the plain BGA plate. The plates were further incubated at 37 °C for 18 h. After the specific enrichment broth steps, up to two pink round colonies emerging on BGA plate were streaked onto blood agar (tryptic soy agar with 5% sheep blood, Remel, Lenexa, KS) and incubated at 37 °C for 18 h. A loop full of culture from each isolate was placed into CryoCare beads (Scientific Device Laboratory, Des Plaines, IL), mixed well and stored at −80 °C for further characterization at a later date.

3.7. Confirmatory tests

Salmonella colonies were subjected to a serum agglutination testing by using *Salmonella* O Antiserum Poly A-I & Vi (factors 1-16, 19, 22-25, 34, Vi) (Difco, Becton Dickinson, Sparks, MD) for initial screening. Approximately 100 µl of antisera was placed on a sterile petri dish and mixed with pure colonies. The reaction was observed for 30 seconds for signs of agglutination. The agglutination positive isolates were accepted as presumptive *Salmonella* isolates. After the preliminary confirmation with antisera, the MALDI-TOF MS method was used for secondary confirmation. Isolates grown on blood agar were removed using a sterile toothpick and applied to the MALDI sample target

(Bruker Daltonics, Bremen, Germany) as a thin layer. Next, one µl of Bruker HCCA (α-Cyano-4-hydroxycinnamic acid) matrix solution was applied to each well containing bacteria and the plate was air-dried. A quality control (bacterial test standard, Bruker Daltonics) and negative control (matrix only) were included in each plate. The Microflex LT instrument (Bruker Daltonics GmbH, Leipzig, Germany) was used to measure the protein-mass. Spectra were analyzed using FlexControl® v.3.4 and MBT Compass software (Bruker Daltonics GmbH, Leipzig, Germany). Genus and species were identified using the main spectra (Bruker Daltonics [BDAL] main spectrum profile [MSP]) library. *Salmonella* isolates scoring ≥ 2.0 were confirmed as *Salmonella.* The isolates confirmed by both confirmation methods were considered as *Salmonella* and were carried to the next step of analysis.

3.8. Phenotypic antibiotic susceptibility

Phenotypic antibiotic susceptibilities of each *Salmonella* isolate were determined by the broth microdilution method on the Sensititre® system (TREK, Thermo Scientific Microbiology, Oakwood Village, OH) using the National Antimicrobial Resistance Monitoring System (NARMS) gram-negative (CMV3AGNF, Thermo Fisher Scientific, Waltham, MA) panel. The NARMS gram-negative panel contains various concentrations of 14 antibiotics (9 classes of antibiotics) including cefoxitin, azithromycin, chloramphenicol, tetracycline, ceftriaxone, amoxicillin-clavulanic acid, ciprofloxacin, gentamicin, nalidixic acid, ceftiofur, sulfisoxazole, trimethoprim-sulfamethoxazole, ampicillin, and streptomycin (Figure 13). The MICs for each antibiotic were determined for each isolate using the same laboratory conditions across all assays.

The isolates were inoculated onto blood agar and incubated at 37 °C for 18 h. Approximately one colony was picked and suspended in 5 ml sterile deionized water to adjust to a 0.5 McFarland equivalence turbidity standard (Remel®, Lenexa, KS) using the Sensititre® Nephelometer (TREK, Thermo Scientific Microbiology, Oakwood Village, OH). Next, 50 µl of the suspension was transferred to 11 ml Mueller-Hinton (MH) broth (Thermo Scientific Microbiology, Oakwood Village, OH), and 50 µl of MH broth was delivered to each well of the Sensititre plate using the TREK Sensititre® Automated Inoculation Delivery System (TREK, Thermo Scientific Microbiology, Oakwood Village, OH).

FOX 32	AZI 8	CHL 16	AXO 64	AXO 0.25	CIP 2	GEN 16	NAL 16	XNL 2	FIS 32	AMP 32	STR 16
FOX 16	AZI 4	CHL 8	AXO 32	AUG2 32/16	CIP 1	GEN 8	NAL 8	XNL 1	FIS 16	AMP 16	STR 8
FOX 8	AZI 2	CHL 4	AXO 16	AUG2 16/8	CIP 0.5	GEN 4	NAL 4	XNL 0.5	SXT 4/76	AMP 8	STR 4
FOX 4	AZI 1	CHL 2	AXO 8	AUG2 8/4	CIP 0.25	GEN 2	NAL 2	XNL 0.25	SXT 2/38	AMP 4	STR 2
FOX 2	AZI 0.5	TET 32	AXO 4	AUG2 4/2	CIP 0.12	GEN 1	NAL 1	XNL 0.12	SXT 1/19	AMP 2	NEG
FOX 1	AZI 0.25	TET 16	AXO 2	AUG2 2/1	CIP 0.06	GEN 0.5	NAL 0.5	FIS 256	SXT 0.5/9.5	AMP 1	POS
FOX 0.5	AZI 0.12	TET 8	AXO 1	AUG2 1/0.5	CIP 0.03	GEN 0.25	XNL 8	FIS 128	SXT 0.25/4.75	STR 64	POS
AZI 16	CHL 32	TET 4	AXO 0.5	CIP 4	CIP 0.015	NAL 32	XNL 4	FIS 64	SXT 0.12/2.38	STR 32	POS

Figure 13. Gram-negative NARMS plate (CMV3AGNF) format

Cefoxitin (FOX), azithromycin (AZI), chloramphenicol (CHL), tetracycline (TET), ceftriaxone (AXO), amoxicillin/clavulanic acid 2:1 ratio (AUG2), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), ceftiofur (XNL), sulfisoxazole (FIS), trimethoprim/sulfamethoxazole (SXT), ampicillin (AMP), and streptomycin (STR). MICs are in µg/ml. Source: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FMBD%2FSpecification-Sheets%2FSensititre-Plate-Layout-GN4F.pdf&title=U2Vuc2l0aXRyZSBHTjRGIFBsYXRlIExheW91dA==

Each new lot number of CMV3AGNF plates was tested against quality control strains of *E. coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeroginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 (American Type Culture Collection, Manassas, VA) as recommended by the CLSI [90]

Plates were sealed and incubated at 37 °C for 18 h and then read using the Sensititre OptiRead™ Automated Fluorometric Plate Reading System (TREK, Thermo Scientific Microbiology). The results were initially interpreted as resistant, intermediate or susceptible based on CLSI methods and referent NARMS MIC breakpoint values where applicable [90]. The CLSI breakpoints for streptomycin in *Salmonella* are not established. NARMS used the breakpoint of ≥ 64 µg/ml for resistance between 1996 and 2013. After examining phenotypic and genotypic resistance mismatches of streptomycin in *Salmonella*, NARMS lowered the resistance breakpoint to ≥ 32 µg/ml and applied this standard to isolates tested since 2014. Azithromycin was included into the NARMS CMV3AGNF panel in 2011. The interpretation criteria for azithromycin are available for only *S.* Typhi, and this criterion was included by CLSI in 2014. Based on the CLSI interpretation, azithromycin susceptibility for *S.* Typhi is ≤ 16 µg/ml and the resistance value is ≥ 32 µg/ml. NARMS uses these breakpoints for non-typhoidal *Salmonella* strains in order to monitor resistance in *Salmonella*; however, these criteria were not considered as valid to predict the clinical efficacy of azithromycin on non-typhoidal *Salmonella* [32].

Since the MIC interpretative criteria for azithromycin and streptomycin are not established for non-typhoidal *Salmonella* by CLSI, the NARMS interpretative criteria (https://www.cdc.gov/narms/antibiotics-tested.html) were used for azithromycin (≥ 32 µg/ml), and streptomycin (≥ 32 µg/ml) in this dissertation [32] (Table 5).

Table 5. Interpretative MIC criteria of antibiotics used in this study

CLSI Class	Antimicrobial Agent	MIC (µg/ml)			
		Sus	Int	Res	Plate
β- Lactam/ β-lactamase inhibitor combinations	Amoxicillin-clavulanic acid	≤8/4	16/8	≥32/16	≥32
Penicillins	Ampicillin	≤8	16	≥32	≥32
Macrolides	Azithromycin	≤16	NA	≥32	>16
	Cefoxitin	≤8	16	≥32	≥32
Cephems	Ceftiofur	≤2	4	≥8	≥8
	Ceftriaxone	≤1	2	≥4	≥4
Phenicols	Chloramphenicol	≤8	16	≥32	≥32
Quinolones	Ciprofloxacin	≤0.06	0.12-0.5	≥1	≥1
Aminoglycosides	Gentamicin	≤4	8	≥16	≥16
Quinolones	Nalidixic acid	≤16	NA	≥32	≥32
Aminoglycosides	Streptomycin	≤16	NA	≥32	≥64
Folate pathway inhibitors	Sulfisoxazole	≤256	NA	≥512	>256
Tetracyclines	Tetracycline	≤4	8	≥16	≥16
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	≤2/38	NA	≥4/76	≥4

Sus (Susceptible), Int (Intermediate), Res (Resistant), Plate (Sensititre panel upper detection limit). Adapted from https://www.cdc.gov/narms/antibiotics-tested.html in September 2019.

3.9. DNA extraction and WGS

The QIAcube® HT (Qiagen, Valencia, CA) platform and QIAamp 96 DNA QIAcube® HT Kit (Qiagen, Valencia, CA) were used to extract the bacterial DNA from a single-colony, according to the manufacturer's instructions. A single-colony was suspended into 5 ml of TSB and incubated at 37 °C for 18-24 h. After incubation, 1 ml of suspension was transferred into a micro-collection tube (1.2 ml) and centrifuged at 4,000 rpm for 15 min at room temperature. After the incubation period, the supernatant was discarded and the remaining pellet was re-suspended in 180 ml of ATL-DX buffer (Qiagen, Valencia, CA) that was previously prepared with 15 ml ATL and 100 µl DX. One tube of small pathogen lysis beads (Qiagen, Valencia, CA) was added into the

suspension and the suspension was disrupted with the Qiagen TissueLyser system (Qiagen, Valencia, CA) for 5 min at 25 Hz. The tubes were briefly centrifuged to remove foam. Next, 40 µl of Proteinase K was added to each tube. The tubes were incubated for 1 h at 900 rpm and 56 °C followed by a heat shock for 10 min at 95 °C using a ThermoMixer (Eppendorf, Hauppauge, NY). The suspension was placed into the fridge at 4 °C for 5 min to cool down. Later, 4 µl of RNAse was added into the suspension and incubated for an additional 5 min at room temperature. After the bench step, samples were set in the QIAcube HT instrument for the DNA wash and elution steps using a modified protocol for gram-negative bacterial DNA extraction.

The DNA purity was assessed at 260 nm/280 nm ratio of absorbance on the FLUOstar® Omega multi-mode microplate reader (BMG LABTECH, Cary, NC). The DNA quantity was confirmed by fluorometric methods using Quant-IT PicoGreen dsDNA Assay kit (Thermo Fisher Scientific, Waltham, MA) on the FLUOstar® Omega multi-mode microplate reader and Qubit 1X dsDNA HS Assay Kit in the Qubit 4 Fluorometer (Life Technologies, Carlsbad, CA) following the manufacturers' instructions.

The Illumina® Nextera XT (or, the Flex) library preparation kit (Illumina, San Diego, USA) was used to generate sequencing libraries following the manufacturer's instructions -. The two kits (XT and Flex) were slightly different in terms of the reagents and the protocols used. However, the main principal steps of each kit were similar and include steps for tagmentation of DNA, amplification of libraries using specific index primers, pooling the libraries, and denaturation of the libraries. For tagmentation, the DNA was tagmented with specific buffers such as: TD®, ATM®, and NT® using the XT Kit, whereas the BLT® and the TB1® were used in the Flex Kit. After the tagmentation

step, a clean-up process was utilized in the Flex Kit protocol using the TSB® and TWB® reagents. The library amplification step was performed via PCR using the index primers (index 1-2 or index oligos) and PCR reagents (NPM® or EPM®). The libraries were cleaned up with ethanol and the reagents of RSB®, AMPure® beads and/or SPB®. To arrange the library sizes, the AMPure beads were applied when the XT Kit was used; whereas in the Flex Kit, this reaction took place in the tagmentation step using the BLT® (a bead-linked transposome reagent).

The fragment sizes of libraries were evaluated using the Standard Sensitivity NGS Fragment Analysis Kit® (Agilent, Santa Clara, CA) and Fragment Analyzer Automated CE System® (Advanced Analytical, Des Moines, IA). The library quantities were validated using the fluorometric methods performed by the FLUOstar® Omega multi-mode microplate reader and Qubit 4 Fluorometer. The fragment smear analysis and the concentration of the individual libraries were assessed based on the manufacturer's instructions. Good quality libraries, with fragments ranging from 250-1,500 bp (base-pair) and an average 500-600 bp length, were carried to the pooling and library denaturation steps. After cleaning the libraries, an additional normalization and denaturation step took place for the XT Kit using the LNA1®, LNB1®, LNW®, LNS®, and the sodium hydroxide reagents. On the other hand, the Flex Kit followed a straight denaturation process with sodium hydroxide, without the normalization step. After the libraries were pooled and denatured, they were loaded on sequencing cartridges with either MiSeq® Reagent v.2 or MiSeq® Reagent v.3 chemistry. The sequencing runs were performed on the Illumina® MiSeq platform (Illumina, San Diego, CA, USA) using Illumina® MiSeq Reagent

(Illumina, San Diego, CA, USA) v.2 chemistry with paired-end 2 × 250-bp reads or else v.3 chemistry with paired-end 2 × 300-bp reads[96].

3.10. Descriptive statistics

The data types obtained from our study were: 1) quantification of $\log_{10}$ CFU *Salmonella* (continuous, normalized), 2) number of samples with *Salmonella* following the enrichment (prevalence, binary), 3) phenotypic antibiotic resistance (binary: CLSI or NARMS ordered interpretive data reclassified as resistant or susceptible (includes the intermediate class)), 4) serotype and STs (multinomial categorical), 5) antimicrobial resistance genes (ARGs) (multinomial categorical), and 6) plasmids (multinomial categorical).

For descriptive statistics, data were initially cross-tabulated across sampling days and treatments by sample type. Overall frequency distributions of the data were determined graphically.

Phenotypic antibiotic resistance data, plus point estimates and confidence intervals (CIs), were examined individually (14 antibiotics) using the Clopper-Pearson exact method that calculates the "binomial exact CIs" based on the beta distribution rather than calculating the approximate CIs, which are based on the normal distribution [238].

Graphical visualizations and tables were generated using Microsoft Excel 2016 (Microsoft Corporation, Redmond, Washington, USA). For statistical analyses, the slaughter days were collapsed to a single-period (Day 112, also called terminal day) for all sample types.

Univariate analysis was performed using the likelihood-ratio chi-square test to address shared pen effects on serotype presence in hide samples. The interrater agreement test (kappa, - *kap*- in Stata) was applied to measure agreement between for quantifiable CFUs and enrichment results. Pairwise comparisons of the total genome assembly length means were conducted by serotype with equal variances hypothesis using Tukey's test.

3.11. Multi-variable regression analyses

Stata version 15.1 (StataCorp LLC, College Station, TX) was used for multi-variable analyses of the enrichment results and CFU counts. The regression analyses were performed separately for each of feces, lymph node and hide samples. For all sample types, the treatment was forced as a fixed effect. Day and treatment by day interactions were only forced as fixed effects in the regression model utilized for the fecal samples.

Clustering effects of pen and animal identifier were included in each model to avoid underestimating variance factors. Categorical pen identifiers were included in all models either as a random effect variable or else robust variance component, regardless of sample type. The individual animal identifiers were forced into each model where there was a repeated measurement (i.e., feces and lymph nodes), either as a random effect or else a robust variance component, except for the hide samples where there were no repeated measurements within animal.

The observations obtained from the control group were considered as baseline observations to measure the effect of either ceftiofur or tulathromycin on a variety of outcomes. In addition, Day 0 observations were considered as the baseline to measure the period effect among fecal observations.

3.11.1. Two-part and interval-linear regression models

Dilution factors were used to back-calculate either the CFUs in one gram of lymph node or fecal sample (recorded as CFU/g); or, on one meter-square of hide surface area (recorded as CFU/m^2). Results obtained from overall CFUs were log_{10} transformed to normalize data more suited to the assumptions of linear regression.

In our study, two types of regression models (two-part regression models or imputation models followed by interval-linear regression) were found to be well suited for different sample types and data features. The decision of selecting the appropriate model for CFUs was made based on two criteria: 1) presence of excess zero counts, and 2) likelihood of CFU zero counts were being either structural (true zero) or else sampled as zero (i.e., not zero, but under the LLQ of the assay).

Two-part models: 1) zero-inflated Poisson (ZIP), 2) zero-inflated negative binomial (ZINB), and 3) Cragg's hurdle (CHURDLE or hurdle) were explored for feces and lymph node observations. The log_{10} transformed CFUs were rounded for ZIP and ZINB regression models to obtain integers, as required for the models. The CHURDLE models directly utilized continuous log_{10} CFUs. Both the binary inflation (part 1) and the selection models (part-2) were built using the fixed effects of individual treatments (and interaction), sampling day (feces only) and pen; meanwhile, clustered robust variances were generated by the individual animal identifier for fecal and lymph node related regression analyses, due to limitations of two-part models to apply multi-level regression analysis (Table 6).

The best-fit model was selected according to the lowest Akaike's information criterion (AIC) or Bayesian information criterion (BIC) for feces and lymph nodes. Later,

the post-estimation marginal means and 95% CIs from the selected model were evaluated and visualized using - *marginsplot* - either in linear connected plot format (for feces) showing the day and treatment effects, or else in bar plots (for lymph nodes) showing the treatment effects. The average marginal effects were also computed using the multiple-pairwise comparisons and these comparisons were adjusted using Bonferroni's method [239] for each contrast of the fixed effects and their interactions.

Table 6. Parameters forced into the two-part regression models

Models	Sample types	Fixed effects	Robust variance
ZIP	Feces	Day, treatment, day#treatment*,Pen	Animal
	Lymph nodes	Treatment, Pen	Animal
ZINB	Feces	Day, treatment, day#treatment*, Pen	Animal
	Lymph nodes	Treatment, Pen	Animal
CHURDLE	Feces	Day, treatment, day#treatment*, Pen	Animal
	Lymph nodes	Treatment, Pen	Animal

*Interaction terms; the random effects forced as categorical observations.

A multiple imputation technique using an interval linear regression model was adapted to impute the only the zero CFUs as they were accepted as missing values (n=27) and the actual missing values (n=2) according to the distribution between zero and the LLQ for observations that were likely the sampling sampled as zero. Briefly, first the multiple imputation datum style was set to wide format, individual treatment and pen variables were added as the regular registered variables, and a new variable was assigned as the imputation variable. The imputation was performed using interval regression (- *mi impute intreg* -) including the new imputation variable as the dependent, and treatment and pen as the independent variables (plus, interactions) (49). The lower interval censoring

limit was set to zero, whereas the upper censoring limit was set to the LLQ value. Twenty imputations and a random seed number of 1234 were selected to begin the process of imputation according to the standards (29, 50). The estimations using the multiple imputation data were performed with multi-level mixed-effects linear regression model using - *mi estimate: mixed* - command with fixed effect of treatment and the random effect of pen. The marginal predictive counts of treatments were analyzed using the -mimargns- and graphically visualized using the - *marginsplot* - commands (51).

3.11.2. Logistic regression models

Multi-level mixed-effect logistic regression (-*melogit*-) was used for the binary coded observations obtained from the confirmed *Salmonella* isolated from the enrichment process separately for feces, lymph nodes, and hides. The default full-factorial model (or main fixed effects) of individual treatments and sampling days were added where appropriate to fecal, lymph node, and hide results (Table 7). The interaction terms for treatment by day were forced into the fecal model.

The variance correlation structures and matrices (- *estat vce, correlation* -) were explored before the final regression analyses. The pen-level and animal-level dependencies were determined to be exchangeable for all sample types; in contrast, individual animal dependencies were set as unstructured for feces (n = 6 per animal) and lymph nodes (n = 2 per animal). The predicted marginal means and the 95% CIs of day (feces only) and treatment were examined.

For the fecal regression analysis, graphical visualizations of the predicted margins and the 95% CIs were presented as a linear connected plot; on the other hand, the lymph

node and hide model results were presented as bar graphs showing only the treatment effects.

Table 7. Parameters forced into the logistic regression models

Sample types	Fixed effects	Random effects
Feces	Day, treatment, day#treatment*	Pen, animal
Lymph nodes	Treatment	Pen, animal
Hides	Treatment	Pen

*The interaction terms.

In addition, each fixed effect or interaction-term was explored using the stepwise comparisons from the full model to the intercept-only model. To obtain the direct effect of day and treatment on the predictive marginal means and the 95% CIs, each parameter was examined from the full model comparing to the intercept-only model by the -2(Log-likelihood), degrees of freedom (df), and *P*-values. The average marginal effects were also computed using multiple-pairwise comparisons and these comparisons were adjusted with Bonferroni's method for each contrast of fixed effect and interaction term. In addition, the residual intra-class correlation coefficients (ICCs) that were reported from the regression analyses were examined to explore the magnitude of the variance that was attributable to the clusters such as pen or/and individual animal.

3.12. Bioinformatic analyses

Web-based and command-line tools on the Texas A&M University High-Performance Research Computer (HPRC) were used for the bioinformatic analyses.

3.12.1. Quality assessments of the sequencing data

Bad quality FASTQ (raw sequence reads) reads (i.e., reads with a PHRED score less than 33 [240]) and less than 36 bp length (- *MINLEN:36* -) were removed using Trimmomatic v.0.36 [241] on the command-line. In addition, we removed at the end of each read the low-quality or N bases that were below-quality 3 using the - *LEADING:3* – and the - *TRAILING:3* - options. The - *SLIDINGWINDOW: 4:15* - option was also included to scan the reads with a 4-base wide sliding window, removing the reads when the average quality per base dropped below a threshold of 15. To clean the Illumina® Nextera kit specific primer adapters listed in the *Nextera-PE-PE.fasta* file with maximum allowable mismatches of 2, palindrome clip (between two 'adapter ligated' reads) threshold of 30 and the simple clip (between any adapter) threshold of 10 settings were adapted using the - *ILLUMINACLIP: NexteraPE-PE.fa:2:30:10* - option. FastQC software v.0.11.7 [242] was used to assess raw-read quality, and reports were aggregated using MultiQC v.1.5 on command-line [243]. Statistics such as per base sequence quality, per tile sequence quality, per sequence quality, per base sequence content, per base N content, sequence length distribution, sequence duplication-level, over-represented sequences, adapter content, and the K-mer content were examined. In addition, the average read lengths were obtained per FASTQ pairs, to calculate the coverage in depth. Sequences passing these quality matrices were carried to the next step: assembly process.

Pair-end trimmed FASTQ files were assembled using SPAdes v.3.11.1 on the command-line with default parameters except - *careful* – option that reduced the number of mismatches and short indels compared to the default settings [244]. The default parameters to generate assembly files included the required phred offset score of 33, the

read error corrections of pairs and, the k-mer assembly sizes set to 21, 33, 55, 77, 99, and 127, since the expected read length was equal to or greater than 250 bp. The assembly qualities of the FASTA (assembled sequence file) files were assessed using the Quality Assessment Tool for Genome Assemblies (QUAST) [245]. The number of the total contigs larger than 5,000 bp, the total read lengths, the N50 values, and the GC (%) contents were obtained. The depth of coverage was calculated for each isolate based on the average sequence length, the number of reads and the genome size using the formula for the depth coverage = ([average sequence length × number of reads] / genome size). The assemblies more than 200 contigs and less than 28X depth of coverage were considered bad quality and re-sequenced.

3.12.2. Serotype and sequence typing

SeqSero v.1.0 software was used for *Salmonella* serotyping from raw sequencing reads (FASTQ files) [85]. The serotyping results obtained from the SeqSero database, which is curated from somatic O-antigen and flagellar H1 (*fli*C), and H2 antigen (*flj*B) encoding genes, is known to corresponds with current antigenic profiles recognized in the Kauffmann-White-LeMinor scheme [82]. STs were determined using SRST2 (Short Read Sequence Typing for Bacterial Pathogens) v.0.2.0 and the *Salmonella* MLST database, curated from Public Databases for Molecular Typing and Microbial Genome Diversity Platform (www.pubmlst.org) and accessed in July 2018. The STs were determined based on sequence matches with the MLST database consisting of the seven housekeeping genes, *aro*C, *dna*N, *hem*D, *his*D, *pur*E, *suc*A, *and thrA* [246].

3.12.3. Antibiotic resistance gene and plasmid detection

Mass screening of contigs harboring ARGs and plasmids was conducted using the pipeline ABRicate v.0.8.5 (https://github.com/tseemann/abricate) and determined using a resistance gene database ResFinder and a plasmid database PlasmidFinder (both updated as of 28 July 2018) [98, 110]. A coverage threshold of 60% and, identity match threshold of 90% were utilized for alignment parameters for each gene detected. In addition, the database accession number (GeneBank accession no.), and the name of the gene matched was reported for each positive inquiry. The ARG that was identified was further explored for a contig-related inquiry to validate the location (chromosomal vs plasmidal) of the ARG using the RAStk annotation tool [247], and the basic local alignment search tool (BLAST) [248]. Later, if the ARG was found to be in a plasmid, this contig was graphically visualized with SnapGene software v.4.1.3. (GSL Biotech; available at www.snapgene.com).

3.12.4. Phylogenetic analyses

SNP analyses were conducted across all serotypes, and for each serotype individually. While a cladogram was adapted mainly showing the clustering and genetic relatedness among all serotypes by ignoring the branch lengths, additional phylograms were generated to calculate the SNP variations represented within the branch lengths observed for each serotype.

Reference genomes were selected using the Similar Genome Finder service on the web-based PATRIC (the Pathosystems Resource Integration Center) platform (available at https://www.patricbrc.org/app/GenomeDistance). The best genome matches were screened

by keeping the threshold values at default (i.e., maximum hit value of 50, *P*-value of 1, and Mash/MinHash distance [estimating the distance based on rate of sequence mutation] value of 0.05) [249, 250]. Using the Similar Genome Finder, the closest genomic distance complete genome reference was selected for each serotype. A reference strain from one of the most prevalent serotypes was selected for the phylogenetic analysis conducted across all serotypes. A complete genome of each reference was obtained from the NCBI (National Center for Biotechnology Information) database in FASTA format.

These selected reference genomes were further uploaded to a web-tool called PHASTER (Phage Search Tool Enhanced Release, available at http://phaster.ca/) to detect the existing prophage regions [251]. The sequences belonging to the phage regions were further masked with the letter of "N" utilizing BEDTools v.2.18 [252]. These masked references were carried to the next step; that is, the alignment process for the phylogenetic analyses.

All *Salmonella* genomes in this study were aligned to the reference strain using the core genome SNP analysis via ParSNP v.1.2 [253] using the assembled (FASTA) sequencing files. The extended multi-fasta files (XMFA) obtained from the core-genome SNP alignment were converted to the multi-fasta file format using HarvestTools v.1.2 [253]. To select the best nucleotide substitution model to include in the maximum-likelihood tree, a model testing tool called Model-test NG v.0.1.5 was adopted [254]. Further, the phylogenetic tree was inferred using the selected best model with IQ-tree v.1.6.10, including bootstrap values with 1,000 iterations [255].

Later, the phylogenetic tree was visualized as a cladogram and the graphics were generated using a web-based Interactive Tree of Life (iTOL, available at https://itol.embl.de) tool [256]. The genotypic relatedness (clade-level) was explored by pen, block, and cattle origin/source among the serotypes and graphically represented by colors.

The phylogenetic comparisons for individual serotypes were determined by the McOutbryk SNP calling pipeline (available under the MIT license at https://github.com/hcdenbakker/McOutbryk) using raw (FASTQ) sequencing reads. This genome graph-based pipeline uses the McCortex tool [257] to build graphs of a reference sequence and the data of the genomes to be queried for SNPs. The SNP calling stage consists of two phases: (Phase 1) an initial phase, which consists of a comparison of the reference graph and each query genome to construct a list of putative variable sites within the population, and (Phase 2) a final SNP calling phase, which calls the allele for each putative SNP site found in the Phase 1. While the SNP calling is done *de novo* at the initial step, the pipeline uses BWA (Burrow-Wheeler aligner)-mem [258] at the final step to place the SNP sites in relation to the reference sequence. In addition to BWA, the pipeline relies on VCFtools [259], and vcflib (available under the MIT license at https://github.com/vcflib/vcflib) for VCF (Variant Call format) file manipulation. The SNPs from prophage regions were excluded by using a referent strain with masked prophage regions. This pipeline was written in Python3, distributed by the Python Software Foundation (http://www.python.org).

The variant SNP sites were later carried to a final filtration using IQ-tree and the variant site output was further evaluated for the best nucleotide substitution model using IQ-tree v.1.6.10 -*m MPF+ASC*- option to construct a maximum-likelihood phylogeny. This option finds the best fit substitution model among the total 22 nucleotide substitution models listed in Table 8 based on the BIC criteria, including the ascertainment bias correction (ASC), where the SNPs data no longer include any constant regions, but includes the variant regions (detailed information can be found in the IQ-tree manual provided at http://www.iqtree.org/doc/iqtree-doc.pdf). The output file containing only variant sites was carried to the next-step: analyses for model testing using best-fit maximum-likelihood approaches.

The phylogenetic tree was inferred using the selected model with IQ-tree, including bootstraps values with 1,000 iterations using the option of - *m*(selected model) *+ASC –alrt 1000* - [255]. Later, the phylogenetic tree was visualized with the branch lengths reflecting individual SNP differences observed within each serotype using iTOL. The tree topology observed in each serotype was visualized and the pen (Pen 7 to 59), source (Source 1 or 2), day (Days 0, 7, 99, 120, 134, or 141), and sample type (fecal, lymph node, or hide) characteristics were shown in the cells with either colors, or else binary formatted, in which were all presented as square.

The sample types were presented as a circle (fecal), star (lymph node), or as a left-pointing triangle (hide). Again, the branches that had the bootstrap support value of 800-1,000 were presented with a grey circle located the middle of the corresponding branch and sized proportional to the given support values.

Table 8. Nucleotide substitution models

Year	Name	Model	Base-rates
1969	Jukes-Cantor[260]	JC or JC69 (e)	AC = AG = AT = CG = CT = GT
1981	Felsenstein[261]	F81 (u)	
1993	Tamura, Nei[262]	TN or TN93 (u)	AC=AT=CG=GT, AG, CT
		TN (e)	
1985	Hasegawa-Kishino-Yano[263]	HKY or HKY85 (u)	AC=AT=CG=GT, AG=CT
1980	Kimura[264, 265]	TPM1, K80 or K2P (e)	
1981		TPM1, K81 or K3P (e)	AC=GT, AG=CT, AT=CG
		TPM1, K81u	
		TPM2 (e)*	AC=AT, CG=GT, AG=CT
		TPM2 (u)*	
		TPM3 (e)*	AC=CG, AT=GT, AG=CT
		TPM3 (u)*	
1990	Transition[266]	TIM1 (u)	AC=GT, AT=CG, AG, CT
		TIM1 (e)	
		TIM2 (u)	AC=AT, CG=GT, AG, CT
		TIM2 (e)	
		TIM3 (u)	AC=CG, AT=GT, AG, CT
		TIM3 (e)	
2003	Transversion[266]	TVM (u)	AC, AT, CG, GT, AG=CT
		TVM (e)	
1986	General time reversible[267]	GTR (e)	AC, AG, AT, CG, CT, GT
1994	Symmetric[268]	SYM (u)	

(e) equal base frequencies, (u) unequal base frequencies. *Year and model information not available. The list of models was obtained from the IQ-tree manual http://www.iqtree.org/doc/Substitution-Models [269]

4. RESULTS[*]

4.1. Descriptive statistics

A total of 1,155 samples was received. Of these, 799 were fecal samples, 224 were lymph nodes (one or two per animal), and 132 were hide samples. Two animals left the study due to illness after Day 28 and thus were lost to follow-up. Therefore, feces, lymph nodes, and hide samples were not obtained from those two cattle subsequent to their loss. One steer that left the study was from a tulathromycin-treated pen (Pen 51) and the other steer was from a ceftiofur-treated pen (Pen 55); these losses were due to foot injury and BRD, respectively. As a result, a total of 132 cattle completed the study.

On Day 0, there was one fecal sample missed due to a steer escaping the chute. In addition, a total of 36 lymph nodes from cattle in Pens 57, 58, and 59 were missing due to a lack of training in sample collection by personnel at the commercial slaughter plant on the first day of slaughter (Day 99). Later, a single-staff member was retrained and we successfully collected the subiliac lymph nodes during the subsequent three slaughter periods without any missed samples. Among the 224 lymph nodes obtained, the mean weight was 22 g per node with a minimum of 4.6 g and a maximum of 70.4 g (Figure 14).

[*] Part of this chapter is reprinted with permission from "Population Dynamics of Salmonella enterica within Beef Cattle Cohorts Followed from Single-Dose Metaphylactic Antibiotic Treatment until Slaughter" Levent G, Schlochtermeier A, Ives SE, Norman KN, Lawhon SD, Loneragan GH, Anderson RC, Vinasco J, Scott HM. 2019 Appl Environ Microbiol 85:e01386-19, Copyright © 2019 American Society for Microbiology.

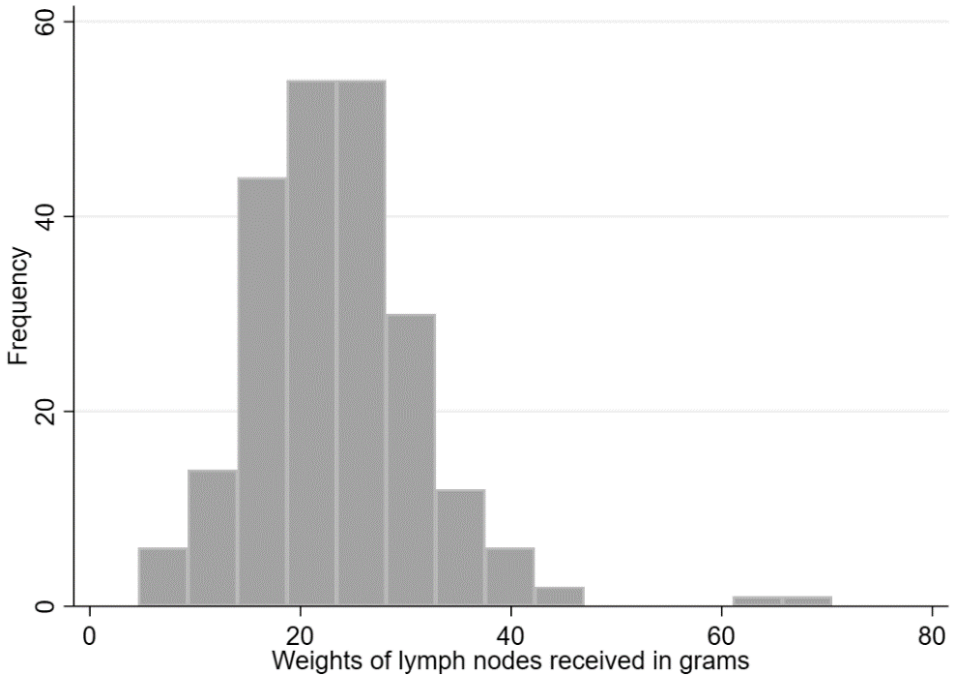


Figure 14. Histogram representing the weight distribution of the lymph nodes

4.1.1. Quantitative results

Salmonella colony counts obtained from the spiral plater were back-calculated using the corresponding dilution factors and converted to CFUs per gram (for feces and lymph nodes) and per meter-squared for hide swabs. The CFUs thus obtained were highly right-skewed. In order to approach to normal distribution, the CFUs were $\log_{10}$ transformed. After the $\log_{10}$ transformation, histograms of CFU frequencies for each sample type were examined and are presented in Figure 15.

Out of a total of 799 fecal samples, 531 observations were recorded as zero counts and the remaining 268 observations were quantifiable. Among these 268 observations, the minimum fecal $\log_{10}$ CFU was 2.64 and the maximum was 7.04. Out of a total of 224 lymph node samples, 81 observations were recorded as zero and the remaining 143 observations were quantifiable. Among these, the minimum lymph node $\log_{10}$ CFU observed was 1.84 and the maximum was 6.34. Out of a total of 132 hide observations, 25 observations were recorded as zero and the remaining 107 observations were quantifiable. Among these, the minimum hide $\log_{10}$ CFU was 3.3 and the maximum was 7.2. The minimum value of the LLQ was observed among the lymph nodes (1.84 $\log_{10}$), followed by the feces (2.64 $\log_{10}$), and the hides exhibited the highest LLQ (3.3 $\log_{10}$).

On Days 0, 7, and 14 the distribution of the $\log_{10}$ CFUs was highly right-skewed with a 75^{th} percentile of zero and means of 0.79, 0.43 and 0.73 $\log_{10}$ CFU per gram feces, respectively. On Day 28, at the 50^{th} percentile the $\log_{10}$ CFUs was zero versus 4.54 at the 75^{th} percentile and with a mean of 1.79 $\log_{10}$ CFU per gram of feces. On Day 56 and at the terminal day (aggregated into Day 112) $\log_{10}$ CFUs at the 50^{th} percentiles were 3.2 and 5.53 with means of 2.71 and 4.08 $\log_{10}$ CFU per gram feces, respectively. The lymph node CFUs at the 50^{th} percentile was 3.48 with a mean of 2.95 $\log_{10}$ CFU per gram of lymph node (Table 9).

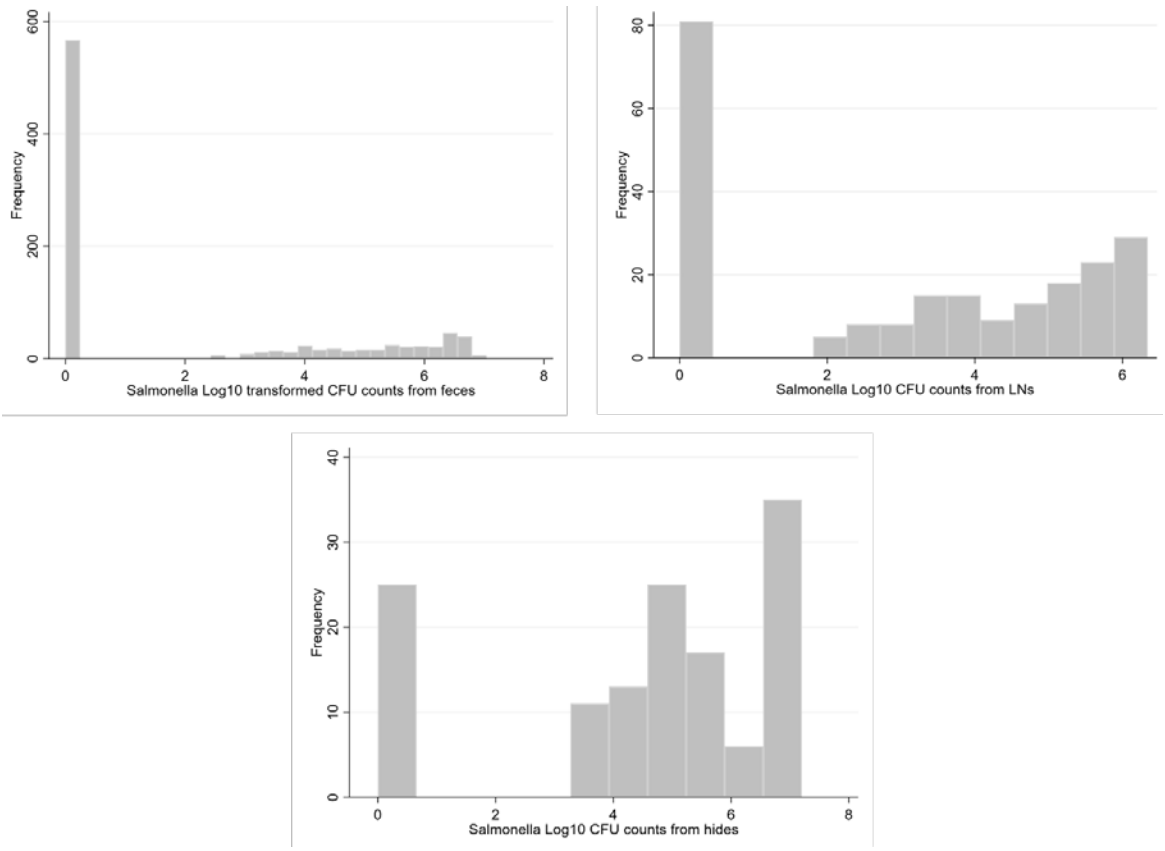


Figure 15. Histograms representing the $\log_{10}$ CFU frequencies for feces, lymph nodes, and hides

Feces are presented on the top left corner, lymph nodes are presented the top right corner, and the hides are presented in the middle.

Table 9. Log_{10} CFU mean, percentiles and standard deviation distribution across days and sample types

Samples	Obs[a]	Percentiles			Mean	Std. Dev.
		25th	50th	75th		
Day 0 feces	133	0	0	0	0.79	1.8
Day 7 feces	134	0	0	0	0.43	1.47
Day 14 feces	134	0	0	0	0.73	1.81
Day 28 feces	134	0	0	4.54	1.79	2.6
Day 56 feces	132	0	3.2	5.2	2.71	2.67
Terminal (feces)	132	0	5.53	6.31	4.08	2.75
Lymph node	224[b]	0	3.48	5.32	2.95	2.44
Hide	132	3.78	4.96	6.61	4.47	2.4

[a] Total number of observations, [b] Two lymph nodes per animal, Std. Dev. (Standard deviation).

4.1.2. Enrichment results

Salmonella prevalences in fecal samples were estimated at: 43.6% (58/133) on Day 0, 20.1% (27/134) on Day 7, 20.1% (27/134) on Day 14, 41.0% (55/134) on Day 28, 57.5% (76/132) on Day 56, and 80.3% (106/132) on the terminal day. Lymph node *Salmonella* prevalence at the carcass-level was 84.2% (96/114 – 20 cattle lymph nodes were missing); whereas, overall lymph node prevalence was 75.4% (169/224). Hide prevalence was 84.8% (112/132) across all cattle and pens.

Among control-, tulathromycin- and ceftiofur-treated animals, terminal fecal prevalences were distributed as 66.6%, 92.3%, and 87.1%, lymph node prevalences were distributed as 77.0%, 88.2%, and 90.6%, and hide prevalences were distributed as 77.7%, 87.1%, and 92.3%, respectively (Table 10).

Table 10. Overall and treatment-level *Salmonella* prevalence distribution by day (feces only) and sample type

	Fecal samples by day						Lymph node*	Hide
	0	7	14	28	56	Terminal		
Cef	32.5 (13/40)	15 (6/40)	17.5 (7/40)	35 (14/40)	66.6 (26/39)	87.1 (34/39)	90.6 (29/32)	92.3 (36/39)
Con	54.7 (29/53)	16.6 (9/54)	22.2 (12/54)	42.5 (23/54)	42.5 (23/54)	66.6 (36/54)	77.0 (37/48)	77.7 (42/54)
Tul	40 (16/40)	30 (12/40)	20 (8/40)	45 (18/40)	69.2 (27/39)	92.3 (36/39)	88.2 (30/34)	87.1 (34/39)
Tot	43.6 (58/133)	20.1 (27/134)	20.1 (27/134)	41 (55/134)	57.5 (76/132)	80.3 (106/132)	84.2 (96/114)	84.8 (112/132)

*Animal-level frequencies are presented for the lymph node data. Overall lymph node prevalence was 75.4% (169/224). Ceftiofur (ceftiofur), Con (control), Tul (tulathromycin), Tot (total).Top values are prevalence represented as percentage. No. of positive/total No. values presented below each percentage. Reprinted with permission from Levent et al. (2019) [236].

4.1.3. Kappa agreement

Overall, among the 1,156 observations, 504 samples resulted in zero CFU counts and were also enrichment negative. In contrast, 491 observations that resulted in quantifiable CFUs were also enrichment positive. The disagreement between the two outcomes was illustrated in 134 observations that were *Salmonella* positive after enrichment but the CFUs of these observations were zero; in contrast, 27 observations were negative after enrichment, but their CFUs were greater than zero (Table 11).

The agreement observed comparing the two methods was classified as good (i.e., for kappa values larger than 70.0%) with 85.7%. The lowest kappa agreement during fecal sampling was observed on Day 0 with 72.1% kappa value, and ranged from 84.3 to 91.7% on the following days. The kappa agreement of the overall fecal observations was 85.8%. The lymph node samples yielded 83.9% kappa value whereas hides represented the lowest kappa value (79.5%) of all sample types. In summary, CFU quantification and enrichment results showed good kappa agreement overall.

Table 11. Agreement between the observations obtained from CFU and enrichment results by day and sample types

	E (p), C(p)	E (n), C(n)	E(p), C(n)	E(n), C (p)	Total
Day 0	22	74	36	1	133
Day 7	9	104	18	3	134
Day 14	18	105	9	2	134
Day 28	43	77	12	2	134
Day 56	68	53	8	3	132
Terminal	90	23	16	3	132
Total (feces)	250	436	99	14	799
Lymph node	138	50	31	5	224
Hide	96	9	16	11	132
Grand total	491	504	134	27	1156

E (enrichment), C (CFU positive [a value larger than zero]), p(positive), n(negative).

4.2. Two-part and interval-linear regression analyses

The LLQs were determined as 2.64 $\log_{10}$ for feces, 1.84 $\log_{10}$ for lymph nodes and 3.3 $\log_{10}$ for hide samples. Two-part models were explored to find the best fit model for excess zero counts in the fecal and lymph node $\log_{10}$ CFU observations. For the hide data, the zero and the missing observations were imputed with the fixed and random effects parameters using interval linear regression between zero and the LLQ (3.3 $\log_{10}$); thereafter, estimates were obtained from the imputed data using multi-level mixed linear regression with the same model parameters.

4.2.1. Two-part regression models

For the fecal and lymph node observations, ZIP, ZINB, and CHURDLE regression models were explored separately. The ZIP and ZINB models provided identical coefficients and similar AIC and BIC values (1,871 versus 1,873 for feces and 796 versus 798 for lymph nodes, respectively) suggesting no over-dispersion in the data (Table 12).

The CHURDLE model was selected as the best fit model for the feces and lymph node observations, since the AIC and the BIC values were significantly lower (1,241 and 437) for both feces and lymph nodes than the ZIP and the ZINB models provided. Therefore, the effects of treatment and day (feces only) are presented based on the CHURDLE model.

Table 12. Akaike's and Bayesian information criteria for two-part regression models

Sample type	Model	No. of observations	ll (null)	ll (model)	df	AIC	BIC
Feces	ZIP	799	-888.39	-877.94	58	1871.88	2143.5
	ZINB	799	-888.39	-877.94	59	1873.88	2150.2
	CHURDLE	799	-715.80	-553.17	58	1222.35	1452.1
Lymph nodes	ZIP	224	-382.00	-370.22	28	796.44	891.9
	ZINB	224	-382.00	-370.22	29	798.44	897.3
	CHURDLE	224	-271.44	-201.80	28	459.60	555.1

ll (log-likelihood).

CHURDLE model considering interaction terms revealed no treatment effects across days (with the P value ranged from 0.068 to 0.919) on *Salmonella* log10 CFUs in feces. However, each of Day 56 and the Day 122 (terminal day)_ had a statistically and significantly higher effect ($P = 0.000$) on CFUs when compared to earlier days (Figure 16). Even though the antibiotic treatments did not significantly affect the *Salmonella* CFUs observed in cattle feces, day had a significant effect (P value ranging from 0.001 to 0.045). In the control group, this effect was observed increasing by day as shown as between Day 0 and Day 122 ($P = 0.001$), between Day 7 and Day 122 ($P < 0.001$), and between Day 14 and Day 122 ($P = 0.001$). We did not observe any period effects among other day-to-day comparisons, whose P values ranged from 0.095 to 1.000. Interestingly, we did not

observe any significant difference among days in the tulathromycin treatment group; the minimum detected P value was 0.295 and the maximum was 1.000 among the pairwise comparisons of days within that group. In the ceftiofur group, similar to the control group, we found a significant increase by the end of the study. In this group, the CFUs on Day 56 were significantly higher than on Day 7 ($P = 0.011$), Day 112 was also significantly higher than each of Days 0, 7, 14, and 59 ($P = 0.002$, $P < 0.001$, $P = 0.002$, $P = 0.045$, respectively). All pairwise CFU period contrasts and the corresponding P-values are provided for each treatment in Table 13.

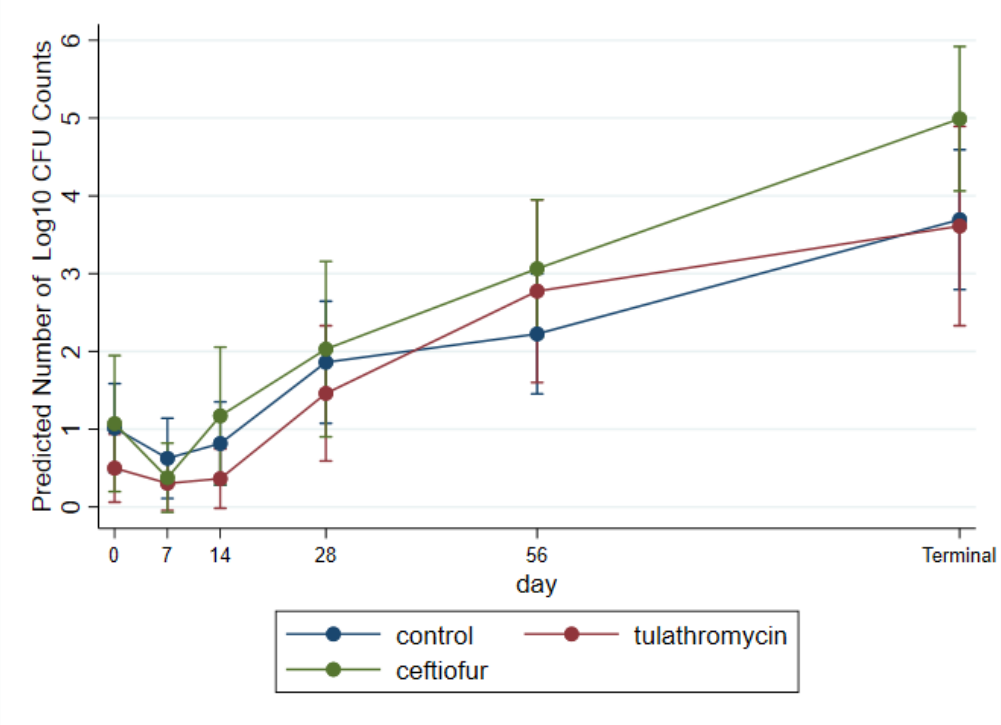


Figure 16. Predictive $\log_{10}$ CFU margins of day and treatment for feces modelled with CHURDLE

Table 13. Bonferroni-adjusted pairwise fecal log_{10} CFU contrasts between day pairs for each treatment group using the CHURDLE model

Trt	Day comparisons		Contrast	Std.Err.	Bonferroni adjusted			
					Z	*P*-value	95% CIs	
Con	7	0	-0.02289	0.133167	-0.17	1	-0.50137	0.455585
	14	0	-0.03997	0.097162	-0.41	1	-0.38908	0.309138
	28	0	0.374496	0.177297	2.11	1	-0.26255	1.011537
	56	0	0.396341	0.162805	2.43	1	-0.18863	0.98131
	112	**0**	**1.190647**	**0.266388**	**4.47**	**0.001**	**0.233494**	**2.147799**
	14	7	-0.01708	0.146016	-0.12	1	-0.54172	0.50757
	28	7	0.397389	0.189166	2.1	1	-0.2823	1.077076
	56	7	0.419235	0.163547	2.56	1	-0.1684	1.006873
	112	**7**	**1.21354**	**0.257619**	**4.71**	**0**	**0.287894**	**2.139186**
	28	14	0.414466	0.174826	2.37	1	-0.2137	1.042629
	56	14	0.436312	0.172207	2.53	1	-0.18244	1.055065
	112	**14**	**1.230617**	**0.276115**	**4.46**	**0.001**	**0.238514**	**2.222719**
	56	28	0.021845	0.202247	0.11	1	-0.70484	0.748534
	112	28	0.816151	0.276643	2.95	0.486	-0.17785	1.810148
	112	56	0.794305	0.253442	3.13	0.264	-0.11633	1.704941
Tul	7	0	-0.02021	0.082613	-0.24	1	-0.31704	0.276626
	14	0	-0.01125	0.079263	-0.14	1	-0.29605	0.273547
	28	0	0.29758	0.158675	1.88	1	-0.27255	0.86771
	56	0	0.733504	0.236523	3.1	0.295	-0.11634	1.583348
	112	0	1.192832	0.39892	2.99	0.427	-0.24052	2.626182
	14	7	0.008958	0.080889	0.11	1	-0.28168	0.299596
	28	7	0.317787	0.165574	1.92	1	-0.27713	0.912708
	56	7	0.753712	0.249378	3.02	0.384	-0.14232	1.649744
	112	7	1.21304	0.42236	2.87	0.624	-0.30453	2.730612
	28	14	0.30883	0.16201	1.91	1	-0.27328	0.890943
	56	14	0.744754	0.244501	3.05	0.355	-0.13376	1.623266
	112	14	1.204083	0.415443	2.9	0.574	-0.28863	2.6968
	56	28	0.435925	0.236221	1.85	1	-0.41284	1.284686
	112	28	0.895253	0.378249	2.37	1	-0.46382	2.25433
	112	56	0.459328	0.347107	1.32	1	-0.78785	1.706511
Cef	7	0	-0.22615	0.136014	-1.66	1	-0.71486	0.262554
	14	0	-0.01497	0.138258	-0.11	1	-0.51174	0.481805
	28	0	0.313079	0.207606	1.51	1	-0.43287	1.059024
	56	0	0.353088	0.15745	2.24	1	-0.21264	0.918819
	112	**0**	**1.416695**	**0.326541**	**4.34**	**0.002**	**0.24341**	**2.589979**
	56	7	0.427233	0.19438	2.2	1	-0.27119	1.125654
	14	7	0.211187	0.113819	1.86	1	-0.19777	0.620148
	28	7	0.539232	0.215582	2.5	1	-0.23537	1.313835
	56	**7**	**0.57924**	**0.145752**	**3.97**	**0.011**	**0.055542**	**1.102939**
	112	**7**	**1.642847**	**0.351315**	**4.68**	**0**	**0.380548**	**2.905147**
	28	14	0.328044	0.198541	1.65	1	-0.38533	1.041417
	56	14	0.368053	0.145998	2.52	1	-0.15653	0.892636
	112	**14**	**1.43166**	**0.328287**	**4.36**	**0.002**	**0.252102**	**2.611218**
	56	28	0.207783	0.264197	0.79	1	-0.7415	1.157061
	112	28	1.27139	0.43238	2.94	0.501	-0.28218	2.824964
	56	28	0.040009	0.191293	0.21	1	-0.64732	0.727337
	112	28	1.103616	0.322548	3.42	0.095	-0.05532	2.262556
	112	**56**	**1.063607**	**0.293552**	**3.62**	**0.045**	**0.008853**	**2.118361**

Trt (Treatment), Con (Control), Tul (Tulathromycin), Cef (Ceftiofur). *P*-values that are less then <0.05 and their corresponding pairwise comparisons are bolded. Std.Err (standard error).

Similarly, no statistically significant effect was observed among log_{10} CFUs in the lymph nodes among the ceftiofur and control groups (P = 0.754), ceftiofur and tulathromycin groups (P = 1.000), and tulathromycin and control groups (P = 0.797).

Bonferroni adjusted contrasts of each two-way treatment comparison are provided in Table 14. In addition, the predicted mean CFUs were graphically presented in Figure 17.

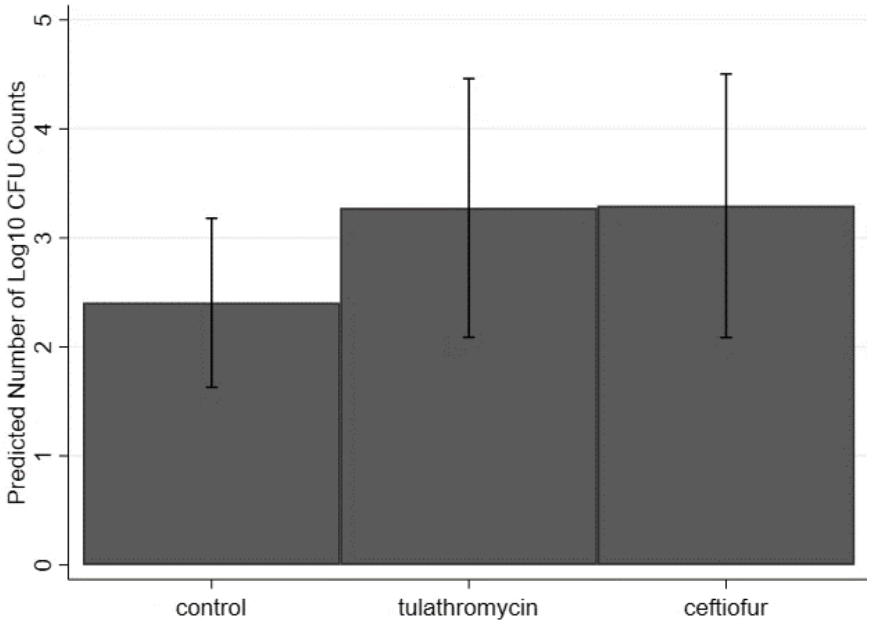


Figure 17. Predictive log_{10} CFU margins by treatment for lymph nodes modelled with CHURDLE

Table 14. Bonferroni-adjusted pairwise lymph node CFU contrasts between treatments using the CHURDLE model

Treatments		Delta method			Bonferroni adjusted		
		Contrast	Std. Err.	Z-value	P-value	95% CIs	
Tul	Cont	0.908712	0.816614	1.11	0.797	-1.04625	2.863669
Cef	Cont	0.815487	0.710859	1.15	0.754	-0.88629	2.517268
Cef	Tul	-0.09322	1.039197	-0.09	1.000	-2.58104	2.394592

Trt (Treatment), Con (Control), Tul (Tulathromycin), Cef (Ceftiofur).

4.2.2. Multiple imputation-based interval-linear regression estimated by linear regression

An imputation method using interval-based linear regression provided $\log_{10}$ CFUs for negative hide samples due to the low-percentage (18.9%) of zero counts in hide samples. In a shared pen environment, hide prevalence by pens varied from 54.5% to 100% with an overall mean of 84.8%. Therefore, the *Salmonella* enrichment negative (n = 25) and the missing (n = 2) observations were accepted as LLQ and imputed across the 0 to 3.3 $\log_{10}$ CFU range to better meet normality and heteroscedasticity assumptions of linear regression. A total of 15 observation from the control group, and six observations from each of ceftiofur and tulathromycin treated groups were imputed using the fixed effect of treatment and the cluster effect of pen. Before and after the imputation, slight mean differences were observed in each treatment group (Table 15).

Table 15. Log_{10} CFU mean and standard deviation differences among treatment groups before and after imputation method

Trt	Number of imputed obs.	Number of imputations (*m*)	Obs.*	Log_{10} CFU			
				Mean	Std. dev.	Min.	Max.
Con	15	0	39	5.3	1.3	3.3	7.1
		20	54	4.5	1.7	1.4	7.1
Tul	6	0	34	5.7	1.0	3.7	7.0
		20	40	5.3	1.5	2.3	7.0
Cef	6	0	34	5.6	1.1	3.6	7.2
		20	40	5.1	1.4	2.5	7.2

*Observations before and after 20 imputations. Trt (Treatment), Con (Control), Tul (Tulathromycin), Cef (Ceftiofur).

The estimates were obtained using the multi-level mixed effects linear regression model, which did not show any statistically significant effect observed in log_{10} CFUs among hide samples of cattle treated with or without ceftiofur or tulathromycin (Figure 18). The marginal predicted means, standard errors and the 95% CIs are presented in Table 16.

Table 16. Marginal predicted hide log_{10} CFU means, standard errors and 95% CIs of imputed interval-regression model

	Margins	Std. Err.	95% CIs	
Control	4.904	0.403	4.114	5.694
Tulathromycin	4.826	0.466	3.911	5.741
Ceftiofur	5.202	0.469	4.283	6.122

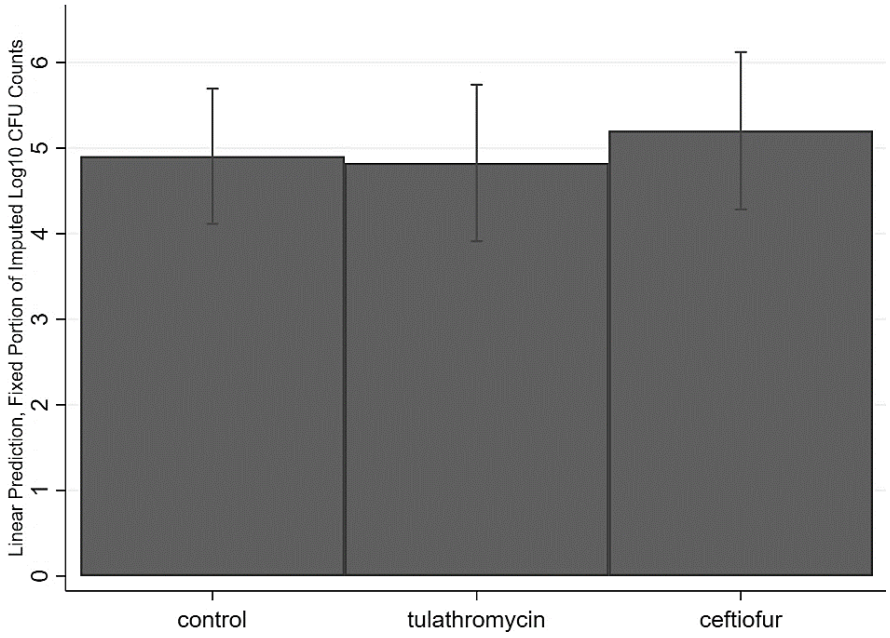


Figure 18. Predictive $\log_{10}$ CFU margins by treatment for hides modeled with interval-based linear regression using a multiple imputation method

4.3. Logistic regression analyses

4.3.1. Feces

The likelihood-ratio based test showed that treatment group did not result in statistically significant differences ($P = 0.825$) among the treatment and control groups for fecal prevalence of *Salmonella* (Figure 19). However, there was a significant ($P < 0.0001$) day effect observed across all treatment groups in fecal prevalence of *Salmonella* (Table 17).

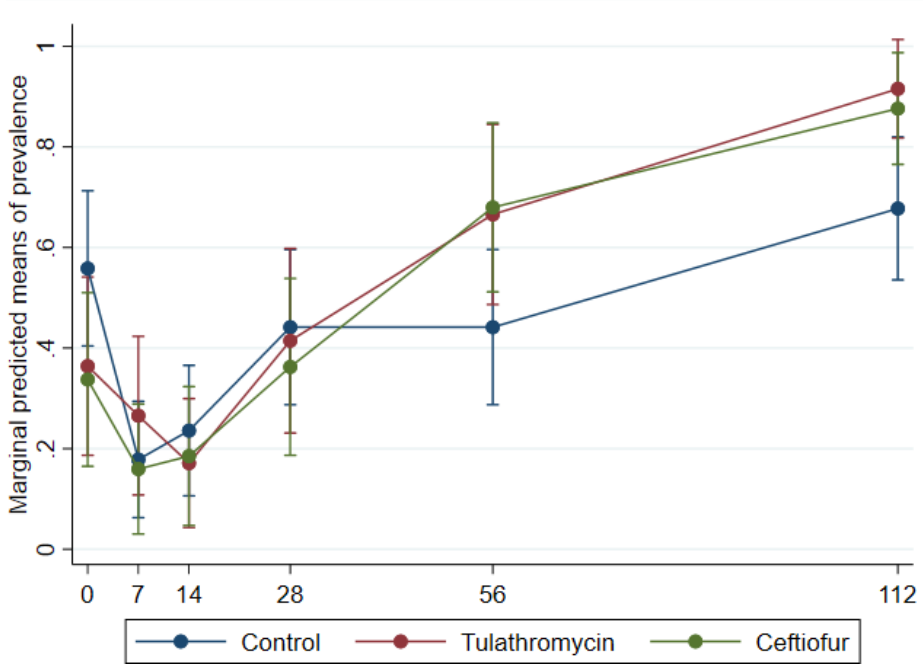


Figure 19. Adjusted fecal marginal prevalence mean predictions and 95% CIs by treatment and day

Reprinted with permission from Levent et al. (2019) [236].

According to pairwise adjusted comparisons of marginal results, *Salmonella* prevalence in antibiotic-treated groups showed similarity across all treatment groups for any given day (Table 18).

Table 17. Stepwise multi-level logistic regression analyses of the fixed effects with corresponding df, -2(log-likelihood) and *P*-values

Sample Type	Model	df	-2(Log-likelihood)	*P*-value**
Feces	Full model*	17	879.40032	0.000
	Day and Treatment	7	907.13836	0.000
	Treatment	2	1074.79028	0.825
	Day	5	907.52280	0.000
	Intercept only	0	1075.10612	.
Lymph nodes	Full model (Treatment)	2	223.14966	0.297
	Intercept only	0	225.74820	.
Hide	Full model (Treatment)	2	102.63036	0.218
	Intercept only	0	105.92971	.

Each mixed model included within-pen and within-animal (feces and lymph nodes only) random effects to account for clustering. * Day, treatment, and interaction term. **Comparisons via likelihood-ratio chi-square tests are versus intercept-only model. Additional comparisons among nested forms of each sample-type model can be determined as the difference between both -2(log-likelihood) and df of the two models compared against the chi-square distribution. Reprinted with permission from Levent et al. (2019) [236].

However, Period (Day)- had a significant effect on the prevalence of *Salmonella* within each treatment group as follows: a significant ($P = 0.001$ and $P = 0.018$, respectively) prevalence decrease was observed from Day 0 to Days 7 and 14 in the control group. Later, for this same group a recovery to baseline value was observed by Days 28 and 56. By Day 112, the prevalence was significantly increased when compared to Day 7 and Day 14 ($P < 0.0001$ for both). The *Salmonella* prevalence in tulathromycin- and ceftiofur-treated groups was similar to the baseline values until Day 56.

A significant increase was observed at Day 56 when compared to Day 7 ($P = 0.006$ and $P < 0.0001$, for tulathromycin and ceftiofur, respectively) and Day 14 ($P < 0.0001$ for both antibiotics).

Table 18. Bonferroni-adjusted pairwise comparisons of prevalence contrasts for feces, lymph node, and hide observations for each treatment and by day (feces only)

Sample type	Day	Treatment comparisons		Contrast	Std.Err.	Bonferroni adjusted			
						Z	*P*-value	95% CIs	
Feces	0	Tul	Con	-0.19439	0.11567	-1.68	1.000	-0.61002	0.22124
		Cef	Con	-0.22092	0.11274	-1.96	1.000	-0.62601	0.18418
		Cef	Tul	-0.02653	0.12635	-0.21	1.000	-0.48053	0.42747
	7	Tul	Con	0.08723	0.09715	0.90	1.000	-0.26185	0.43631
		Cef	Con	-0.01891	0.08514	-0.22	1.000	-0.32484	0.28702
		Cef	Tul	-0.10614	0.10433	-1.02	1.000	-0.48103	0.26874
	14	Tul	Con	-0.06459	0.09042	-0.71	1.000	-0.3895	0.26032
		Cef	Con	-0.05065	0.09273	-0.55	1.000	-0.38385	0.28255
		Cef	Tul	0.01394	0.09630	0.14	1.000	-0.3321	0.35998
	28	Tul	Con	-0.02696	0.11817	-0.23	1.000	-0.45158	0.39766
		Cef	Con	-0.07899	0.11372	-0.69	1.000	-0.48760	0.32961
		Cef	Tul	-0.05204	0.12971	-0.40	1.000	-0.51813	0.41405
	56	Tul	Con	0.22424	0.11694	1.92	1.000	-0.19595	0.64443
		Cef	Con	0.23825	0.11130	2.14	1.000	-0.16168	0.63819
		Cef	Tul	0.01401	0.12475	0.11	1.000	-0.43425	0.46227
	Ter	Tul	Con	0.23805	0.08615	2.76	0.876	-0.07151	0.54761
		Cef	Con	0.19878	0.08903	2.23	1.000	-0.12111	0.51868
		Cef	Tul	-0.03927	0.07521	-0.52	1.000	-0.30951	0.23097
LNs	Ter	Tul	Con	0.15891	0.10072	1.58	0.344	-0.08223	0.40005
		Cef	Con	0.10003	0.10445	0.96	1.000	-0.15002	0.35009
		Cef	Tul	-0.05887	0.10873	-0.54	1.000	-0.31918	0.20143
Hides	Ter	Tul	Con	0.09080	0.10217	0.89	1.000	-0.15380	0.33541
		Cef	Con	0.15815	0.08749	1.81	0.212	-0.05130	0.36761
		Cef	Tul	0.06734	0.09153	0.74	1.000	-0.15177	0.28646

LNs (lymph nodes), Con (Control), Cef (Ceftiofur), Tul (Tulathromycin), Std.Err. (Standard errors). Reprinted with permission from Levent et al. (2019) [236].

This increase continued through Days 56 and 122 ($P < 0.0001$ for both), and by the end of study *Salmonella* prevalence in both the ceftiofur and tulathromycin treated groups was significantly higher ($P < 0.05$) than the earlier days (Table 19).

Table 19**.** Bonferroni-adjusted pairwise fecal prevalence contrasts between days for each treatment group

Trt	Day comparisons		Contrast	Std.Err.	Bonferroni adjusted			
					Z	P-value	95% CIs	
	7	**0**	**-0.38012**	**0.08208**	**-4.63**	**0.001**	**-0.67505**	**-0.08520**
	14	**0**	**-0.32257**	**0.08378**	**-3.85**	**0.018**	**-0.62362**	**-0.02153**
	28	0	-0.11688	0.08746	-1.34	1.000	-0.43115	0.19739
	56	0	-0.11688	0.08746	-1.34	1.000	-0.43115	0.19739
	112	0	0.11918	0.08490	1.40	1.000	-0.18587	0.42423
	14	7	0.05755	0.07424	0.78	1.000	-0.20921	0.32431
	28	7	0.26324	0.08164	3.22	0.193	-0.03012	0.55661
Con	56	7	0.26324	0.08164	3.22	0.193	-0.03012	0.55661
	112	**7**	**0.49930**	**0.07845**	**6.36**	**0.000**	**0.21741**	**0.78119**
	28	14	0.20569	0.08334	2.47	1.000	-0.09378	0.50517
	56	14	0.20569	0.08334	2.47	1.000	-0.09378	0.50517
	112	**14**	**0.44175**	**0.08064**	**5.48**	**0.000**	**0.152007**	**0.73150**
	56	28	-0.00000	0.08722	-0.00	1.000	-0.31340	0.31339
	112	28	0.23605	0.08499	2.78	0.838	-0.06932	0.54143
	112	56	0.23605	0.08499	2.78	0.838	-0.06932	0.54143
	7	0	-0.09850	0.09500	-1.04	1.000	-0.43987	0.24286
	14	0	-0.19278	0.09076	-2.12	1.000	-0.51891	0.13335
	28	0	0.05055	0.10051	0.50	1.000	-0.31060	0.41170
	56	0	0.30175	0.10119	2.98	0.438	-0.06184	0.66534
	112	**0**	**0.55162**	**0.09121**	**6.05**	**0.000**	**0.22389**	**0.87934**
	14	7	-0.09427	0.08417	-1.12	1.000	-0.39671	0.20816
	28	7	0.14905	0.09686	1.54	1.000	-0.19899	0.49709
Tul	**56**	**7**	**0.40025**	**0.09750**	**4.10**	**0.006**	**0.04991**	**0.75059**
	112	**7**	**0.65012**	**0.08368**	**7.77**	**0.000**	**0.34945**	**0.95079**
	28	14	0.24332	0.09307	2.61	1.000	-0.09111	0.57775
	56	**14**	**0.49452**	**0.09328**	**5.30**	**0.000**	**0.15935**	**0.82970**
	112	**14**	**0.74439**	**0.07303**	**10.19**	**0.000**	**0.48197**	**1.00681**
	56	28	0.25120	0.10248	2.45	1.000	-0.11702	0.61942
	112	**28**	**0.50107**	**0.09360**	**5.35**	**0.000**	**0.16473**	**0.83740**
	112	56	0.24987	0.09154	2.73	0.970	-0.07904	0.57878

Table 19. Continued

Trt	Day comparisons		Contrast	Std.Err.	Bonferroni adjusted			
					Z	P-value	95% CIs	
Cef	7	0	-0.15921	0.10195	-1.56	1.000	-0.52554	0.20711
	7	0	-0.17812	0.09079	-1.96	1.000	-0.50437	0.14812
	14	0	-0.15231	0.09185	-1.66	1.000	-0.48235	0.17773
	28	0	0.02504	0.09815	0.26	1.000	-0.32764	0.37773
	56	0	0.34229	0.09736	3.52	0.067	-0.00755	0.69213
	112	**0**	**0.53888**	**0.08990**	**5.99**	**0.000**	**0.21586**	**0.86190**
	14	7	0.02581	0.08072	0.32	1.000	-0.26425	0.31587
	28	7	0.20316	0.09187	2.21	1.000	-0.12696	0.53327
	56	**7**	**0.520412**	**0.09049**	**5.75**	**0.000**	**0.195273**	**0.84555**
	112	**7**	**0.717002**	**0.07608**	**9.42**	**0.000**	**0.443634**	**0.99037**
	28	14	0.177348	0.09285	1.91	1.000	-0.15628	0.51098
	56	**14**	**0.494599**	**0.09166**	**5.40**	**0.000**	**0.165241**	**0.82395**
	112	**14**	**0.691189**	**0.07873**	**8.78**	**0.000**	**0.408273**	**0.97410**
	56	28	0.317251	0.09796	3.24	0.184	-0.03475	0.66924
	112	**28**	**0.513841**	**0.09098**	**5.65**	**0.000**	**0.186937**	**0.84074**
	112	56	0.196590	0.08718	2.25	1.000	-0.11668	0.50985

Trt (Treatment), Con (Control), Tul (Tulathromycin), Cef (Ceftiofur). *P*-values that are less then <0.05 and their corresponding pairwise comparisons are bolded. Reprinted with permission from Levent et al. (2019) [236].

4.3.2. Lymph nodes

A total of 224 observations were included in the model. The marginal predicted means of *Salmonella* prevalence in lymph nodes for the control group were estimated as 0.66 (0.51-0.81 95% CI), the tulathromycin group was 0.82 (0.68-0.96 95% CI), and the ceftiofur group was 0.76 (0.60-0.92 95% CI) (Figure 20).

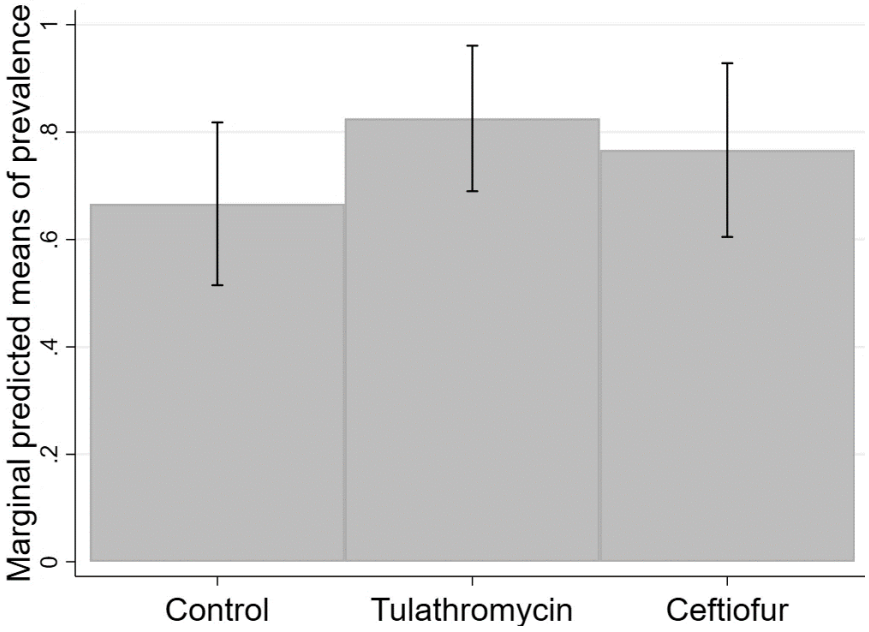


Figure 20. Adjusted lymph node marginal mean prevalence predictions and 95% CIs by treatment

4.3.3. Hides

For the hide observations (n = 132), only the fixed effect of treatment was included in the regression analysis, since hide swab samples were also only collected at slaughter age. Pens were included as the single-random effect and animal identifiers were excluded from the random effects because a single-observation obtained from each animal. The predicted means were 0.77 (0.61-0.93 95% CI) for control-, 0.86 (0.71-1.00 95% CI) for tulathromycin-, and 0.93 (0.83-1 95% CI) for ceftiofur-treatment groups (Figure 21).

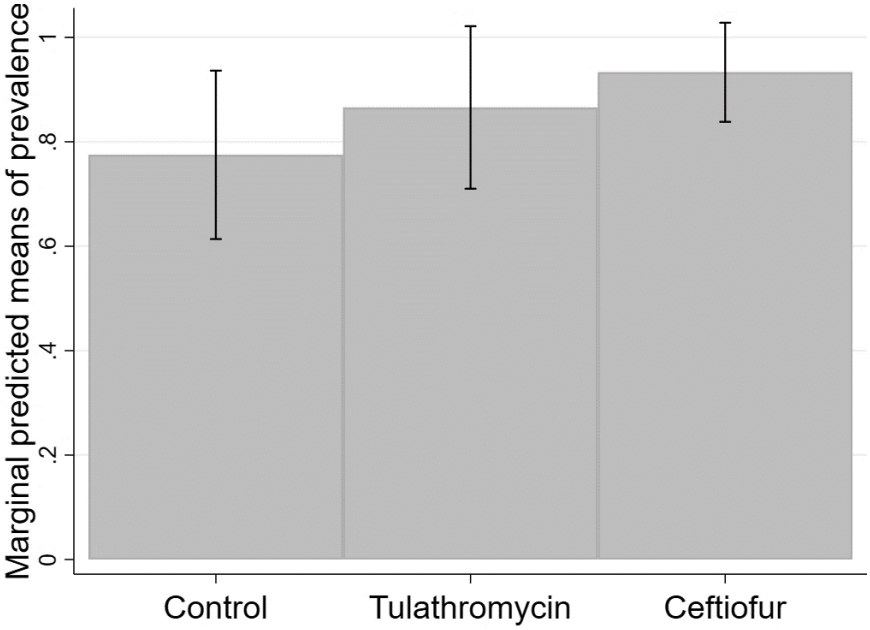


Figure 21. Adjusted hide marginal prevalence mean predictions and 95% CIs by treatment

Overall, likelihood-ratio based tests did not reveal any statistically significant differences in *Salmonella* prevalence among the treatment groups for lymph node and hide samples ($P = 0.297$ and $P = 0.218$, respectively) (Table 17). *Salmonella* prevalence in the control group was not significantly different than the tulathromycin and ceftiofur group among the lymph nodes ($P = 0.344$ and $P = 1.000$, respectively) (Table 18). Similarly, *Salmonella* prevalence in the control cattle was not significantly different from tulathromycin and ceftiofur groups among the hide samples ($P = 1.000$ and $P = 0.212$, respectively). A detailed summary of pairwise contrasts of predicted margin comparisons are provided in Table 18.

Pen-level ICCs for *Salmonella* prevalence were reported as 0.21 (0.12-0.35 95% CI) for feces, 0.13 (0.02-0.44 95% CI) for lymph nodes and 0.30 (0.06 -0.73 95% CI) for hide samples. The animal-level ICCs for *Salmonella* prevalence were 0.21 (0.12- 0.35 95% CI) for temporally dependent fecal samples within animals, and 0.64 (0.37- 0.84 95% CI) for contemporaneously sampled bilateral sub-iliac lymph nodes within animals.

4.4. Quality metrics of the sequencing data

The WGS was performed on all fecal isolates (n=191) recovered from Days 0, 7 and the terminal day, one lymph node isolate from each animal (n=96), and all hide isolates (n=112) in order to explore the population dynamics of *Salmonella* before treatment, immediately after treatment and at slaughter age. Run parameters were evaluated after genome assembly of the 399 isolates. The average number of contigs was 43 (range 17-95), the N50 value was 423,770 bp (range 101,429 - 966,986 bp), and the average depth of coverage was 54X (range 28X-190X). The average genome length of the

Salmonella isolates was 4,775,057 bp (range 4,541,106 - 5,072,387 bp) (Figure 22). The average GC content was 52.1% (range 51.8- 52.3). Sequencing data from this project can be found under NCBI BioProject accession number PRJNA521731.

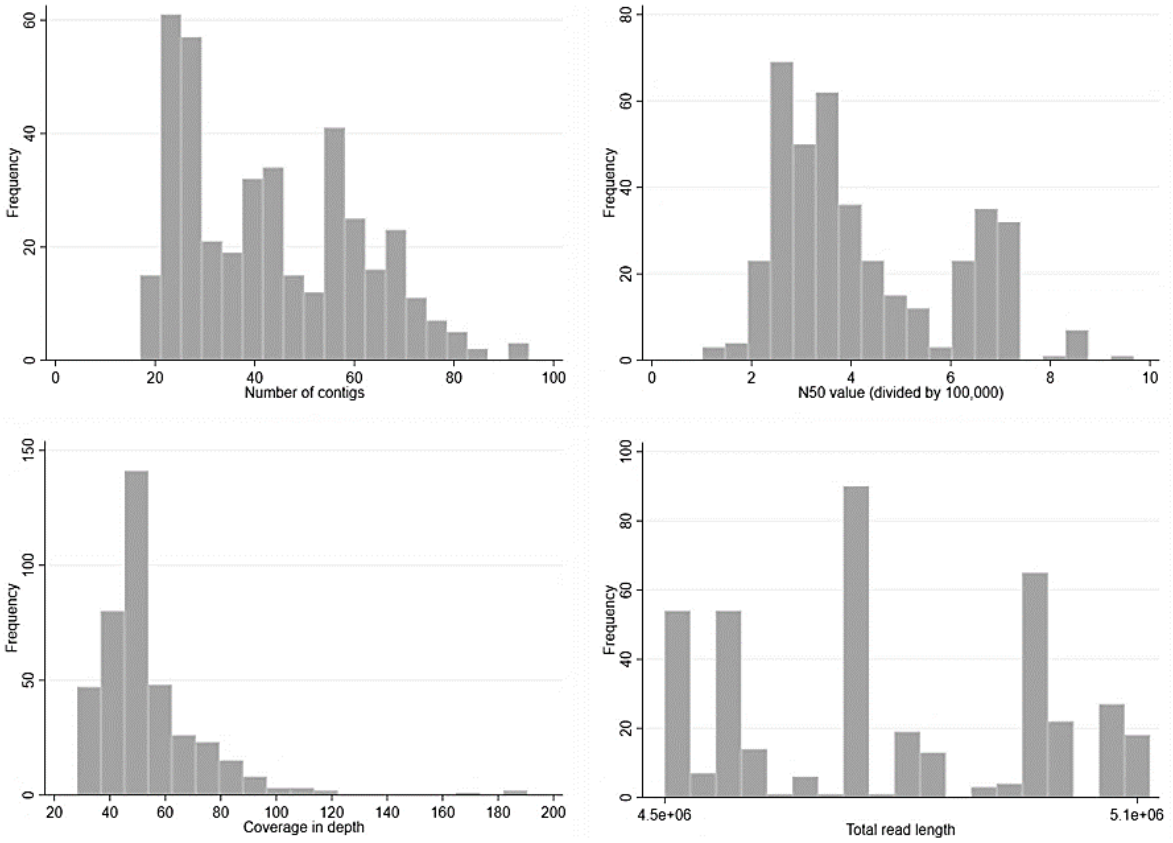


Figure 22. Histograms of descriptive assembly data

4.5. Serotypes and STs

A total of seven different serotypes was identified during the study. Serotypes found in this study were *S.* Anatum (n = 113), *S.* Cerro (n = 64), *S.* Kentucky (n = 11), *S.* Lubbock (n = 136), *S.* Montevideo (n = 68), *S.* Newport (n = 6), and *S.* Norwich (n = 1).

Among these seven serotypes, *S*. Cerro and *S*. Montevideo had only one flagellar antigen (H1); therefore, these serotypes are also called mono-phasic, while the remaining serotypes had both H1 and H2 flagellar antigens. *S*. Lubbock, *S*. Montevideo, and *S*. Norwich were in the O:7 while *S*. Kentucky and *S*. Newport were in the O:8 antigen group. *S*. Anatum and *S*. Cerro were in the O:3 and O:18 antigen groups, respectively. The antigenic formulas of these serotypes are presented in Table 20.

Table 20. O, H1, and H2 antigenic profiles of serotypes found in this study

	O[a]	O	H1	H2	Antigenic formula
Anatum	E1	3	e, h	1, 6	3,10:e,h:1,6
Cerro	K	18	z4	-	18:z4,z23:-
Lubbock	C1	7	g, m, s	e, n, z15	7:g,m,s:e,n,z15
Montevideo	C1	7	g, m	-	7:g,m,s:-
Kentucky	C2	8	i	z6	8:i:z6
Newport	C2	8	e, h	1, 2	8:e,h:1,2
Norwich	C1	7	e, h	1, 6	7:e,h:1,6

[a] Historical O group designation by letters [82].

Serotype-level genome length was compared via one-way analysis of variance (ANOVA) after dropping the single-isolate observation of *S*. Norwich. A significant ($P < 0.005$) length difference was observed among isolates at the serotype-level. However, no significant difference was observed regarding the genome length of the isolates within the same serotype. Pairwise comparisons of the marginal linear predictions by serotypes and WGS assembly lengths showed that *S.* Cerro, *S.* Lubbock, and *S.* Montevideo WGS assembly lengths were statistically significantly different from each other and the remainder of the serotypes. *S.* Anatum was statistically different from all serotypes except *S.* Kentucky (Table 21).

Table 21. Pairwise comparisons of mean assembly lengths of each serotype

No. of observation	Serotype	Mean	Std.Err	95% CI		Tukey group
113	Anatum	4764826	3895.418	4757168	4772484	B
64	Cerro	4551648	2738.685	4546264	4557032	
136	Lubbock	4974442	4051.287	4966477	4982407	
68	Montevideo	4614955	4788.598	4605541	4624369	
11	Kentucky	4746997	407.2556	4746197	4747798	AB
6	Newport	4689851	5607.97	4678826	4700875	A
1	Norwich	4819889	0	-	-	-

Means sharing a letter in the Tukey group label are not significantly different at the 5% level.

Based on pairwise comparisons of the total length of sequence assemblies per serotype, *S*. Cerro, *S*. Lubbock, and *S*. Montevideo were significantly ($P < 0.05$) different from the rest of the serotypes. *S*. Anatum was significantly different ($P < 0.05$) from *S*. Newport; however, both serotypes were not significantly different from *S*. Kentucky.

All *Salmonella* serotypes that were screened for seven legacy MLST genes (*aro*C, *dna*N, *hem*D, *his*D, *pur*E, *suc*A, *thr*A) belonged to a single-ST classification per serotype. The STs were identified as follows: 1) ST 64 (*S*. Anatum), 2) ST 367 (*S*. Cerro), 3) ST 413 (*S*. Lubbock), 4) ST 138 (*S*. Montevideo), 5) ST 118 (*S*. Newport), 6) ST 2119 (*S*. Norwich), and 7) ST 152 (*S*. Kentucky) (Table 22).

Table 22**.** Serotype and legacy MLST allele gene distributions of the serotypes

	ST	*aro*C	*dna*N	*hem*D	*his*D	*pur*E	*suc*A	*thr*A
Anatum	64	10	14	15	31	25	20	33
Cerro	367	14	112	43	123	118	115	120
Lubbock	413	15	70	93	78	113	6	68
Montevideo	138	11	41	55	42	34	58	4
Newport	118	16	2	45	43	36	39	42
Norwich	2119	2	31	10	62	14	19	34
Kentucky	152	62	53	54	60	5	53	54

Reprinted with permission from Levent et al. (2019) [236].

4.6. Population structure of *Salmonella*

When the distribution of *Salmonella* serotypes was examined across sampling days and sample types, distinct distributions of serotypes were identified. Specifically, the unique patterns were made clear when the distribution of serotypes was examined considering cattle source, block designation of pens, and their corresponding slaughter days: Block 1 (Pen 7-9) on Day 134, Block 2 (Pen 51-53) on Day 141, Block 3 (Pen 54-56) on Day 120, Block 4 (Pen 57-59) on Day 99. Block 1 was assigned to the cattle from Source 1, the remaining blocks were from Source 2.

4.6.1. By sample type and day

All seven serotypes (*S*. Anatum, *S*. Cerro, *S*. Kentucky, *S*. Lubbock, *S*. Montevideo, *S*. Newport, and *S*. Norwich) were isolated from fecal samples. Five different serotypes (*S*. Anatum, *S*. Cerro, *S*. Lubbock, *S*. Montevideo, and *S*. Newport) were isolated from lymph nodes, and six different serotypes (*S*. Anatum, *S*. Cerro, *S*. Lubbock, *S*. Montevideo, *S*. Newport, and *S*. Norwich) were isolated from hide samples [236]. *S*. Anatum, *S*. Lubbock, and *S*. Montevideo were recovered across all sample types and sampling days (i.e., 0, 7, terminal). The *Salmonella* serotype distribution observed among the sampling day and sample types are represented in Figure 23.

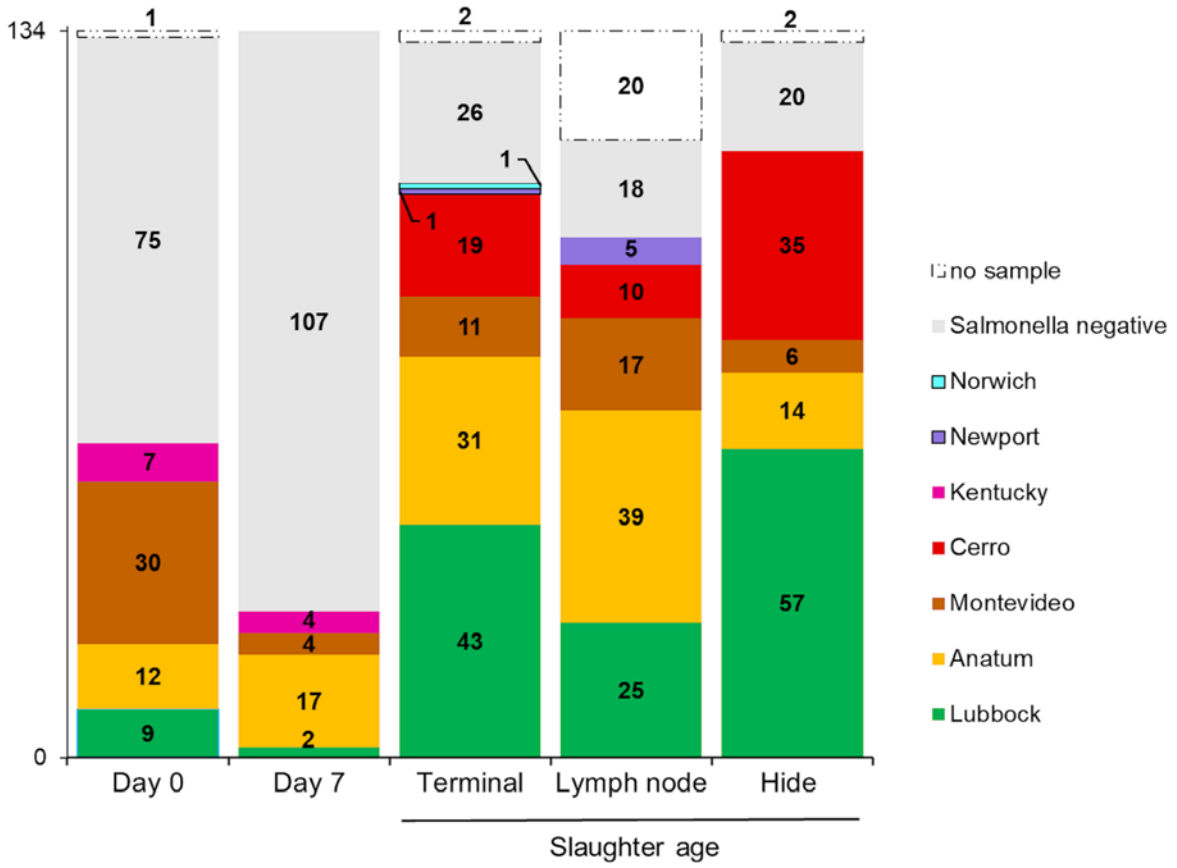


Figure 23. Serotype distribution of *Salmonella* across days and sample types

Reprinted with permission from Levent et al. (2019) [236].

In contingency table analysis, unadjusted likelihood-ratio chi-square tests showed significant associations among the 12 pens when comparing detected serotypes isolated from hide samples. The unadjusted crude likelihood ratio chi-square statistic was 168.9 with 33 df (P <0.0001) for the effect of pen. The unadjusted crude likelihood-ratio chi-square was 32.2 with 6 df (P <0.0001) for pen-level treatments (Figure 24). Note however, the effect of treatment disappeared when accounting for pen alone.

	Ceftiofur				Control				Tulathromycin				
	9	53	55	57	8	52	54	59	7	51	56	58	Total
Anatum	1	0	1	0	0	3	8	0	0	0	0	1	14
Cerro	3	0	9	0	2	0	0	1	7	1	11	1	35
Lubbock	1	9	0	11	4	5	0	10	0	8	0	9	57
Montevideo	4	0	0	0	0	2	0	0	0	0	0	0	6
Salmonella negative cattle hides	3	2	0	0	5	1	3	0	5	1	0	0	20
Total number of cattle hides	12	11	10	11	11	11	11	11	12	10	11	11	132

Figure 24. Hide-level unadjusted frequencies and prevalence proportions of *Salmonella* serotypes by pen

Frequencies of serotypes are indicated with grey color scale. Reprinted with permission from Levent et al. (2019) [236].

S. Anatum was prevalent across all days that were sequenced, but mainly found on Days 0, 7 and 120, and not the other slaughter days (Table 23). Interestingly, at slaughter age, all isolates recovered from feces, lymph nodes, and hides were in complete serotype agreement and all were identified as *S.* Anatum in cattle from Pen 54.

In contrast, the *S.* Cerro serotype was only observed on terminal slaughter days across multiple types of samples and the majority of the pens, except this serotype was not isolated from Pen 53, Pen 54, or Pen 57. Interestingly, all hide isolates recovered from cattle in Pens 7 and 56 were identified as *S.* Cerro. This serotype was observed across all slaughter days, but was found mainly on Days 120 and 134 (Table 23).

Half (34/68) of the *S.* Montevideo isolates recovered in this study were found in Day 0 fecal samples. However, this serotype was also prevalent (32/68) on Day 134 and observed in all types of samples. *S.* Montevideo was not found in any lymph node of cattle

in Blocks 2, 3, or 4 but was found in the lymph nodes of cattle in Block 1, which went to the slaughter on Day 134 (Table 23). Besides Block 1, this serotype was also only identified on the hides from two cattle in Pen 52, which was in Block 2 and slaughtered on Day 141. This serotype was observed in all types of treatment pens.

S. Lubbock was the most prevalent serotype across the study. On Day 0, this serotype was only isolated from fecal samples collected from Pens 9, 52, 55, 58 and 59 and on Day 7, this serotype was only identified in Pen 9. At slaughter age, this serotype was recovered from all sample types, especially on Days 99 and 141 (Table 23). Interestingly, all isolates from the hide samples collected from Pen 53 and 57 were of the *S.* Lubbock serotype.

S. Kentucky serotype was observed only on Day 0 (n = 7) and 7 (n = 4) fecal samples, and only in pens where cattle originated from a single-source (Source 1) and thus were in Block1. This serotype was not identified at slaughter age from any of the fecal, lymph node or hide samples (Table 23).

Table 23. Serotype distribution by day and sample type

Day	Anatum	Cerro	Kentucky	Lubbock	Montevideo	Newport	Norwich
0	**12**	0	**7**	**9**	**30**	0	0
7	**17**	0	**4**	**2**	**4**	0	0
99	**7**	**3**	0	**19**	0	0	0
	1	**2**	0	**30**	0	0	0
	6	**1**	0	**6**	0	0	0
120	**10**	**11**	0	**2**	0	0	0
	9	**20**	0	0	0	0	0
	25	**2**	0	0	0	0	0
134	**7**	**4**	0	**4**	**11**	0	0
	1	**12**	0	**5**	**4**	0	0
	3	**7**	0	**7**	**17**	0	0
141	**7**	**1**	0	**18**	0	**1**	**1**
	3	**1**	0	**22**	**2**	0	0
	5	0	0	**12**	0	**5**	0
Total	**113**	**64**	**11**	**136**	**68**	**6**	**1**

Observations from feces are indicated by the white, hide are indicated by the darker gray, and the lymph nodes are indicated by the lighter gray. Terminal isolates that were collected at the slaughter age were Days 99, 120, 134, and 141. Values larger than zero are bolded in the cells.

Six *S*. Newport isolates were found in cattle placed in one pen (Pen 53); of those, five were in lymph nodes and one was in the final fecal sample collected from one of the steers. This serotype was only observed at slaughter age on Day 141 (Table 23). The single pen that harbored *S*. Newport isolates was in the ceftiofur-treated group. A single *S*. Norwich isolate was isolated once from a terminal day fecal sample of a steer located in Pen 52 and that was slaughtered on Day 141 (Table 23).

The distribution of the *Salmonella* serotypes is presented in Figure 25

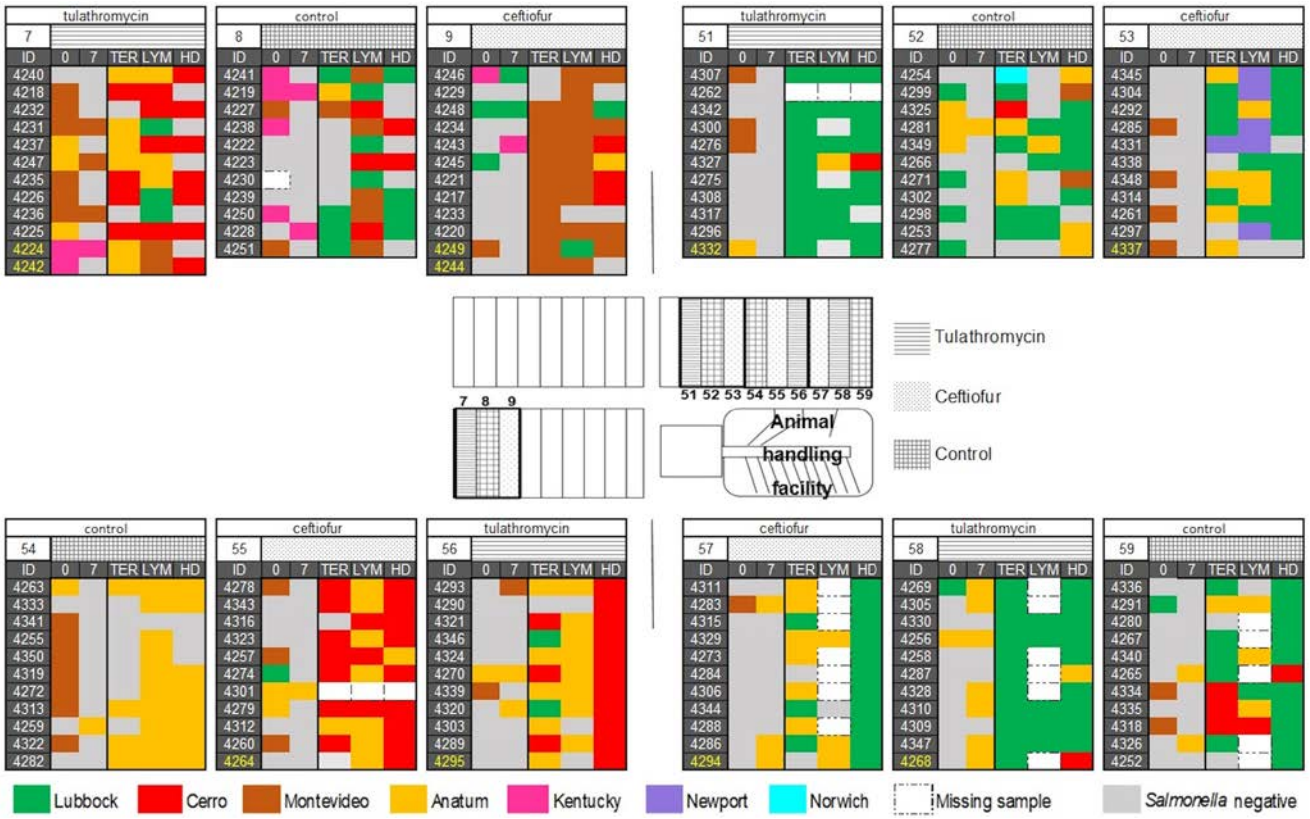


Figure 25. *Salmonella* serotype distribution observed in individual animals by pen, day, sample type and treatment

ID; unique animal identifier, 0; Day 0, 7; Day 7, TER; Terminal fecal isolates, LYM: lymph node isolates, HD: hide isolates. Each block of pens, (three pens on each corner) was harvested on the same day: Block 1 (Pen 7-9) on Day 134, Block 2 (Pen 51-53) on Day 141, Block 3 (Pen 54-56) on Day 120, Block 4 (Pen 57-59) on Day 99. Block 1 is from Source 1; the remaining blocks are from Source 2. The numbers in the upper left corner are pen IDs. Pen treatments are located at the top of each pen. Geographic locations of pens and treatments are schematized in the middle. Reprinted with permission from Levent et al. (2019) [236].

4.6.2. By pen, block, source, and treatments

The serotype distribution by pen is presented in Table 24, and by source and blocks is presented in Table 25, while the treatment distributions are presented in Table 26.

Table 24. Serotype distribution by pen

	Pen											
	7	8	9	51	52	53	54	55	56	57	58	59
Anatum	**13**	**1**	**2**	**2**	**14**	**9**	**26**	**15**	**18**	**14**	**12**	**8**
Cerro	**15**	**6**	**3**	**3**	**1**	0	0	**19**	**14**	0	**1**	**6**
Lubbock	**3**	**11**	**6**	**24**	**17**	**15**	0	**1**	**2**	**14**	**26**	**17**
Montevideo	**14**	**9**	**25**	**3**	**2**	**4**	**7**	**3**	**2**	**1**	0	**2**
Kentucky	**3**	**6**	**2**	0	0	0	0	0	0	0	0	0
Newport	0	0	0	0	0	**6**	0	0	0	0	0	0
Norwich	0	0	0	0	**1**	0	0	0	0	0	0	0

Values larger than zero are bolded in the cells.

Table 25. Serotype distribution by source and block

	Source 1	Source 2		
	Block 1	Block 2	Block 3	Block 4
Anatum	**16**	**25**	**59**	**34**
Cerro	**24**	**4**	**33**	**7**
Lubbock	**20**	**56**	**3**	**57**
Montevideo	**48**	**9**	**12**	**3**
Kentucky	**11**	0	0	0
Newport	0	**6**	0	0
Norwich	0	**1**	0	0

Values larger than zero are bolded in the cells.

Table 26. Serotype distribution by pen-level treatment

	Pen-level Treatment		
	9-53-55-57	8-52-54-59	7-51-56-58
	Ceftiofur	Control	Tulathromycin
Anatum	**40**	**49**	**45**
Cerro	**22**	**13**	**33**
Lubbock	**36**	**45**	**55**
Montevideo	**33**	**20**	**19**
Kentucky	**2**	**6**	**3**
Newport	**6**	0	0
Norwich	0	**1**	0

Assigned pen IDs are placed above the treatment names. Values larger than zero are bolded in the cells.

S. Anatum was the only serotype observed across all blocks, sources, and pens. *S.* Cerro was observed in all sources, blocks and pens except Pen 53, 54, and 57; this serotype was less prevalent (< 8) in Block 2 and 4 compared to Block 1 and 3 (> 23). *S.* Montevideo was observed in all sources, blocks and pens except Pen 58; however, it was mainly prevalent in Source 1 (n=48) as compared to Source 2 (n=24). *S.* Kentucky was only observed in Source 1 and Block 1 in Pens 7, 8 and 9. *S.* Lubbock was observed in all sources, blocks and pens except Pen 54. *S.* Newport was observed in only Source 2, Block 2 and Pen 53. The single *S.* Norwich serotype was observed in Source 2, Block 2 and Pen 52.

When the serotype distributions were examined across pen-level treatments, all serotypes were observed across treatments, except *S.* Newport that was only observed in a single ceftiofur treated pen, and the single S. Norwich which was recovered from a control pen (Table 26).

4.7. Phenotypic antibiotic resistance

Among 630 *Salmonella* isolates, the majority were pan-susceptible (79.0%). The remaining isolates presented with either single (20.4%) or double (0.4%) phenotypical antibiotic resistance. Most of the isolates resistant to a single-antibiotic (n=108) exhibited resistance to tetracycline, while 21 other isolates were resistant to streptomycin. Three isolates were resistant to both tetracycline and streptomycin. Isolates tested in this study did not show any phenotypic resistance to the remaining 12 antibiotics tested including, but not limited to: azithromycin, ceftiofur, ceftriaxone, and ceftiofur.

In this panel, amoxicillin/clavulanic acid (a β-lactam combination antibiotic) was tested at concentrations of 1, 2, 4, 8, 16, and 32 µg/ml. These values were listed based on the first antibiotic (amoxicillin) concentration in the combination that was at a 2:1 ratio. The cut-off value for amoxicillin/clavulanic acid resistance was ≥ 32 µg/ml. Among the isolates tested, the observed MIC range was 1 to 4 µg/ml; therefore, all isolates were classified as susceptible to amoxicillin/clavulanic acid. The majority of the *Salmonella* isolates (92.8%) in this study showed an MIC at ≤ 1 µg/ml. Only 6.9% of the isolates showed an MIC at 2 µg/ml, and 0.1% of the isolates had an MIC at 4 µg/ml. No isolates were found with MIC at the levels of 8, 16, and 32 µg/ml.

Similarly, ampicillin (a penicillin class antibiotic) was tested at concentrations of 1, 2, 4, 8, 16, and 32 µg/ml. The cut-off value for ampicillin resistance was ≥ 32 µg/ml. Among the isolates tested, the observed MICs were between 1-16 µg/ml, and all isolates were susceptible to ampicillin. The majority of the isolates (92.8%) showed an MIC at ≤ 1 µg/ml. Only 6.8% of the isolates had an MIC at 2 µg/ml, and 0.1% of the isolates had an MIC at 4 µg/ml or 16 µg/ml.

Azithromycin (a macrolide class antibiotic) was tested at concentrations of 0.12, 0.25, 0.5, 1, 2, 4, 8, and 16 µg/ml. The cut-off value for azithromycin resistance was >16 µg/ml. The majority of the isolates (80.3%) showed an MIC of 4 µg/ml. The MICs of 2 and 8 µg/ml were observed in 8.7% and 8.8% of the isolates, respectively. In addition, MICs at 1, 0.5, and 0.12 µg/ml were observed in 1.1%, 0.3%, and 0.4% of the isolates, respectively. The remaining 0.1% of isolates had an MIC equal to 16 µg/ml. There was no isolate resistant to azithromycin with an MIC >16 µg/ml (Table 27).

Table 27. Azithromycin MIC distribution by individual-treatment

MICs (µg/ml)	0.12	0.25	0.5	1	2	4	8	16
Ceftiofur	**2**	0	0	**3**	**11**	**157**	**14**	0
Control	**1**	0	0	**3**	**29**	**184**	**21**	0
Tulathromycin	0	0	**2**	**1**	**15**	**165**	**21**	**1**
Total	**3**	0	**2**	**7**	**55**	**506**	**56**	**1**

Total number of isolates tested = 630. Values larger than zero are bolded in the cells.

Cefoxitin (a cephem class antibiotic) was tested at concentrations of 0.5, 1, 2, 4, 8, 16, and 32 µg/ml. The cut-off value for cefoxitin resistance was ≥ 32 µg/ml. The MICs at levels of 2, 4, and 8 µg/ml were observed in 25.2%, 68.5%, and 5.4% of the isolates, respectively. The remaining isolates had MICs of 0.5 µg/ml (0.3%), 1 µg/ml (0.3%), and 16 µg/ml (0.1%). No MIC was observed at ≥ 32 µg/ml.

Ceftiofur (a third-generation cephalosporin – another cephem class antibiotic) was tested at the MIC concentrations of 0.12, 0.25, 0.5, 1, 2, 4, and 8 µg/ml. The NARMS cut-off value for ceftiofur resistance was ≥ 8 µg/ml. The majority of the isolates (92.5%) showed an MIC at 1 µg/ml. Another 5.0% and 1.5% of isolates exhibited MICs of 0.5 and

2 µg/ml, respectively. The MICs of 0.12 and 0.25 µg/ml were observed only in 0.3% and 0.4% of isolates, respectively. The MICs at 4 and 8 µg/ml were not observed in any isolates (Table 28). Therefore, in our study all isolates were determined to be susceptible to ceftiofur.

Table 28. Ceftiofur MIC distribution by individual-treatment

MICs (µg/ml)	0.12	0.25	0.5	1	2	4	8
Ceftiofur	**1**	**1**	**8**	**174**	**3**	0	0
Control	**1**	**1**	**15**	**216**	**5**	0	0
Tulathromycin	0	**1**	**9**	**193**	**2**	0	0
Total	**2**	**3**	**32**	**583**	**10**	0	0

Total number of isolates tested = 630. Values larger than zero are bolded in the cells.

Ceftriaxone (a third-generation cephalosporin – another cephem class antibiotic) was tested at concentrations of 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 µg/ml. The cut-off value for ceftriaxone resistance was ≥ 4 µg/ml. All isolates (100.0%) exhibited MIC at ≤ 0.25 µg/ml, which meant they were phenotypically susceptible to ceftriaxone.

Chloramphenicol (a phenicol class antibiotic) was tested at concentrations of 2, 4, 8, 16 and 32 µg/ml. The cut-off value for ceftriaxone resistance was ≥ 32 µg/ml. The MICs were observed at 2, 4, 8, and 16 µg/ml among 3.9%, 30.0%, 65.7%, and 0.3% of the isolates, respectively. No MIC was observed above 16 µg/ml. Therefore, all isolates were phenotypically susceptible to chloramphenicol.

Ciprofloxacin (a fluoroquinolone class antibiotic) was tested at concentrations of 0.015, 0.03, 0.06, 0.12, 0.25, 0.5, 0.1, 2, and 4 µg/ml. The cut-off value for ciprofloxacin resistance was ≥ 1 µg/ml. The majority of the isolates (95.5%) showed an MIC at ≤ 0.015

µg/ml. The remaining 4.2% and 0.1% of isolates showed MICs at 0.03 and 0.06 µg/ml, respectively. No MIC was detected above the concentration of 0.06 µg/ml. Therefore, all isolates were phenotypically susceptible to ciprofloxacin. We also did not identify any isolates with reduced susceptibility (MICs of 0.12– 1 µg/ml) to ciprofloxacin (Table 29).

Table 29. Ciprofloxacin MIC distribution by individual-treatment

MICs (µg/ml)	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4
Ceftiofur	**175**	**12**	0	0	0	0	0	0	0
Control	**231**	**7**	0	0	0	0	0	0	0
Tulathromycin	**196**	**8**	**1**	0	0	0	0	0	0
Total	**602**	**27**	**1**	0	0	0	0	0	0

Total number of isolates tested = 630. Values larger than zero are bolded in the cells.

Nalidixic acid (a quinolone class antibiotic) was tested at concentrations of 0.5, 0.1, 2, 4, 16, and 32 µg/ml. The cut-off value for nalidixic acid resistance was ≥ 32 µg/ml. The majority of the susceptible isolates (50.1% and 49.6%, respectively) had MICs at 2 and 4 µg/ml. Only 0.1% of the isolates exhibited 0.5 µg/ml MIC for nalidixic acid. No MIC was observed at concentrations higher than 4 µg/ml. Therefore, all isolates were susceptible to nalidixic acid.

Gentamicin (an aminoglycoside class antibiotic) was tested at concentrations of 0.25, 0.5, 1, 2, 4, 16, and 32 µg/ml. The cut-off value for gentamicin resistance was ≥16 µg/ml. The MICs of 0.25, 0.5, 1, and 2 µg/ml were observed in 22.3%, 57.7%, 19.2% and 0.5% of the isolates. No MIC was observed above 2 µg/ml; therefore, all isolates were susceptible to gentamicin.

Streptomycin (another aminoglycoside class antibiotic) was tested at concentrations of 2, 4, 16, 32, and 64 µg/ml. The cut-off value for streptomycin resistance was ≥ 16 µg/ml. Among the *Salmonella* isolates tested, 46.0% and 41.7% of the isolates exhibited MICs at 8 and 16 µg/ml, respectively. The remaining 0.6% of the isolates showed an MIC at 2 µg/ml and 7.7% of the isolates showed the MIC at 4 µg/ml. Streptomycin-resistant isolates were observed in 3.1% (20/630) and 0.6% (4/630) of the total isolates, which represented MICs at 16 and 64 µg/ml, respectively.

Sulfisoxazole (a folate pathway antagonist class antibiotic) was tested at concentrations of 16, 32, 64, 128, and 256 µg/ml. The cut-off value for sulfisoxazole resistance was > 256 µg/ml. The majority (40.4%) of the isolates showed the MIC at ≤ 16 µg/ml, followed by 32 µg/ml (20.4%), 64 µg/ml (25.0%), 128 µg/ml (13.1%) and = 256 µg/ml (0.7%) of the isolates. All isolates were susceptible to sulfisoxazole in this study.

Trimethoprim/sulfamethoxazole (another potentiated folate pathway antagonist class antibiotic) was tested at concentrations of 0.12, 0.25, 0.5, 0.1, 2 and 4 µg/ml (trimethoprim only). The cut-off value for trimethoprim/sulfamethoxazole resistance was ≥ 4 µg/ml. The majority (99.8%) of the isolates showed the MIC at ≤ 0.12 µg/ml. The remaining (0.1%) isolates exhibited the MIC at 0.25 µg/ml. No MICs were observed at 0.5, 1, 2, and 4 µg/ml. All isolates were susceptible to the trimethoprim/sulfamethoxazole combination.

Tetracycline (a tetracycline class antibiotic) was tested at concentrations of 4, 16, and 32 µg/ml. The cut-off value for tetracycline resistance was ≥ 16 µg/ml. The majority of the isolates (82.3%) showed the MIC at ≤ 4 µg/ml. The remaining isolates (17.7%)

were phenotypically resistant to tetracycline either at 32 µg/ml (0.2% [1/630]) or above 32 µg/ml (17.4% [110/630]) such that they were right-censored on this plate.

Overall results suggest that among the 630 *Salmonella* isolates tested for 14 antibiotics, 79.0% of these isolates were pan-susceptible. The remaining isolates represented single (20.4%) or double (0.4%) phenotypical antibiotic resistance against either tetracycline, or streptomycin. The majority of these isolates (n = 108) exhibited single-resistance to tetracycline (17.6%, 4.7- 20.8 [95% CI]), while 21 isolates were resistant only to streptomycin (3.8%, 2.4 - 5.6 [95% CI]). Three isolates were resistant to both tetracycline and streptomycin. No resistance was observed against amoxicillin/ clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, nalidixic acid, gentamicin and trimethoprim/sulfamethoxazole [236] (Table 30).

Table 30. Overall MICs distribution and antibiotic resistance classification of *Salmonella* isolates

Antibiotics	Exact (Clopper-Pearson)		MIC (µg/ml) distribution and classification of resistance (%)														
	R (%)	95% CI	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Amoxicillin/ clavulanic acid[a]	0.00	0.00-0.05*							92.8	6.9	0.1	0.0	0.0	0.0				
Ampicillin	0.00	0.00-0.05*							92.8	6.8	0.1	0.0	0.1	0.0				
Azithromycin[b]	0.00	0.00-0.05*				0.4	0.0	0.3	1.1	8.7	80.3	8.8	0.1					
Cefoxitin	0.00	0.00-0.05*						0.3	0.3	25.2	68.5	5.3	0.1	0.0				
Ceftiofur	0.00	0.00-0.05*				0.3	0.4	5.0	92.5	1.5	0.0	0.0						
Ceftriaxone	0.00	0.00-0.05*					100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
Chloramphenicol	0.00	0.00-0.05*								3.9	30.0	65.7	0.3	0.0				
Ciprofloxacin	0.00	0.00-0.05*	95.5	4.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0							
Nalidixic acid	0.00	0.00-0.05*						0.1	0.0	50.1	49.6	0.0	0.0	0.0				
Gentamicin	0.00	0.00-0.05*					22.3	57.7	19.2	0.6	0.0	0.0	0.0					
Streptomycin[b]	3.80	2.40-5.60								0.6	7.7	46.0	41.7	3.1	0.6			
Sulfisoxazole	0.00	0.00-0.05*											40.4	20.4	25.0	13.1	0.7	0.0
Trimethoprim/ sulfamethoxazole[a]	0.00	0.00-0.05*				99.8	0.1	0.0	0.0	0.0	0.0							
Tetracycline	17.60	14.70-20.80									82.3	0.0	0.0	0.2	17.4			

[a]MIC to the first antibiotic in each combination is listed. [b]NARMS breakpoints used for classification. *One-sided 97.5% CI was used when the prevalence estimate was zero. R: Resistance. Grey areas indicate out-of-dilution range of the Sensititre® plate. Breakpoints for resistance classification are indicated using vertical lines. Numbers in grey areas are right-censored MIC concentrations. Resistant values are indicated in red. Reprinted with permission from Levent et al. (2019) [236].

Cross-tabulations of serotype by individual phenotypic antibiotic resistance data showed strong associations. Among *S*. Montevideo isolates, 95.5% (63/68) exhibited tetracycline resistance. In contrast, the remaining six serotypes (*S*. Anatum, *S*. Kentucky, *S.* Lubbock, *S.* Newport, and *S*. Norwich) exhibited either complete susceptibility to tetracycline or had a limited number of tetracycline resistant isolates (i.e., one *S*. Cerro and two *S*. Lubbock isolates). Phenotypic streptomycin resistance was found in seven *S*. Anatum, six *S*. Lubbock, one *S*. Montevideo, and one *S*. Kentucky isolates (Table 31).

Table 31. Antimicrobial resistance phenotype distributions by serotype

	Str[a]	Tet[b]	Tet-Str	Pan-sus[c]	Total
Anatum	**7**	0	0	106	113
Cerro	0	**1**	0	63	64
Lubbock	**6**	**2**	0	128	136
Montevideo	**1**	**63**	**2**	2	68
Kentucky	**1**	0	0	10	11
Newport	0	0	0	6	6
Norwich	0	0	0	1	1

[a] Streptomycin, [b] tetracycline, [c] pan-susceptible. Values representing phenotypic antibiotic resistance larger than zero are bolded in the cells. Reprinted with permission from Levent et al. (2019) [236].

Only the MICs of 4 and 32 µg/ml were observed against tetracycline in the isolates. All *S*. Anatum, *S*. Newport, and *S*. Cerro isolates showed an MIC at 4 µg/ml. The majority of *S*. Lubbock isolates (134/136) and *S*. Cerro (63/64) showed an MIC at 4 µg/ml.

4.8. Antibiotic resistance genes and plasmids

A total of 399 isolates were screened with 95% identity match and 60% of coverage threshold for ARG identification using the ResFinder database in ABRicate. Among all *Salmonella* isolates that were sequenced and screened for ARGs, all isolates

harbored a single-aminoglycoside resistance gene *aac*(6')-*Iaa,* A total of 67 isolates harbored the tetracycline resistance gene *tet*(A) and also the fosfomycin resistance gene *fos*A7 (Table 32). All these isolates were also screened with 95% identity match and 60% of coverage threshold for plasmid identification using the PlasmidFinder database in ABRicate. Among these, 67 isolates had the IncN plasmid and 64 isolates had the IncI1 plasmid (Table 33).

Table 32. Antibiotic resistance genes identified in the study

Gene name	Gene product	Coverage (%)	ID (%)	GeneBank No.
*fos*A	*fos*A7_1	65.9	96.06	LAPJ01000014
aac(6')-*Iaa*	*aac*(6')-*Iaa*_1	100	97.0-99.0	NC_003197
tet(A)	*tet*(A)_6	97.8	100	AF534183

ID: Identity match, Coverage: Coverage of the actual sequence length. GeneBank No: Accession numbers in GenBank database.

Table 33. Plasmids identified in the study

Plasmid	Gene name	Gene product	Coverage	ID	GeneBank No.
IncI1	IncI1	IncI1_1_Alpha	100	99.3	AP005147
IncN	IncN	IncN_1	100	100	AY046276

ID: Identity match, Coverage: Coverage of the actual sequence length. GeneBank No: Accession numbers in GenBank database.

All of the *Salmonella* isolates had the *aac*(6')-*Iaa* aminoglycoside gene regardless of the serotype. The specific ARG profiles that were found in all *Salmonella* isolates were fosfomycin resistance gene *fos*A and tetracycline resistance gene *tet*(A) that was in *S.* Montevideo serotype (Table 34). A detailed information about the ARGs found in this study was provided in Table 32.

Table 34. Resistance genes and plasmidal distributions by serotype

	Resistance genes			Plasmids	
	aac(*6*')-*Iaa*	*tet*(A)	*fos*A	IncN	IncI1
Anatum	**113**	0	0	0	**16**
Cerro	**64**	0	0	0	**3**
Kentucky	**11**	0	0	0	0
Lubbock	**136**	0	0	0	**43**
Montevideo	**68**	**67**	**68**	**67**	**2**
Newport	**6**	0	0	0	0
Norwich	**1**	0	0	0	0

Values larger than zero were bolded in the cells.

In terms of comparing the genotypic and phenotypic antibiotic resistance observed in isolates, S. Montevideo isolates that were phenotypically resistant to tetracycline all harbored *tet*(A) gene (65/65). Among the remaining three *S.* Montevideo isolates, one did not harbor the *tet*(A) gene and IncN plasmid, and did not show any phenotypic tetracycline resistance whereas another one carried both *tet*(A) gene and IncN plasmid but was phenotypically susceptible to tetracycline; interestingly, this isolate had phenotypic streptomycin resistance. The remining one did not harbor the *tet*(A) gene and IncN plasmid but also did not have phenotypic tetracycline resistance.

In this study, we did not test for fosfomycin resistance; therefore, we could not observe the expression of *fos*A gene with a phenotype; moreover, this gene was detected at the relatively low (65.7%) coverage that was close to the threshold value (60.0%). The isolates that harbored aac(*6'*)-*Iaa* gene were susceptible to the aminoglycoside class antibiotics tested in this study.

Among the 399 sequenced isolates, a total of 129 of isolates had a minimum of one plasmid. There were only two type of plasmids observed among those isolates: IncI and

IncN plasmids (Table 33). A total of 64 isolates harbored the IncI plasmid, whereas 68 isolates harbored the IncN plasmid. Three isolates had both the IncN and the IncI1 plasmids. The distribution of these plasmids was highly serotype specific.

IncN plasmid was found in 67 of the 68 *S.* Montevideo isolates. Later, with the BLAST and RAStk analyses, the *tet*(A) gene along with its repressor *tet*(R) gene) was identified in a contig annotated to an IncN plasmid, which proved this gene was in the IncN plasmid (Figure 26).

The IncI1 plasmid was commonly found in the *S.* Lubbock (43/136) serotype, followed by *S.* Anatum (16/113) and *S.* Cerro (3/64) serotypes, but did not harbor any ARGs. Among those, only two *S.* Montevideo isolates that harbored the IncI1 plasmid also carried the second IncN plasmid. *S.* Newport and *S.* Norwich were the only serotypes that did not harbor any identified plasmids. Detailed information about the plasmids found in this study are provided in Table 33.

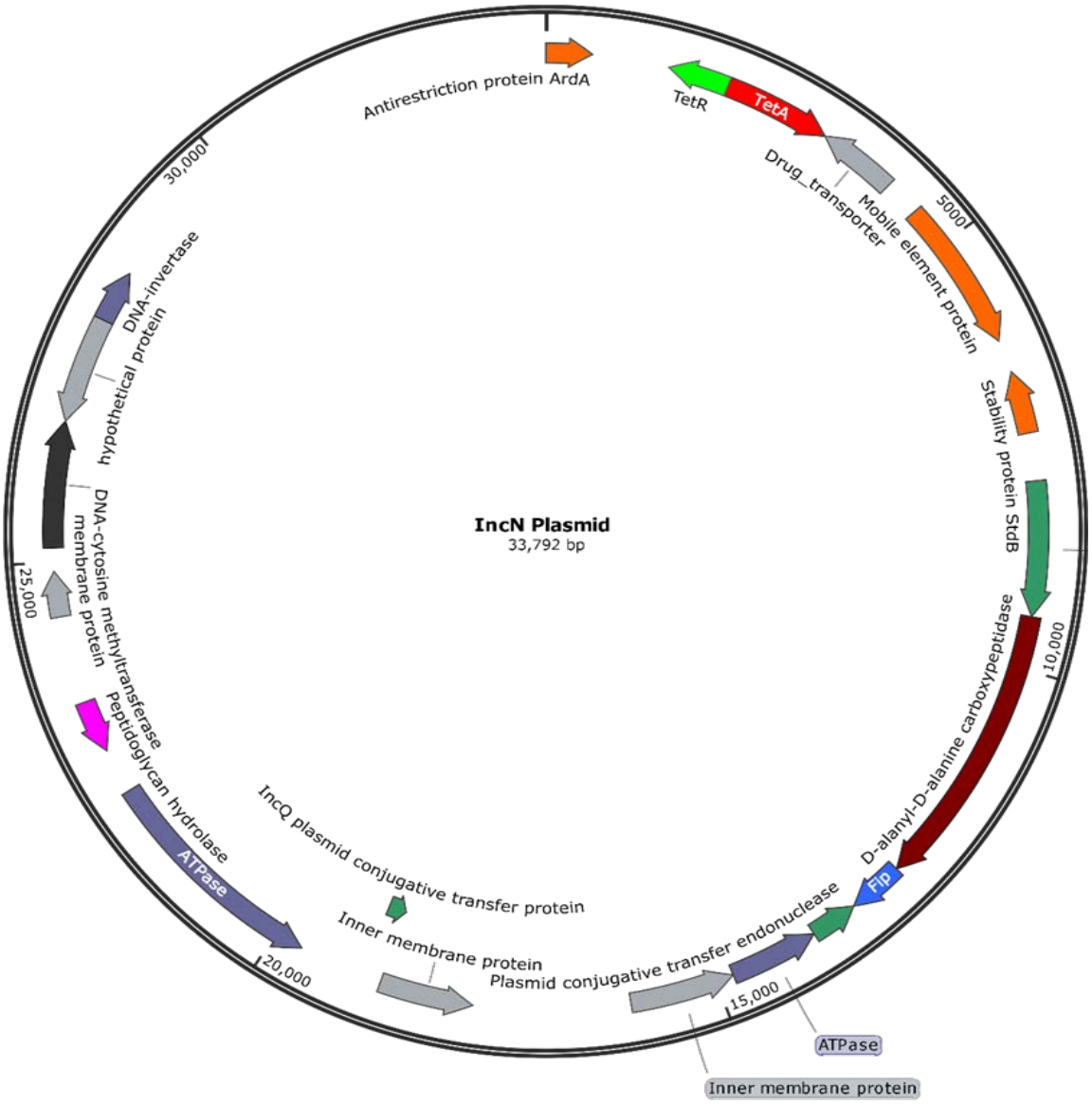


Figure 26. Circular representation of the IncN plasmidal contig harboring the *tet*(A) gene

The *tet*(A) gene is colored in red, and the repressor *tet*(R) gene is colored in light green.

4.9. Cladogenetic relationships

The serotype *S.* Anatum was one of the most prevalent serotypes found in our study. Therefore, a complete genome of an *S.* Anatum (GenBank: CP007483.2) was selected as the reference to perform the genome alignments of all *Salmonella* serotypes and strains observed in this study. This reference was initially isolated from a ground beef product from Beef Industry Food Safety Council (BIFSCo) Region-3, which was located in the southern USA (unpublished data), and this isolate was submitted by USDA-Meat Animal Research Center in 2014. This strain was the closet genome to the *S.* Anatum isolates recovered from our study, with a k-mer count coverage of 959/1,000, a Mash/MinHash distance of 1.00 e^{-3}, and a *P*-value of 0.00. A total of 5 prophage regions were detected and masked in this reference genome, regardless of their score. Of those, three were intact regions and the remaining two regions were either questionable or incomplete (Table 35).

A Generalized Time Reversible (GTR)+G4 (gamma parameter 4) model was selected from Model-test NG based on the best AIC value. This model only counts for SNP regions and generates the phylogeny using those sites. The tree was further inferred using IQ-tree with 1,000 bootstrap iterations. Later, the tree was rooted on the reference strain described above for graphical visualizations. The cladogram branch that was found in the same phylogenetic structure of the tree for 800-1,000 sampling times (iterations) was accepted as highly confident for its position. Those branches are graphically shown with grey circles in the middle of each branch with the size proportional to the bootstrap value.

Table 35. Prophage regions masked in the *S.* Anatum reference genome

Phage Name	RefSeq No.	Score	Size (kb)	Position (bp)
Salmon_Fels_2	NCC_010463	Intact	44.5	1,145,366-1,189,915
Salmon_Fels_1	NCC_01039	Intact	16.2	2,040,464-2,056,760
Salmon_118970_sal3	NCC_031940	Intact	45.3	2,691,803-2,737,129
Entero_P4	NCC_001609	Questionable	11.8	4,099,187-4,111,081
Burkho_BcepMu	NCC_005882	Incomplete	18.1	4,390,249-4,408,403

RefSeq No: Reference sequence number in NCBI.

The cladogram was accompanied with the graphical input as the unique colors representing each serotype with a closely related scale of colors to distinguish each block/cattle origin related metadata easily. Pens (n = 12) were colored individually while blocks (1-4) were colored by shades of blue, pink/purple, red/orange and green, respectively. The sources were indicated by shades of blue (for Source 1) versus the shades of remaining block colors (Source 2) (Figure 27).

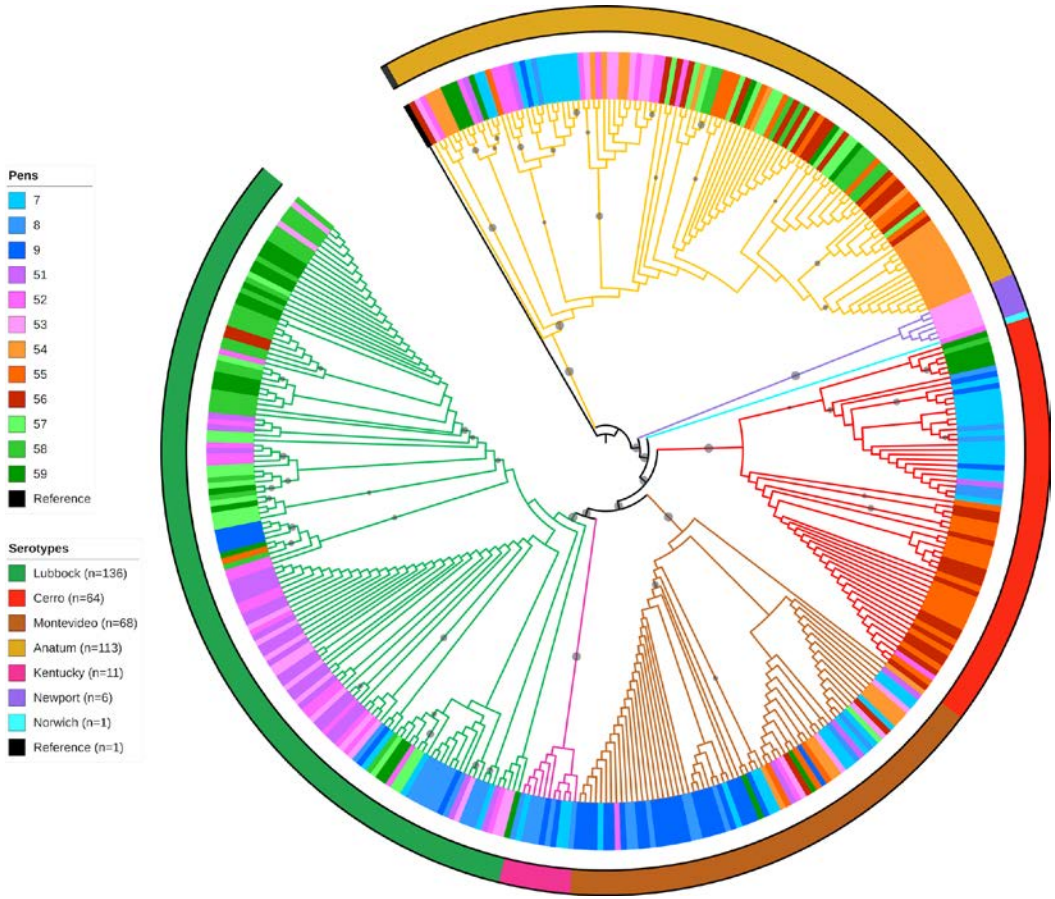


Figure 27. Cladogram representing the pen-level overall population structure of *Salmonella* serotypes

The maximum-likelihood cladogram was generated using GTR+G4 Model. Serotype colors are represented in the cladogram branches and outer circle. Colors in the inner circle represent pen distribution. Block 1 (pens with shades of blue), Block 2 (pens with shades of pink/purple), Block 3 (pens with shades of red/orange), Block 4 (pens with shades of green). Source 1 (shades of blue), Source 2 (shades of pink, red, green. Reprinted with permission from Levent et al. (2019) [236].

4.10. Phylogenetic relationships

Serotype-level SNP analyses were conducted only for the serotypes of *S.* Lubbock, *S.* Anatum, *S.* Montevideo, and *S.* Cerro. The *S.* Kentucky, *S.* Newport, and *S.* Norwich serotypes were excluded from these analyses due to small numbers of observations (< 12 isolates) within each of these serotypes.

4.10.1. *S.* Lubbock

A complete genome of an *S.* Lubbock (GenBank: CP032814.1) isolate, which was obtained from the sub-iliac lymph node of a cow located in Texas, United States was found to be the closet genome to the *S.* Lubbock isolates recovered from our study; that is, with k-mer count coverage of 999/1,000, Mash/MinHash distance of $2.38e^{-5}$ and a *P* value of 0 [15, 270]. A total of 11 prophage regions were detected and masked in this reference genome, regardless of their score. Of those, nine were intact regions and the remaining two regions were incomplete prophage regions (Table 36).

Table 36. Prophage regions masked in the *S.* Lubbock reference genome

Phage Name	RefSeq No.	Score	Size (kb)	Position (bp)
Haemop_HP1	NC_001697	Intact	54.5	1,103,664-1,158,175
Salmon_Fels_2	NC_010463	Intact	34.3	1,148,048-1,182,440
Entero_PsP3	NC_005340	Intact	34.2	1,212,067-1,246,302
Salmon_g341c	NC_013059	Intact	40.6	1,505,665-1,546,354
Edward_GF_2	NC_026611	Intact	41.6	2,084,146-2,125,762
Riemer_RAP44	NC_019490	Intact	35.8	2,111,701-2,147,564
Entero_mEp235	NC_019708	Intact	55.5	2,674,744-2,730,306
Salmon_SEN34	NC_028699	Intact	47.9	2,963,014-3,010,960
Salmon_ST160	NC_014900	Incomplete	42.4	3,240,527-3,283,000
Burkho_BcepMu	NC_005882	Incomplete	20.9	4,569416-4,590,395
Escher_pro483	NC_028943	Intact	40.3	4,710353-4,750,711

RefSeq No: Reference sequence number in NCBI.

SNP analysis of 136 *S*. Lubbock isolates was performed in the McOutbryk SNP calling pipeline. One of the 136 isolates could not be genotyped using the McCortex genotyping algorithm, and was excluded from the analysis. Therefore, 135 isolates were carried forward for SNP analysis. After filtration of variant sites by IQ-tree, a total of 84 variant (SNP) sites with 76 distinct patterns was detected (Figure 28). Among the 84 SNP sites, 36 were parsimony-informative, and the remaining 48 were singleton sites.

The phylogenetic tree was inferred using the K2P+ASC model, which was the best nucleotide substitution model according to the BIC value. The log-likelihood of the tree was -509.6744. The total tree length (sum of branch lengths) was 0.0027 with a total of 84 SNP sites. Therefore, a single-SNP was equivalent to a 0.000032 branch length. The tree scale (0.000099) was equivalent to approximately three nucleotide substitutions per site (Figure 29).

The phylogenetic tree was divided into two distinct clades (Clades I and II) showing distinct patterns. While Pens 57, 58, and 59 were commonly observed in Clade I with significant branching, Pens 7, 8, and 9 (Source 1) were only observed in Clade II, along with the majority of cattle in Pens 51-53. When the day distribution was assessed, Days 0 and 7 *S*. Lubbock isolates were only observed in Clade II; that is, there were no early day isolates observed in Clade I. The phylogeny of *S*. Lubbock had the highest number of SNPs (n = 84) and distinct day, source, and pen patterns. There were no sample-type-related dynamics observed among the *S*. Lubbock isolates (Figure 29).

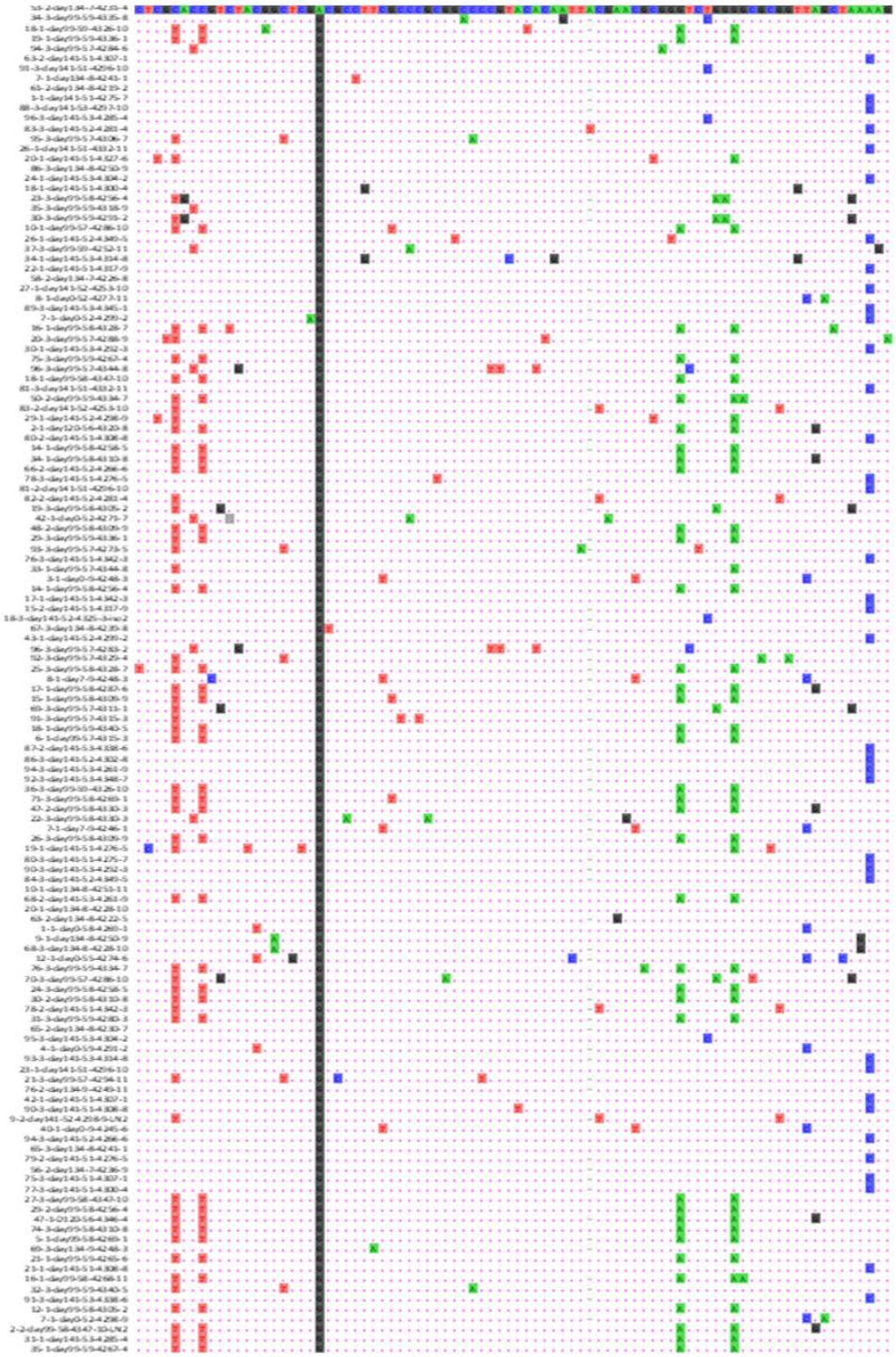


Figure 28. SNP variations and distributions observed among *S.* Lubbock isolates

Colors refer to the bases as follows: A (green), G (Black), C (blue), and T (red). The referent isolate that was chosen for SNP variation analysis is in the first row. Isolate IDs listed on the left are included for reference purposes.

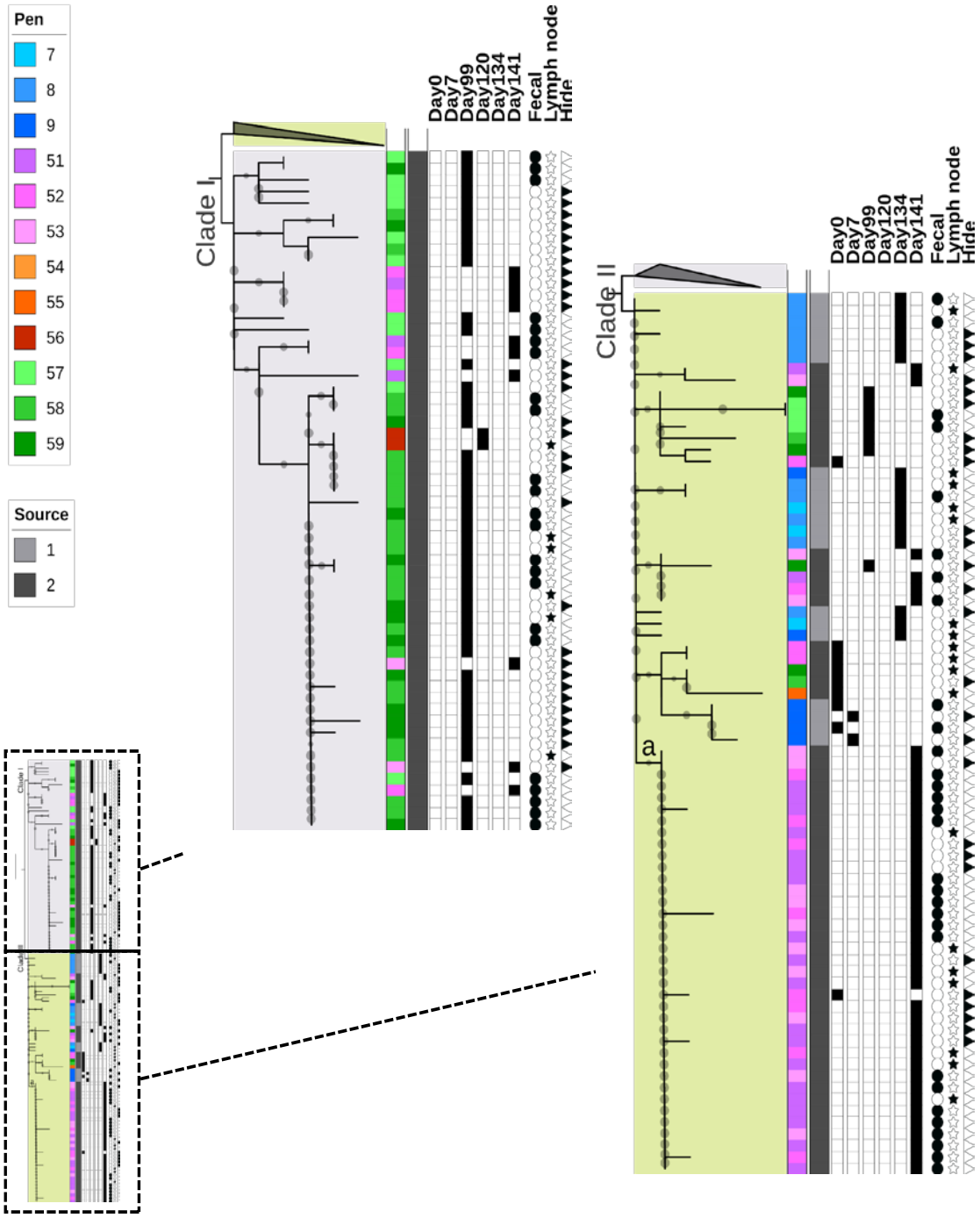


Figure 29. Phylogram representing *S.* Lubbock isolates

The maximum-likelihood phylogram was generated using the K2P+ASC nucleotide substitution model. Pen (first column) and source (second column) are presented in the legends; Days are represented in the following 6 columns. The sample types are indicated in circles (fecal), stars (lymph node) or left-sided triangles (hides) in the last 3 columns. The bootstrap support values are presented as grey circles of proportionate sizes in the middle of each branch.

4.10.2. *S.* Anatum

The SNP analysis of 113 *S.* Anatum isolates was performed in the McOutbryk pipeline. One isolate could not be genotyped using the McCortex genotyping algorithm and was excluded from the analysis. Therefore, 112 isolates were carried forward to SNP analysis. The reference genome of *S.* Anatum was also used as the reference in this phylogenetic analysis. The masked prophage regions of this reference were previously presented in Table 35. After filtration of the variant sites, a total of 65 SNP sites with 61 distinct patterns was detected (Figure 30). Among the 65 SNP sites, 35 were parsimony-informative and the remaining 30 were singleton sites. The best-fit nucleotide substitution model was selected as K3P+ASC according to the lowest BIC value. The log-likelihood of the tree was -373.633. The total tree length (sum of branch lengths) of 0.0028 was equivalent to 65 SNP sites. Therefore, one SNP in this tree was calculated as equal to 0.000043 branch length. The tree scale (0.000099) shows approximately 2.3 nucleotide substitution/per site (Figure 31).

The phylogenetic tree was divided by two significant clades (Clade I and II) showing distinct patterns. While Pens 7, 8, and 9 (Source 1 and Day 134) was only observed in Clade I with significant branching, Pen 56 was only observed in Clade II. When the day distribution was explored, there was no Day 7 isolate observed in Clade I; meanwhile, the *S.* Anatum isolates from the remaining days in this clade remained intact with significant branch support values. There were no sample-type-related dynamics observed among the *S.* Anatum isolates (Figure 31).

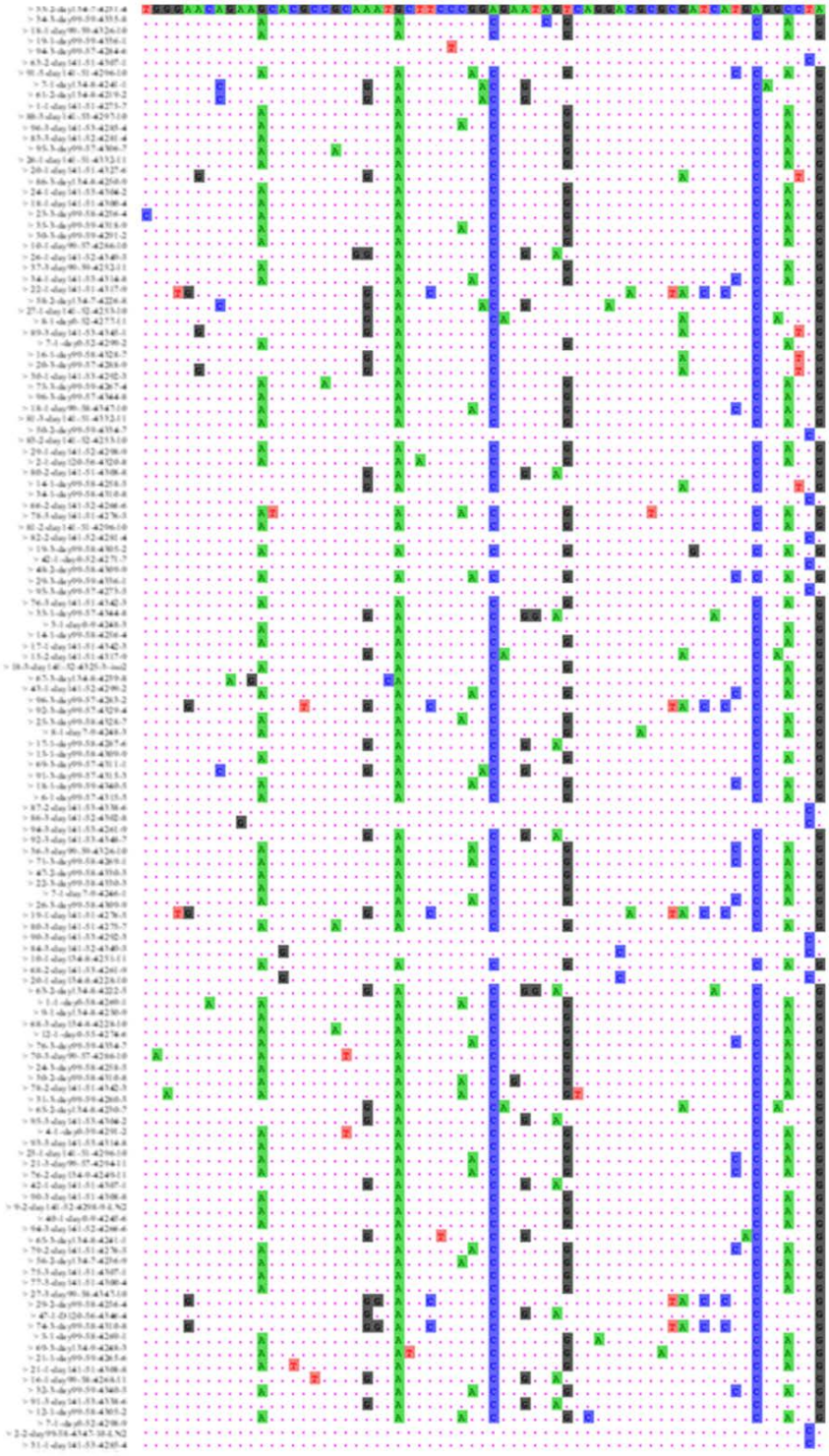


Figure 30. SNP variations and distributions observed among *S.* Anatum isolates

Colors refer to the bases as follows: A (green), G (Black), C (blue), and T (red). The referent isolate that was chosen for SNP variations is in the first row. Isolate IDs listed on the left are included for reference purposes.

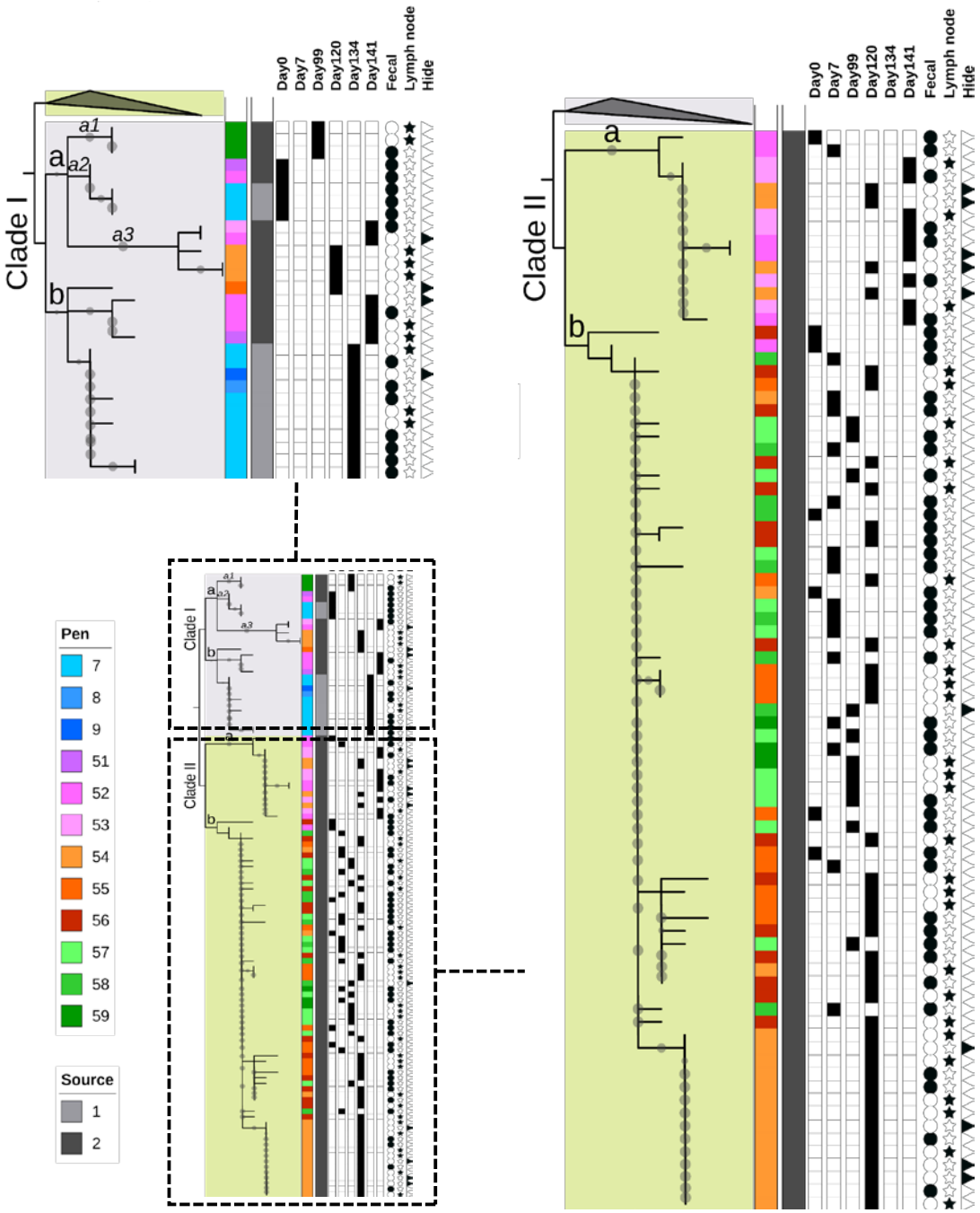


Figure 31. Phylogram representing *S.* Anatum isolates

The maximum-likelihood phylogram was generated using a K3P+ASC nucleotide substitution model. Pen (first column) and source (second column) are represented in the legends. Days are represented in the following 6 columns. Sample types are indicated in circles (fecal), stars (lymph nodes) or left-sided triangles (hides) in the last 3 columns. Bootstrap support values in the range of min. 80.1 and max. 100 are represented with grey circles in the middle of each corresponding branch.

4.10.3. *S*. Montevideo

A complete genome of *S*. Montevideo (GenBank: CP032816.1) was obtained from the same study [15, 270] that we used for the *S*. Lubbock reference genome. This genome was the closet genome to the *S*. Montevideo isolates recovered from our study with k-mer count coverage of 957/1,000, Mash/MinHash distance of 1.00 e^{-3} and a *P* value of 0.00. A total of 4 prophage regions were detected and masked in this reference genome. Of those, two were intact regions and the remaining two regions were incomplete (Table 37).

Table 37. Prophage regions masked in the *S*. Montevideo reference genome

Phage Name	RefSeq No.	Score	Size (kb)	Position (bp)
Salmon_Fels_2	NC_010463	Intact	42.4	1,108,750-1,151,168
Cronob_vB_CsaM_GAP32	NC_019401	Incomplete	19.8	1,948,518-1,968,382
Entero_cdtI	NC_009514	Incomplete	6.1	2,595,886-2,602,054
Entero_P2	NC_001895	Intact	31.3	4,332,529-4,363,916

RefSeq No: Reference sequence number in NCBI.

SNP analysis of 68 *S. Montevideo* isolates conducted by McOutbryk SNP pipeline, showed a total of 12 SNP sites (Figure 32) that all had distinct patterns. Among the 12 SNP sites, only one was parsimony-informative and the remaining 11 were singleton sites. The best-fit nucleotide substitution model was selected as K2P+ASC according to the lowest BIC value. The log-likelihood of the tree was -53.849. The total tree length (sum of branch lengths) was 0.003 with a total of 12 SNPs site. Therefore, one SNP in this tree was equal to 0.00025 branch length. The tree scale of 0.0001 is equivalent to approx. 0.4 nucleotide substitution/per site (Figure 33).

The phylogenetic tree was divided by two clades (Clade I and II) for *S*. Montevideo isolates. Clade I consisted of a single distinct isolate from Pen 52 that was from Day 141. In Clade II, there was a significant sub-branch (Clade II/a). The majority of Source 1 was located in this clade. In Clade II/a, there were only slaughter age isolates isolated from only Pens 7, 8, 9 and 52. Isolates from the remaining days (Day 0 and 7) were all located in the other sub-clades, along with the additional pens (except Pen 52 and Pen 9). The phylogeny of *S*. Montevideo had the lowest number of SNPs (n = 12) and distinct day and pen patterns were observed among these isolates. There was no sample-type-related genetic relatedness observed among the *S*. Montevideo isolates (Figure 33).

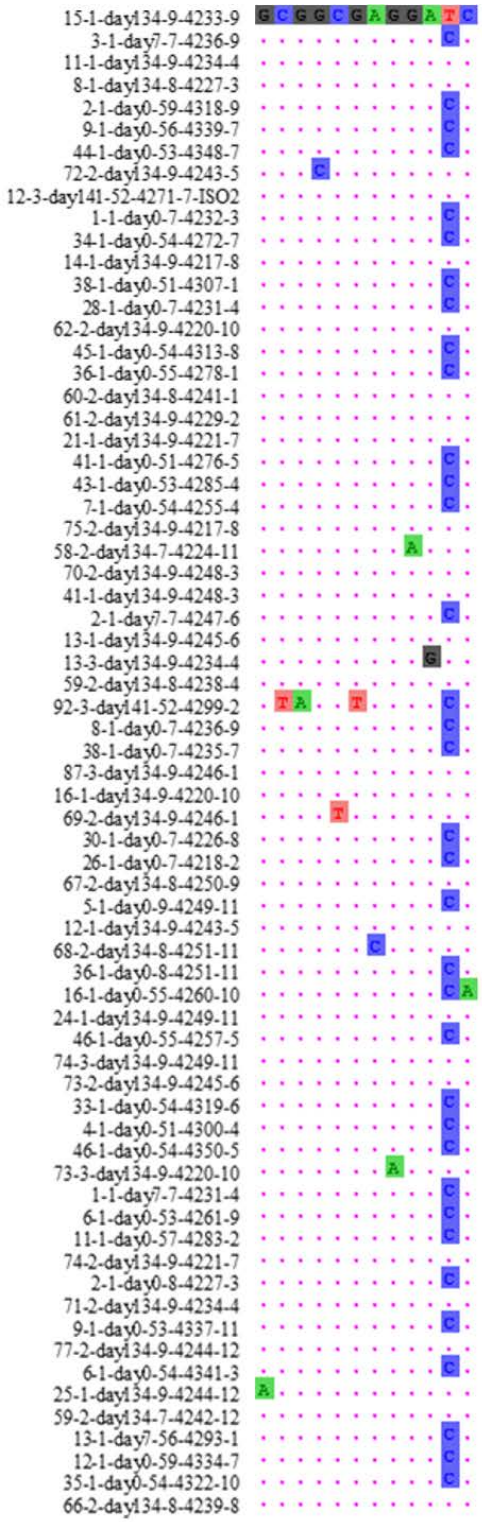


Figure 32. SNP variations and distributions observed among *S*. Montevideo isolates

Colors refer to the bases as follows: A (green), G (Black), C (blue), and T (red). The referent isolate chosen for SNP variations is in the first row.

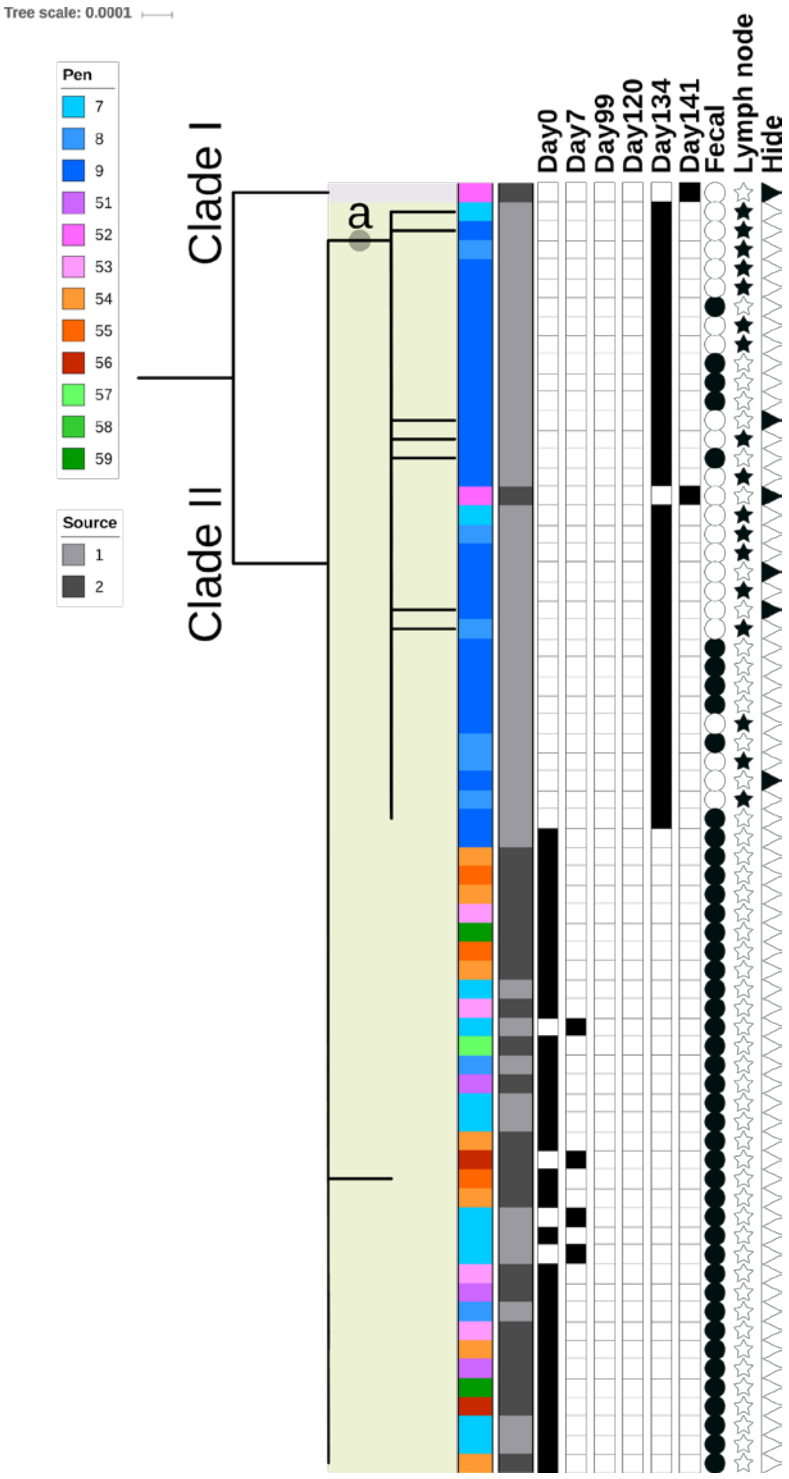


Figure 33. Phylogram representing *S.* Montevideo isolates

The maximum-likelihood phylogram was generated using a K2P+ASC nucleotide substitution model. Pen (first column) and source (second column) are represented in the legends. Days are represented in the following 6 columns. Sample types are indicated in circles (fecal), stars (lymph nodes) or left-sided triangles (hides) in the last 3 columns. A single bootstrap support value of 84.3 is represented with a grey circle in the middle of the corresponding branch.

4.10.4. *S.* Cerro

The complete genome of an *S.* Cerro strain (wild-type serovar 87, GenBank: CP008925.1) that was isolated in China was selected as the reference genome for the phylogenetic analysis. This genome found to be the closest available genome to the *S.* Cerro isolates recovered from our study, with a k-mer count coverage of 997/1000, Mash/MinHash distance of 7.16 e^{-5} and a *P* value of 0.00. A total of 5 prophage regions was detected and masked in this reference genome. Of those, one was intact, three were questionable and the remaining one had incomplete prophage regions (Table 38)

Table 38. Prophage regions masked in the *S.* Cerro reference genome

Phage Name	RefSeq no.	Scoring	Size (kb)	Position (bp)
Salmon_vB_SosS_Oslo	NC_018279	Intact	50.2	130,352-180,614
Gifsy_2	NC_010393	Questionable	11.3	234,157-245,537
Salmon_SEN34	NC_028699	Questionable	11.5	910,517-922,099
Salmon_RE_2010	NC_019488	Incomplete	7.0	1,730,341-1,737,418
Entero_UAB_Phi20	NC_031019	Questionable	44.8	4,167,324-4,212,148

RefSeq No: Reference sequence number in NCBI.

Phylogenetic analysis of 64 *S.* Cerro isolates resulted in 17 SNP sites with 16 distinct patterns (Figure 34). Among the 17 SNP sites, nine were parsimony-informative and the remaining eight were singleton sites. When the SNP alignment was tested for the best-fit nucleotide substitution model, the best-fit model was TIMe+ASC according to the lowest BIC value. The log-likelihood of the tree was -78.5521. The total tree length (sum of branch lengths) was 0.0038 with a total of 17 SNP sites. One SNP in this tree was equal to 0.00022 branch length. The tree scale (0.0001) shows approximately 0.4 nucleotide substitutions/per site (see Figure 35).

The phylogenetic tree was divided into two significant clades (Clade I and II) for *S.* Cerro isolates. Since this serotype was limited to slaughter days, there were no earlier observations. Clade I had unique day, source, and pen patterns. For example, all slaughter day isolates except from Day 120 were located in this clade. Also, Source 1 (Pens 7,8 and 9) isolates were only found in this clade, along with isolates from Pens 51,58, and 59. In Clade II, only isolates from Source 2 and Pens 56 and 57 were found. One additional isolate from Pen 52 also was located in this clade and that unique isolate was the only isolate from Day 141. All remaining isolates from this clade were from Day 120 and its associated block. The phylogeny of *S.* Cerro revealed distinct day, source, and pen patterns. There were no sample-type-related dynamics observed among the *S.* Cerro isolates (Figure 35).

When the overall patterns were explored for the phylogenetic trees using the SNP analysis, the largest variations were observed among the *S.* Lubbock isolates (84 SNPs/135 isolates); this tree was inferred with the K2P model, which assumes equal base frequencies and unequal transition/transversion rates. The second largest variations were observed among *S.* Anatum isolates (65 SNPs/112 isolates). That tree was inferred using the K3P model, which also results from equal base frequencies; however, equal substitution rates occur across all bases. *S.* Cerro had the third largest variations (17 SNPs/ 68 isolates); this tree was inferred with the TIM1 model, which assumes equal base frequencies and equal transition rates. The smallest variations were observed in S. Montevideo isolates (12 SNPs/68 isolates); this tree was inferred with the same model selected for *S.* Lubbock.

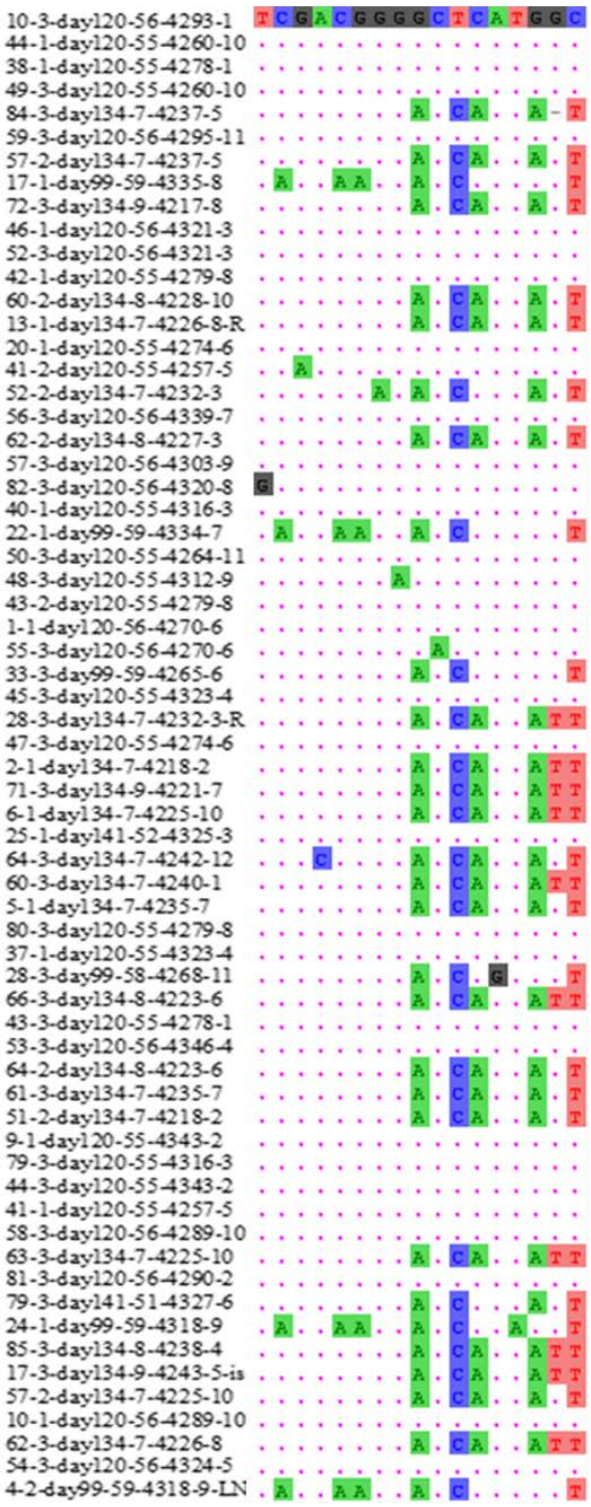


Figure 34. SNP variations and distributions observed among *S*. Cerro isolates

Colors refer to the bases as follows: A (green), G (Black), C (blue), and T (red). The referent isolate chosen for SNP variations is in the first row.

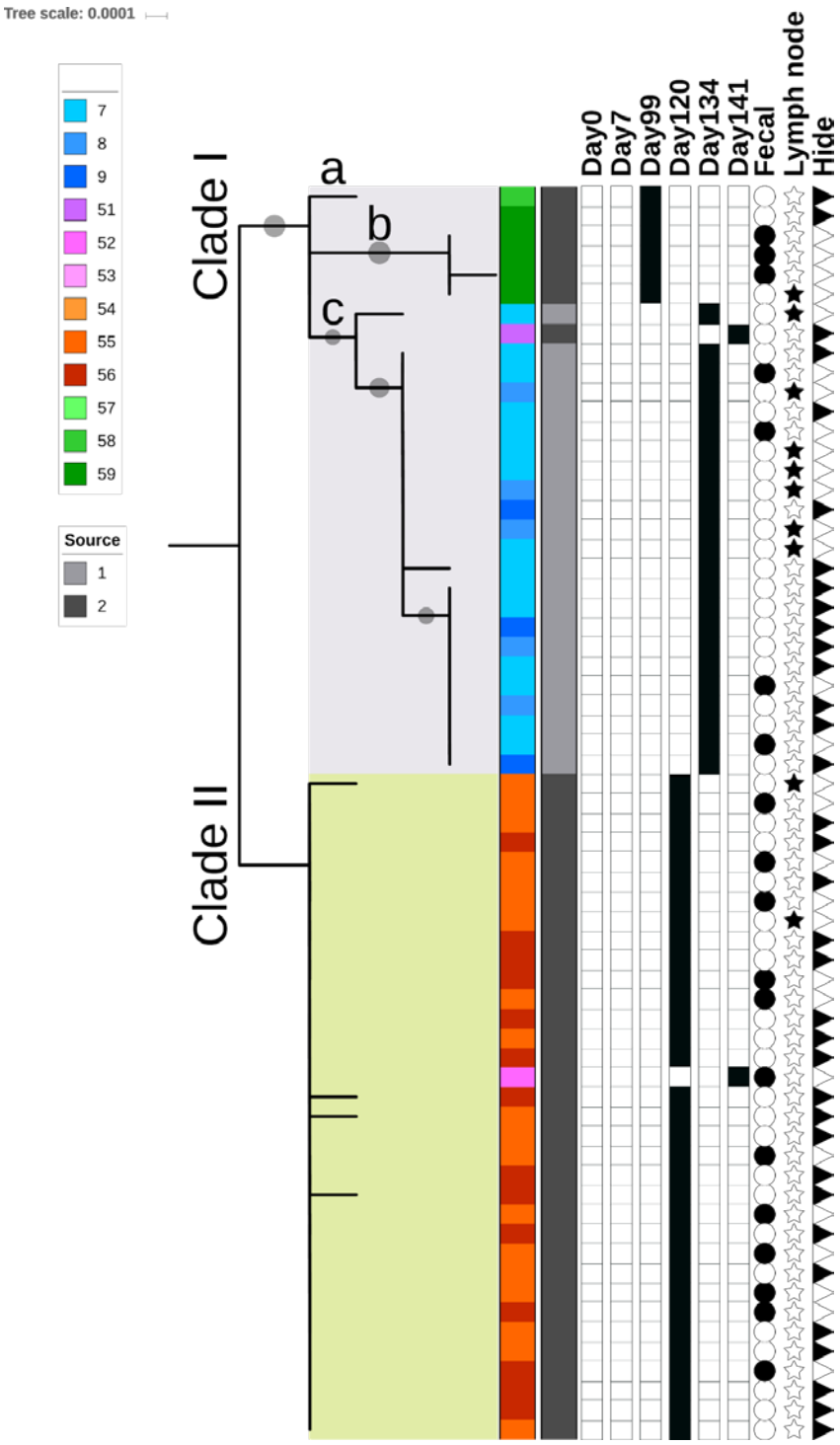


Figure 35. Phylogram representing *S.* Cerro isolates

Maximum-likelihood phylogram was generated using the TIMe+ASC model. Pen (first column) and source (second column) are represented in the legends. Days are represented in the following 6 columns. Sample types are indicated in circles (fecal), stars (lymph nodes) or left-sided-triangles (hides) in the last 3 columns. Bootstrap support values in the range from min. 85.0 to max. 98.1 are presented as grey circles in the middle of each corresponding branch.

5. DISCUSSION*

The judicious use of antibiotics in veterinary medicine has been widely discussed due to the potential risks of cross-resistance to antibiotics used in human medicine. Public health authorities and agencies have been applying restrictions to control and decrease the use of medically important antibiotics and their analogs in feedlot cattle since 1969 [205, 217, 219]. A subset of these antibiotics is still frequently administered to cattle arriving to the feedlots; thus, may pose a selection pressure on cattle origin AR bacterial pathogens that may enter to the food-chain or else spread in the environment up until the slaughter date at 90+ days. This situation can further cause a public health problem by resulting in the treatment failure of antibiotics in humans that are infected by a cattle origin AR pathogen. Therefore, the longer-term effects of antibiotic administrations early in the feeding period needed to be better evaluated to gauge the public health risks associated with antibiotic use in cattle feedlots and their effects on foodborne pathogens in cattle at slaughter.

Our study was the first randomized, controlled, and longitudinal field trial that monitored cattle origin *Salmonella* from the time of the antibiotic treatment (Day 0) up until the slaughter (Day 99+). We achieved this by focusing on fecal, lymph node, and hide samples; each sample type represented a potential source of *Salmonella* contamination in beef products. We employed standard microbiological and advanced molecular methods for initial isolation and confirmation of *Salmonella*. The phenotypic antibiotic

* Part of this chapter is reprinted with permission from "Population Dynamics of *Salmonella enterica* within Beef Cattle Cohorts Followed from Single-Dose Metaphylactic Antibiotic Treatment until Slaughter" Levent G, Schlochtermeier A, Ives SE, Norman KN, Lawhon SD, Loneragan GH, Anderson RC, Vinasco J, Scott HM. 2019 Appl Environ Microbiol 85:e01386-19, Copyright © 2019 American Society for Microbiology.

susceptibility profiles of *Salmonella* isolates were determined using the broth microdilution method. In addition, we also determined the serotype, ST, resistance genes, and plasmidal profiles of these isolates using WGS-based analyses and with commonly accepted *in silico* tools. We also aimed to understand the cattle, environment, and time related population dynamics of *Salmonella*, which represent a public health problem regardless of antibiotic resistance profiles. Therefore, we explored the genetic population dynamics of fecal, lymph node and hide origin *Salmonella* isolated from cattle coming from different sources, pens, and treatment groups using WGS data and SNP analyses.

5.1. Model selection for quantitative data

Using a linear regression model for a quantitative linear dataset is appropriate when the observations are normally distributed (also, without the excess zero counts and those below the LLQ), the variance is constant, and the measured outcomes of observations are assumed independent of one another [271]. Often, in microbiology the CFU observations are $\log_{10}$ transformed in order to better fit to the normal distribution prior to linear regression model application. However, when the quantitative observations have the excess zero counts or/and zero values due to the limits of quantification of the method used, the transformation often does not help the data to entirely meet the assumptions of the linear regression. These excess zero counts are usually the products of the LLQ of the microbiological method chosen for the quantification, but also may reflect underlying variability and relatively low counts in the targeted ecological niche. The excess zero counts in the data are often problematic, since they pose an uncertainty about the

observations that can be either structural or else artefactual sampling zero (i.e., not zero but under the LLQ) counts.

Handling the excess zero counts that are part of an overdispersed and correlated quantitative data is considered one of the more difficult decisions to make by researchers [272]. Each dataset has it is own unique nature and biology, each of which plays a key role for the selection of the appropriate method to handle excess zero counts in such data. For example, if the excess zero counts are obtained from a count observation of a commensal bacterial species, that is almost always found in a sample, it is reasonable to assume that these observations are likely sampling zeros. However, when the zero counts arise from a bacterial species that may or may not regularly be found in a sample, the sampling zero assumption can be problematic. This uncertainty needs to be evaluated carefully based on biological reasoning, along with the other observations obtained from the data. Otherwise, a biased assumption could be made on the nature of the zero counts, which can result in Type-I or Type-II errors for the tested hypothesis.

Decreasing the LLQ and conducting accurate estimates for exposure is highly important for risk analyses [273]. There are various solutions to avoid this kind of biases. A few solutions focus on decreasing the LLQ, either before the analyses at the sample collection stage or in the laboratory, using highly sensitive but also specific methods [272, 273]. Several solutions can be also applied at the statistical analysis stage using methods appropriate to handle the excess zeros [272-276].

One way to avoid excessive zero counts at the sample collection stage is to determine the proper sampling area, time, and transport method that can increase the likelihood of bacterial quantification [274]. Another way is improving the laboratory-

based methods by using lower-dilution factors (e.g., using a spiral plater), retesting the sample, increasing the number of replicates, or utilizing a pre-enrichment or amplification method that provides a relative abundance (usually combined with molecular quantification methods). Another way of handling the zero-count uncertainty is using the statistical methods at the data analysis stage using different statistical approaches based on observations in the data and biological reasoning [273].

The statistical solutions that are used to handle the excess zero counts at the analysis stage can be listed as: 1) omitting the zero counts and applying a parametric model [272, 274], 2) substituting the zero counts with a value related to the LLQ (e.g., the LLQ, 1/2 the LLQ, or LLQ$\sqrt{2}$) and applying a parametric model [274, 275], 3) assuming the all zero counts are structural, the data are zero-truncated and then applying a zero-truncated regression model [277], 4) assuming tall the zero counts are sampling zeros (left-censored) and applying a non-parametric survival analyses [278], 5) assuming zero counts are either missing or left-censored observations; therefore, assigning unique values to the zero counts based on the probability of the parental distribution using a multiple imputation based regression model [276], 7) assuming zero counts can be either structural or sampling and using zero-inflated models (two-part models) [279], and 8) assuming the zero counts are structural using hurdle (also, a two-part) models [280].

The two-part models are less biased compared to other methods when the true nature of data is unknown; however, usually the selection of the model is made by testing several two-part models with the same parameters and choosing the lowest AIC and BIC values when selecting the best model. However, when the zero counts are more likely to be sampling zeros, assigning values based on the probability function of the parental

distribution using an imputation regression model is the most accurate and least biased [281].

Two-part regression models are adopted when the zero counts that are not likely sampling errors and are of unknown structure; meanwhile, the observations often are overdispersed and highly correlated to potential clusters [281]. The zero counts for these kinds of data are usually handed with: 1) ZIP models when the variance is equal to the mean, 2) ZINB models when the variance is not equal to the mean, and 3) CHURDLE models using appropriate models based on the distribution of the observations [279-281]. These regression models consist of two model parts: 1) a binary probability model using either logit or probit distribution for the zero counts, and 2) inflation models or selection models that account for i) continuously coded observations (linear) or ii) integer observations using either Poisson or negative-binomial probability distributions.

Two-part Poisson and negative-binomial models are usually preferred when zero counts are assumed to be either structural zero or else sampling zero counts (these also are called zero-inflated models). Therefore, these types of models include the zero count observations in both first and second part of the model. On the other hand, Cragg's hurdle model assumes that all zero counts are structural (true) zeros and these counts are not included in the second part of the model; that is, they are included only in the first part of the model [282]. The statistical hurdle model was first proposed in 1971 as an alternative model for handling excess zero counts when there is the probability that these zero counts are not sampling zero [283]. Soon after, the hurdle models became widely used to handle excess zero count data in the econometric literature. In recent years, this model type has

begun to be preferred to handle excess zero counts of data in microbiological fields [279, 284].

To be able to improve the LLQ in our study, we collected feces that is a sample type more likely to harbor intestinal origin *Salmonella*. We also sampled the sub-iliac lymph nodes that have previously been reported to harbor higher numbers of *Salmonella* compared to the other lymph nodes of cattle. We swabbed a large surface of brisket area (1,000 cm^2) of the ventral cattle hide that was more likely to reflect the environmental *Salmonella* in high numbers [15, 121, 285-287]. This study also was purposely started in March and ended in early August to increase the likelihood of quantifying *Salmonella* at slaughter due to previously conducted studies reported increasing count and prevalence of *Salmonella* in summer periods [69, 132]. To avoid the possible reduction of numbers of viable and quantifiable bacteria during transportation, all samples were delivered to our laboratory via overnight shipment in coolers maintained at +4 °C.

In our study, we utilized the same quantitative methods across all samples and days to better be able to compare our findings. We also utilized a spiral-plating method, which significantly decreases the LLQ for CFU data [119]. However, we also utilized a non-selective (TSB) media incubation for 3 h as a pre-enrichment step in order increase the likelihood of obtaining a CFU value from the samples that were *Salmonella* positive.

Even though we aimed to apply the best possible practices to decrease the LLQ of our quantification method, our fecal and lymph node CFU data had excess zero counts in all types of samples. After the $\log_{10}$ transformation, only the quantifiable hide observations approached normality; however, the fecal and lymph node observations were still not normally distributed (Figure 15). Therefore, we examined our fecal and lymph

node CFU data with zero-inflated and hurdle models. The marginal trends and coefficients were similar for all models, and especially were nearly identical for ZIP and ZINB models, suggesting the mean was equal to the variance. These results suggest that all three models provided a good-fit for data yielding excess zero counts. However, CHURDLE significantly improved the model fit (based on the AIC and BIC values), which is most likely due to using non-rounded observations or perhaps the higher probability of zeros being structural when compared to hides. The structural zero hypothesis was also supported by the kappa agreement, showing that only a minor proportion of these zero counts were positive after a much more extensive *Salmonella* enrichment process (Table 11).

Since enrichment results were obtained with *Salmonella* specific enrichment media and this step eliminates the non-*Salmonella* colonies during the incubation stage, and all these isolates were confirmed with serum O-antigen and MALDI-TOF MS methods, they were more likely to reflect the closest value to the true prevalence when *Salmonella* prevalence is low or else the background microbiota are complex. Therefore, an animal-level assessment has also been conducted assuming if an observation that had CFU was zero but the prevalence was positive, it was more likely the CFU was under the limit of detection for CFU. Similarly, if an enrichment result was negative but the CFU number was a positive value, it was accepted to be more likely those colonies may not be true *Salmonella* colonies but another species with similar morphological characteristics as *Salmonella* on selective agar plate. When we examined the kappa agreements between the CFU data of either negative (below-LLQ) or positive (above the LLQ), and the binary coded enrichment results across sample types, the agreements were classified as good.

This also supports the idea that zero counts observed in the study were likely structural zero counts (assuming the enrichment method was accurate); therefore, the hurdle model was the best-fit model for our data.

Beside handling the uncertainty of zero counts observed in fecal and lymph node results, we assumed the zero counts obtained from hide samples were the result of an LLQ error, since it was highly unlikely to obtain structural zero *Salmonella* counts in any given pen where up to 80% of animals tested as positive on hide and fecal samples. Therefore, imputing CFUs between the zero and the LLQ was assumed reasonable. This model first imputes the missing or zero observations between the zero and LLQ values for a given number of iterations under the parenteral distribution of an interval-based regression with the selected model parameters; later, these iterations were included in the selected regression model analyses with the same parameters. This method is widely used to deal with missing observations in a given dataset [288, 289]. We applied multiple imputation along with the interval-linear regression followed by a multi-level linear regression to estimate $\log_{10}$ CFUs of the "zero counts", which were assumed to not truly be zero but instead remained under the LLQ for hide samples. Following the imputations, the distribution of hide $\log_{10}$ CFUs better met the assumptions of linear regression, including normalized residuals and a more constant variance. Even though imputation is usually suggested for data containing up to 80% missing or zero counts, in our study we had only 32% (43/132) of the data points that were imputed; moreover, the equal distribution of these data among the treatments (14-15 observations in each treatment group), provided better validity to impute these observations under the treatment, pen and animal ID effects.

5.2. Quantitative *Salmonella* results

Previous studies investigating the effects of seasonal and cattle age-related factors on fecal *Salmonella* shedding have shown that shedding often is higher in summer and in older feeder cattle when compared to other seasons and in calves [290, 291]. In our study, we also observed increasing CFUs during sampling periods approaching summer (Figure 17). In particular, there was a significant increase of fecal *Salmonella* CFUs in ceftiofur and control groups observed after Days 56 and 112, respectively, when compared to earlier sampling days. Interestingly, this significant increase was not observed in feces from the tulathromycin-treated cattle. When the overall CFU distribution comparing the tulathromycin and the other treatment groups was examined on Day 0, the tulathromycin group CFUs were relatively lower than the other groups and, after treatment, remained almost the same on Day 7 while the other groups were decreasing; therefore, this difference among groups by day may be related to the gradual increase observed in the tulathromycin over the entire study length. Overall, our study did not show any significant effects of ceftiofur and tulathromycin metaphylactic treatments on *Salmonella* CFUs estimated from feedlot cattle feces, lymph nodes and hide samples.

The smallest value for the LLQ was observed in lymph nodes, followed by feces and hides (Figure 15). One possible explanation for these differences could be the competition by the background microbiota during the 3 h pre-enrichment process, since possibly a less complex microbiota is expected to be found in lymph nodes compared to feces, and with feces compared to hide samples. Within this discussion, we mainly focused on the LLQ as other authors have done; however, when samples are overloaded with bacterial colonies, the higher limit of detection can also be problematic. In our study,

we did not observe a high limit of detection in feces and the lymph nodes. However, we observed several right-censoring observations on the hides at the possible upper limit of quantification of 7.2 $\log_{10}$ (Figure 14), which could potentially affect the underlying distribution, and consequently our interpretation of the results. However, considering no treatment effects were observed in the other sample types, and hide origin *Salmonella* were less likely to have been affected by the antibiotics injected subcutaneously to the cattle, this would probably not change our final conclusion regarding the effects of ceftiofur and tulathromycin on cattle hide origin *Salmonella* quantities at slaughter age.

A 3 h pre-enrichment incubation applied to our samples prior to quantification provided the ability to increase the proportion of zero counts that were structural zeros; meanwhile, applying the same method across sampling days and all sample types also provided an internal validity to compare the treatment and period effects on *Salmonella* CFUs obtained from the study. However, utilizing a pre-enrichment step results in reduced external validity; that is, the ability to be able compare our results with those from other laboratories. A laboratory-based method is therefore also needed to better estimate the initial CFUs in these samples prior to enrichment, perhaps by simulating this experiment with a known *Salmonella* concentration spiked into a subset of samples constructing a standard curve to back-calculate enriched CFU values to their initial CFUs before the 3 h enrichment process.

5.3. Prevalence of *Salmonella*

Our findings suggest that neither ceftiofur nor tulathromycin used for control of BRD early in the feeding period affects *Salmonella* prevalence at slaughter, whether observed in cattle feces, lymph nodes or hides. However, the sampling day had a significant (P <0.05) effect on the fecal prevalence of *Salmonella* that was reflected as an increasing prevalence associated with the shift from spring through to the summer months. Similar to the CFUs, this increase could be related to the seasonal effect on *Salmonella* persistence in animals and in the environment due to the increase of the ambient temperature from March 2016 to August 2016. However, this change also can be linked to cattle related factors associated with time such as age, immune status, or other environmentally related factors besides temperature. This finding is similar to those in other studies reporting significant effects of the summer season on fecal shedding of *Salmonella* when compared to the winter or spring seasons; this includes a survey conducted by USDA-APHIS in 1999 [138]. Similar findings also were observed in cattle feces by Vikram et al. (2017), who observed a significant increase (P < 0.01) of the prevalence of *Salmonella* recovered from 719 beef cattle in summer when compared to fall, winter, and spring [136].

In our study, the mean fecal prevalence observed in March was 43.6%, which increased to 80.3% by the beginning of August. However, the peak prevalence that was observed in summer time in the USDA-APHIS study (1999) was 11.4% and in winter time was 4.0%; however, the APHIS results were obtained from fecal samples collected from the pen floors whereas in our study we collected fecal samples *per rectum*. In addition, the USDA-APHIS researchers sampled feedlots located in 11 different states, including those

in more northern latitudes, whereas we were more limited with a single-feedlot in the panhandle of Texas.

On the other hand, Vikram et al. (2017) [136] reported the *Salmonella* prevalence obtained from the fecal samples directly collected by the incision from the colon of the cattle at harvest was around 2.5% in March, and increased to 22-47% by July-August. These samples were collected from a single-beef processing plant in the United States; however, the location (i.e., state) of this plant was not provided. It has been previously shown that *Salmonella* prevalence in cattle is higher in the southern regions compared to other U.S. regions; therefore, the obvious prevalence difference that was observed in our study can most likely be attributed to the locational (latitude) or feedlot dynamics of *Salmonella*.

Gragg et al. (2013) [15] showed a significant regional effect on the sub-iliac lymph node *Salmonella* prevalence obtained from slaughterhouses located in the southern United States, including Texas. In their study they longitudinally sampled sub-iliac lymph nodes at slaughter throughout the year; this way, they also showed a significant increase in the prevalence in summer months. Webb et al. (2017) also observed similar effects of season and geographically regional *Salmonella* prevalence differences in cattle sub-iliac lymph nodes in their repeated cross-sectional study [132]. Their results were also similar to our study, showing significantly higher prevalence in the summer, and southern United States when compared to the winter and midwestern United States. In contrast, the findings of Haneklaus et al. (2012) [16] showed that the *Salmonella* prevalence in cattle superficial cervical and iliofemoral lymph nodes varied significantly among the seven different feedlots located in the southern United States in summer months; this was somewhat

unusual for that region. However, this contradictory finding may be due to the *Salmonella* prevalence variations observed in different lymph node types of cattle [68] [285], other host immunity and age-related factors [286], or the route, amount and time of *Salmonella* exposure in the feedlots [133, 285]. Perhaps seasonal and regional effects reflect more on *Salmonella* prevalence in the sub-iliac lymph nodes than other peripheral lymph nodes. In our study, lymph nodes were only collected during the summer months so no seasonal variability was observed outside of that seen in the fecal samples. Overall, these comparative results support our finding of high *Salmonella* prevalence in the sub-iliac lymph nodes of cattle located in Texas in the summer. However, in our study we only sampled lymph nodes from cattle within a 42-day period during the middle of summer. If our study was conducted in the northern or western parts of United States, sampled other lymph node types, or cattle in winter, we would most likely observe major differences in t *Salmonella* prevalence in lymph nodes.

In our study, at slaughter age the highest *Salmonella* prevalence was found in the hide (84.8%) samples followed by the lymph nodes (84.2%), and then fecal (80.3%) samples. However, it is important to note that in our study hide swab and lymph node samples were processed fresh, while the fecal samples were initially frozen and later thawed and processed. Such dramatic temperature differences can impact numbers of viable bacteria in fecal samples that can lower the rate of isolation from feces, even though samples were stored in a cryo-protectant (glycerol). Both Kunze et al. (2008) [69], and Gragg et al. (2013) [68] also found higher *Salmonella* prevalence on hide swabs when compared to feces and sub-iliac lymph nodes. The higher *Salmonella* prevalence on hide surfaces compared to feces and lymph nodes may be because *Salmonella* were persistent in

the farm environment (e.g., soil and manure) and replenished frequently through the feces of the cattle to the environment [166]. Even though *Salmonella* prevalence is usually higher on hides when compared to other potential beef product contamination sources (i.e., feces and lymph nodes) of cattle, it has been shown that slaughter-plant carcass and hide interventions significantly reduce *Salmonella* carcass prevalence both pre- and post-chill [122, 143] (Table 1).

ICC reveals the magnitude of the effect of clustering within the nested components of variance observed among observations. The ICC has absolute values between zero and one. While an ICC value of zero suggests no correlation among observations within clusters, positive values suggest a positive correlation among observations within clusters [271]. The ICC values can play an important role in understanding the ecological and animal aggregating factors (such as pen, or repeated observations within animal) related to the overall ecology and epidemiology of *Salmonella* in feedlot settings. In our study, 30% of the variance relating to *Salmonella* presence on hides, 21% of the variance in feces and 13% of the variance in lymph nodes were attributed to pen-level variability. This result suggests a strong role played by environmental versus animal-related factors, since at the pen-level *Salmonella* presence/absence is more likely to be similar within a pen than for the lymph nodes obtained from each animal. Animal-level dependencies were measurable only for fecal and lymph node samples. The ICC for animal level clustering showed that 64% of the variance in lymph nodes and 21% of the variance in feces observed for *Salmonella* prevalence were attributed to within-animal dependencies. The lymph node prevalence was likely influenced by fact that the two nodes from individual animal carcasses were contemporaneously sampled at slaughter in the summer while fecal sample

dependencies arose longitudinally over multiple weeks, starting in the spring and ending in the summer. In our study, overall results show that pen and animal level clustering had the greatest impact on *Salmonella* prevalence regardless of assigned treatment groups. To the best of our knowledge, pen-level and animal-level ICC estimates for *Salmonella* prevalence have not been previously reported in randomized cohorts of beef cattle. However, Cull et al. (2017) [292], reported the ICC of enterohemorrhagic *E. coli* from different pens and feedlots. Our estimates were similar to the recent cross-sectional study published by Cull et al. (2017), measuring feedlot and pen-level ICC of *E. coli* collecting fecal cattle samples across eight commercial feedlots in a region near the Texas Panhandle. In their study, *E. coli* ICCs ranged from 4% to 8% among the cattle feces and feedlots whereas at the pen-level ICC ranged from 26% to 31%.

5.4. Antibiotic resistance dynamics

Our study did not reveal any selection effect of ceftiofur or tulathromycin on phenotypic or genotypic ceftriaxone or azithromycin resistance in cattle *Salmonella* populations immediately after the antibiotic administration, as well as at slaughter. In our study, we tested a total of 630 *Salmonella* isolates recovered from feces, lymph nodes and hides on non-antibiotic-selective media. Approximately 80% of the *Salmonella* isolates were pan-susceptible; the remaining 20% of the *Salmonella* isolates exhibited tetracycline (≥ 16 µg/ml) and/or streptomycin (≥ 32 µg/ml) resistance (Table 30). In this study, all *Salmonella* isolates were susceptible to ceftiofur, ceftriaxone, and azithromycin. The MIC distribution for ceftiofur (Table 28), ceftriaxone (all isolates had an MIC of 0.25 µg/ml), and azithromycin (Table 27) did not differ among *Salmonella* isolates recovered from the

ceftiofur, tulathromycin or control cattle. The absence of resistance to beta-lactams and macrolides among these *Salmonella* means their selection was not possible. In a parallel study, a master's thesis published in 2017 showed there were AmpC and ESBL beta-lactamase-producing genes present and selected for among the *E. coli* in this same cattle population. Since these genes were not present in the *Salmonella*, they could not be selected. Our results should be interpreted in line with that important caveat.

The most prevalent phenotypic resistance found in this study was tetracycline. Phenotypic tetracycline resistance is the one of the most commonly observed among *Salmonella* isolates originating from humans [32], cattle [68, 132, 137, 144], and beef products [13, 149]. Tetracycline is classified as highly important for human medicine by the WHO [54]. Based on the comparisons of phenotypic antibiotic resistance data and serotypes, we found that phenotypic tetracycline resistance was observed almost exclusively among *S*. Montevideo serotypes found in feces, lymph nodes and on hides throughout the study; that is, without any known direct selection pressure caused by using tetracycline. Our findings also demonstrated that serotype and antibiotic resistance patterns were closely associated (Table 31) as previously suggested by numerous studies [15, 36, 69, 149, 192]. In our study, we observed the *tet*(A) gene conferring phenotypic tetracycline resistance at an MIC of ≥ 16 µg/ml. This is not unusual, since the *tet*(A) gene is commonly observed in *Salmonella* isolates resistant to tetracycline [192]. Our annotation results showed that the *tet*(A) gene was on a contig that was part of the IncN plasmid; this result also confirms why almost all *S*. Montevideo isolates were resistant to tetracycline and at the same time harbored the IncN plasmid. *S*. Montevideo was prevalent from the beginning until the end of our study in feces, lymph nodes and on hides (Figure

25). When the distribution of this serotype was examined, cattle from Source 1 had the majority of the *S.* Montevideo isolates and these were the single group of cattle had *S.* Montevideo in their lymph nodes and on hides at slaughter age. The IncN plasmid harboring a *bla* gene (specifically, the *bla*$_{\text{CTX-M-1}}$ or *bla* $_{\text{CTX-M-32}}$ genes) was previously reported in the Enterobacteriaceae family [293]. This plasmid harboring *bla* genes was also isolated from *E. coli* recovered from beef cattle [294]. In our study, we did not identify any *bla* gene on this plasmid; likely, this reflects the variation in the STs of the IncN plasmids recovered from different locations and times [295].

Tetracycline is the most commonly used medically important antibiotic in cattle [223, 224]; perhaps, the dissemination of *S.* Montevideo harboring IncN plasmid in our study could be attributable to the cattle source, which consequently could be related to the historical use of the tetracycline in that location [209]. However, the prior antibiotic exposure history of our study cattle was unknown; therefore, such conclusions should be avoided. Another possible explanation for the persistence and dissemination of the *tet*(A) gene on an IncN plasmid could be a possible reduction of fitness cost that can provide survival selection for the *S.* Montevideo isolates, especially if the IncN plasmid is carrying functional genes (e.g., virulence genes) [108]. However, this was not explored in our study.

Even though tetracyclines are not typically preferred for the treatment of *Salmonella* infections in human, they are classified as medically important antibiotics for humans [54]. In our study, we identified *S.* Montevideo harboring a tetracycline resistance gene on a plasmid in 11 fecal, 17 lymph node and six hide samples from the cattle that were sent to the slaughter (Table 23). Clearly, by the application of carcass or hide

interventions, the fecal and hide origin *S*. Montevideo population will be reduced before these beef products are offered for human consumption (Table 1). However, the lymph nodes harboring *S*. Montevideo that are resistant to tetracycline still pose a risk of final transmission of this pathogen to humans through the consumption of undercooked and contaminated ground beef products.

Another ARG that also was observed in all *S*. Montevideo isolates was a chromosomally located *fos*A7 gene. Fosfomycin is a broad-spectrum antibiotic discovered in 1961 that inhibits the MurA enzyme, which plays a role in peptidoglycan synthesis of the bacterial cell wall [296]. Even though fosfomycin is commonly used for the treatment of urinary tract infections in humans, it is not used in food-producing animals in the United States [223]. There are three major classes of fosfomycin modifying enzymes (FosA, FosB, and FosX). The first FosA enzyme expressing fosfomycin resistance gene (*fos*A) was described in 1988 [297]. Since then, numerous *fos*A genes have been described (*fos*A3, *fos*A4, *fos*A5, and *fos*C2) in the literature [298]. In 2016, the *fos*A6 gene was first identified in an ESBL-producing *E. coli* strain [299]. Recently, in 2017 a novel *fos*A7 gene was also identified in a *S.* Heidelberg isolate recovered from chicken [300]. The sequence comparisons between the *fos*A7 gene found in our study versus the *fos*A7 gene isolated from *S*. Heidelberg isolates demonstrated that all *S*. Montevideo isolates harbored *fos*A7 gene that showed 96% similarity to the first reported *fos*A7 gene (data not shown). Moreover, when we also examined the *S*. Montevideo isolates from Ohta et al. (2017 – though in same experimental feedlot), with the updated ARG database, we identified this gene in the *S*. Montevideo isolated recovered from the cattle feces in 2009. This suggests that this gene was not a novel gene that has recently been acquired by *S.* Montevideo

isolates. Fosfomycin is not an antibiotic tested in the NARMS gram-negative Sensititre plate; therefore, the phenotypic conferred resistance to fosfomycin of *S*. Montevideo isolates remains unknown in our study. For comparison, we tested a subset of (n = 10) *S.* Montevideo isolates against phenotypic fosfomycin resistance (the EUCAST MIC interpretation criterium for resistance zone-diameter is ≤ 12 mm) using the disk-diffusion method (data not shown here). All tested isolates were found to be susceptible to fosfomycin. Although we did not use any positive control strain in our approach, if we assume that the *fos*A7 gene in *S*. Montevideo isolates is not expressed, this could be likely that this gene has a different function in the genome or else is cryptic, or it is expressed when it is on a plasmid rather than on a chromosome as suggested by Rehman et al. (2017) [300].

In this study, we detected 21 isolates with phenotypic streptomycin resistance, which was not associated with any particular serotype. However, WGS-based analyses of our study did not detect any streptomycin resistance genes such as *str*, *aad*, *aph*, or *arm*. This could be due to the uncertainty of the MIC breakpoint for streptomycin resistance. In our study, we interpreted the phenotypic resistance using the most up-to-date NARMS MIC breakpoint for streptomycin (≥ 32 µg/ml), which was updated and changed from ≥ 64 µg/ml in 2014. This update was rendered due to the previously observed inconsistencies in the genotypic and phenotypic resistance profiles [301]. When we apply here the previous breakpoint of ≥ 64 µg/ml, we observed an approximately 7-fold decrease in the number of resistant isolates. Therefore, utilizing the lowered break-point of ≥ 32 µg/ml might be biased, and potentially lead us to obtain false-positive results based on a lack of mechanistic gene explanations. On the other hand, this result could also be related to an

as-yet unidentified streptomycin resistance gene that is not in the ARG database. This hypothesis is also supported by Tyson et al. (2016) who reported isolates' lack of a known resistance gene exhibiting phenotypic streptomycin resistance at the MIC of 32 µg/ml [301]. Clearly, more studies are needed to understand both phenotypic and genotypic insights into streptomycin resistance. Updating the breakpoints based on genetic resistance traits may not be suitable without ensuring such genetic traits are expressed at certain levels to encode and express a phenotypic resistance for the antibiotic of interest.

We also observed an aminoglycoside resistance gene (*aac*(*6'*)-*Iaa*) in all *Salmonella* isolates, regardless of the serotype profiles. This gene is often found in *Salmonella* isolates; further, based on what is known to date the *aac*(*6'*)-*Iaa* gene is not known to confer any aminoglycoside resistance in *Salmonella* [302-304]. Previously, similar to *aac*(*6'*)-*Iaa*, the cryptic *aac*(*6'*)-*Iy* aminoglycoside gene on *Salmonella* was activated for aminoglycoside resistance by a transcriptional fusion resulting from a chromosomal deletion [184]. A study contacted by Salipante et al. (2003)[303] also generated artificial mutations (with a possible 2,165 single-amino acid substitutions and 1,699,110 possible double amino acid substitutions) *in vitro* in a *S.* Typhimurium LT2 strain harboring *aac*(*6'*)-*Iaa* gene on its chromosome, in order to understand if there was any evolutionary potential of this gene to express phenotypic aminoglycoside resistance such as to tobramycin, kanamycin, amikacin or gentamicin. The artificial mutations resulted in one or two possible mutations that were likely to occur on *aac*(*6'*)-*Iaa* gene located in the chromosome of the *S.* Typhimurium LT2 strain. Their result showed with 99.9% confidence that any mutation that could potentially occur on this gene would not result in aminoglycoside resistance. Perhaps this gene has another unknown function in

the *S.* Montevideo chromosome that provides a positive selection for the isolates and that has not yet been identified.

In our study, we also identified an IncI1 plasmid that was not associated with any particular serotype. The IncI1 plasmid that was found in our study also did not carry any known ARGs, and the isolates carrying the IncI1 plasmid did not show any phenotypic resistance against the 14 antibiotics tested. However, this plasmid was previously reported to harbor the *bla*$_{\text{CMY-2}}$ gene in *S.* Typhimurium, *S.* Newport, *S.* Heidelberg, and *S.* Infantis serotypes that also expressed phenotypic ceftriaxone resistance. Among these serotypes, only *S.* Typhimurium serotype harboring the IncI1 plasmid with *bla*$_{\text{CMY-2}}$ resistance gene was recovered from a ground beef/cattle source [190]. The IncI1 plasmid is an incompatibility group plasmid more frequently isolated from *Salmonella* compared to the IncN plasmid [109]. This was not the case in our study, since we determined a higher number of IncN plasmids (n= 68) compared to the IncI1 plasmid (n = 64) (Table 34). This difference was related to the vertical transfer of the IncN plasmids among *S.* Montevideo isolates throughout the study time (and their prolific numbers) versus the IncI1 plasmid that was widely shared and spontaneously found and horizontally transferred among many different serotypes found in feces, lymph nodes and on hides of the cattle housed in different pens [305]. Other than in our study with high prevalence of *S.* Montevideo, it is therefore more likely that the IncI1 plasmid would predominate.

The IncI1 plasmid harboring ARGs has often been found in clinically important *Salmonella* serotypes, both in human and animals [306]. Based on a recent study conducted in 2019, the IncI1 plasmid was reported as not being fully associated with biofilm formation or bacteriocin production in cattle origin *S.* Newport, *S.* Anatum, *S.*

Cerro, *S*. Montevideo, and *S*. Heidelberg serotypes [302]. Clearly, the potential functions of the IncI1 plasmid, that is not responsible for any ARGs when located in cattle origin *Salmonella* strains, are not fully addressed yet; therefore, further *in vitro* studies are needed to determine its functions.

5.5. Findings related to the BRD metaphylaxis

In our study, ceftriaxone, ceftiofur or azithromycin resistance genes and phenotypes were not selected in cattle origin *Salmonella* under the selection pressure of single-dose ceftiofur or azithromycin. Our result was in direct contrast with the study conducted by Ohta et al. (2017), which reported a significant increase ($P < 0.05$) in phenotypic ceftiofur and ceftriaxone resistance among *Salmonella* on Day 4, following a single-dose of CCFA administered to cattle on Day 0. However, in that study the 3rd generation cephalosporin resistance was found only in *S*. Reading isolates. The MDR profile (ACSSuT+Cef) was only identified in the *S*. Reading serotype. Interestingly this serotype was not identified on Day 0; perhaps, the *S*. Reading was present in the feces, but was not prevalent without the selection pressure of the antibiotic and therefore was not identified. This finding was parallel with other studies that reported *S*. Reading was associated with certain MDR profiles including ACSSuT or MDR-AmpC profile in cattle [15, 69]. This could potentially explain the absence of ceftriaxone and ceftiofur resistant or MDR *Salmonella* in our study, since we failed to identify this serotype. It is possible that the cattle group in the study of Ohta et al. (2017) had harbored *S*. Reading prior to placement in the experimental pens, since we identified certain serotypes from cattle from certain sources. In our study, if we had a *Salmonella* serotype that was resistant to either ceftiofur or tulathromycin on Day

0, the antibiotic treatments would more likely select for that resistant population; thus, our findings could be dramatically different with different starting serotypes. Similarly, if Ohta's study was conducted up until the date of slaughter, the earlier observed declining prevalence of *S*. Reading isolates, starting from Day 14 to Day 26, would likely continue and eventually disappear without the selection pressure of antibiotics. We base this argument on the time-related dynamics of *Salmonella* that have been shown in our study [236] and also time-related reductions observed on the antibiotic selection of other AR bacterial populations after single-dose antibiotic administration [37-39].

In Ohta et al. (2017), the *Salmonella* prevalence significantly ($P < 0.05$) decreased after the ceftiofur administration on Day 4, and this decrease was recovered back to Day 0 values by Day 14. In our study we did not observe any significant effect of ceftiofur treatment on *Salmonella* prevalence observed in cattle feces. The differences can be related to the initial prevalence of *Salmonella* observed in feces that were most likely affected by the seasonal variations in the sample collection periods of the two studies. Ohta et al. (2017), conducted their study in early August with a starting *Salmonella* prevalence of approximately 80.0%; in our study, we observed a 32.5% *Salmonella* prevalence on Day 0 starting in March. Later, both studies reported almost the half of the reduction in *Salmonella* prevalence (to 34.1% and 15%, respectively) on Day 4 and 7, respectively. In addition, since Ohta et al. (2017), sampled their animals 4-day after the initial ceftiofur treatment, the 3-day difference in our study may also contribute to observed differences in effects on *Salmonella* prevalence.

Previously, it has been documented that there can be certain dissimilarities observed in the fecal microbiota of the same cattle regardless of the antibiotic treatments;

that is, before transportation and after transportation up to 19 days [51]. In Ohta's (2017) study the cattle came from a single-origin and were provided a one-month acclimatization period before the first sample collection. In our study, only 3 days of acclimatization was provided to cattle from two different origins; therefore, it is possible that the transport and environment related stress factors of cattle in our study carried forward and this potentially increased the dissimilarities in the fecal microbiome that manifested as highly variable *Salmonella* serotypes. This could also possibly favor the fecal *Salmonella* population following the treatment, either by reducing the prevalence of ceftiofur susceptible microbiota or else with the help of certain *Bacillus* spp. and *Bacteroides* spp. that can degrade ceftiofur in cattle intestine [234].

Alali et al. (2009) quantified the *bla*$_{\text{CMY-2}}$ gene in feces (collected on Days 0, 3, 7, 10, 14, 18, 21, and 28 after treatment) of beef cattle during a 28-day period, following the single (on Day 0) or multiple (on days 0, 6 and 13) administration of two different doses of ceftiofur (4.4 mg/kg and 6.6 mg/kg) [41]. They found that administration of ceftiofur increased the absolute and normalized number of *bla*$_{\text{CMY-2}}$ genes detected in fecal samples compared to the control group throughout the 28-day period. The observed increase in *bla*$_{\text{CMY-2}}$ genes found by Alali et al. (2009) was most likely related to ceftiofur-resistant *E. coli*, which was phenotypically observed in the same cattle study in a study published by Lowrance et al. (2007) [39]. According to their findings, ceftiofur treatment increased resistant *E. coli* populations after treatment; however, they observed the population returning to pre-administration resistance levels after a two-week period. Weinroth et al. (2017) also investigated the effects of ceftiofur use on the resistome of cattle feces, focusing on the *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{CTX-M-24}}$ genes in the fecal microbiome on Day 0 and Day

26 [47]. In contrast to Alali et al. (2009), but similar to our results, their results did not indicate any significant changes in genes encoding ceftriaxone resistance over a 26-day period, though they did not analyze the samples taken during peak antibiotic activity (i.e., Days 4- 12) and shotgun metagenomics are of notoriously poor sensitivity for all but the most prevalent and high abundance ARGs.

Doster et al. (2018) found that there was no significant difference in the cattle resistome and microbiome among tulathromycin treated and control groups; however, their results may be related to their small sample size (n=15 per treatment group by day) [46]. The authors, however, reported a significant increase in the 16S rRNA normalized ARG abundance and the average relative abundance of microbial taxa between Days 1 and 11 [46]. Similar to the study of Weintroth et al. (2018) [47], Doster et al. (2018) ignored the likely peak period of antibiotic effect on enteric populations; importantly, their study did not measure the long-term effects of tulathromycin treatment out to slaughter eligibility. The study of Doster et al. (2018) was also supported by Holman et al. (2019) [51] showing no significant difference in the cattle resistome and microbiome among tulathromycin-treated and control groups; however, similar to Doster et al. (2018), this may also be related to their small sample size (n = 15 per treatment). However, unlike the Doster et al. (2018), Holman et al. (2019) reported a decrease in OTU after the before transport (day -5) after transport (Days 5, 12, and 19) and by the end of the study (Day 32). In contrast to Weintroth et al. (2018), [47] and Doster et al. (2018), Holman et al. (2019) sampled their group of study cattle on Day 5, which was the peak day to observe the potential effects of the antibiotic; however, they did not observe any resistome differences reflecting azithromycin resistance on that day. It is important to note that Holman et al. (2019), only

screened the macrolide resistance genes of *erm*A and *erm*X (not *erm*B) in their sample, and failed to identify the common azithromycin resistance genes usually observed in *Salmonella*, such as *mph*A/*mph*E, *ere*A, *lnu*F, *mef*B, *and msr*E genes [32, 182].

In our study, isolates were not resistant to azithromycin, ceftiofur or ceftriaxone, either before or after either ceftiofur or tulathromycin treatments. This result was aligned with metagenomic data analyses conducted by Weinroth et al. (2018) and Doster et al. (2018) but countered the findings of Alali et al. (2009) [41, 46, 47]. These studies measured the effect of either ceftiofur or tulathromycin treatment on the microbiome or/and resistome of the fecal community of feces in the beef cattle; however, none of the studies focused directly on culturable bacteria, let alone *Salmonella* as the outcome bacteria of interest.

5.6. Potential public health risks

In our study, animal-level *Salmonella* prevalence in lymph nodes was over 80% at slaughter; given the high probability these *Salmonella* were likely to be trimmed into ground beef, the public health risks and consequences need to be extensively evaluated. Importantly, we isolated *S.* Newport from cattle lymph nodes at slaughter. *Salmonella* Newport is a highly virulent serotype that is consistently listed as one of the top three *Salmonella* serotypes causing clinical human salmonellosis [157]. Interestingly, all these *S.* Newport serotypes were recovered from cattle lymph nodes from a single-pen (Pen 53), only at slaughter age and all were pan-susceptible to 14 antibiotics. At first glance, this is somewhat unusual for this serotype, especially given its historical association with the MDR *S.* Newport outbreaks between 2004 and 2013 [309]. This finding is important from

a public health perspective, since one of the most recent *Salmonella* outbreaks involved a pan-susceptible *S.* Newport contaminating ground beef products resulting in 403 reported salmonellosis cases, 117 hospitalizations and the recall of 5,488 tons of beef products (accessed at http://www.outbreakdatabase.com/reports/2018-2019_JBS_CDC_Marc_22_2019.pdf in August 2019).

The second most important serotype we isolated from the lymph nodes was *S.* Montevideo, even though this serotype has not been seen as being as virulent as *S.* Newport serotype, it is still one of the ten clinically most important serotypes for human infections [157]. We identified this serotype in cattle feces, lymph nodes and hides, and all were carrying plasmidal tetracycline resistance and were phenotypically resistant to tetracycline.

In addition to these serotypes, interestingly we isolated a single *S.* Norwich isolate from a single-fecal sample at slaughter. *S.* Norwich is also reported as a clinically important serotype; however, is not as commonly identified as the other two serotype listed above and this serotype is not usually found with an MDR profile [157]. This was the least worrying clinically important serotype we found, since only one isolate was identified and it was a fecal origin *Salmonella*.

Slaughterhouse interventions such as exposing carcasses to steam vacuuming, lactic acid, and hot water are highly efficacious at reducing the prevalence of fecal or hide origin *Salmonella* on carcass surfaces. However, infected lymph nodes are likely to be incorporated into ground beef during meat processing, thus contaminating the finished retail product and potentially leading to salmonellosis in humans through consumption of under-cooked meat.

A commercially available *Salmonella* vaccine (*Salmonella* Newport bacterial extract SRP [siderophore receptors and porin proteins] vaccine; Zoetis, LLC) was tested previously and was shown to reduce *Salmonella* carriage in cattle feces and on hide samples [237]; however, this hypothesis was tested against the *Salmonella* carriage in lymph nodes in another study that showed no significant effects [296]. Another study exploring the effect of a direct-fed microbial supplement on *Salmonella* carriage in cattle lymph nodes reported that *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* administration to beef cattle during the feeding period can reduce *Salmonella* carriage in sub-iliac lymph nodes [297]. Clearly, to reduce *Salmonella* colonization in the lymph nodes an intervention which can be applied during the feeding period is necessary. Therefore, further studies need to be conducted to develop new interventions targeting virulent human *Salmonella* serotypes in cattle lymph nodes.

5.7. Population dynamics of *Salmonella*

In our study, the most prevalent serotype was *S.* Lubbock (34.1%), followed by *S.* Anatum (28.3%) and *S.* Montevideo (17.0%). In the study by Ohta et al., (2017) conducted in 2009 in the same feedlot, the most prevalent serotype was found to be *S.* Mbandaka (ST 413) with 37.9% of prevalence, followed by 19.1% *S.* Give (ST 654), 15.2% *S.* Reading (ST 1628), 13.6% *S.* Kentucky (ST 198), 13.4% *S.* Montevideo (ST 138), and 0.7% *S.* Anatum (ST 64) [36]. When common serotypes identified in both studies were observed, *S.* Montevideo and *S.* Anatum shared identical STs whereas *S.* Kentucky (ST 152) differed.

In our study, we did not identify any *S.* Give, *S.* Mbandaka, or *S.* Reading. In contrast, in our study we identify *S.* Lubbock (ST 413), *S.* Cerro (ST 367), *S.* Newport (ST 118), and *S.* Norwich (ST 2119). It is also important note that we purposely used the same methods and parameters (with only difference being a shorter TSB incubation time in our study) as Ohta et al. (2017) for *Salmonella* isolation and identification; therefore, these results were comparable between the two research projects that were also conducted in the same experimental feedlot. However, it is important to consider the potential serotype selection bias of the methods used, when comparisons are made among studies using different isolation methods [74].

S. Lubbock is a recently named serotype first isolated from a peripheral cattle lymph node and reported in 2015 [307]. Interestingly, the highly prevalent serotype *S.* Mbandaka in 2009 (identified by Ohta et al. [2017]) completely disappeared and was replaced by a new serotype *S.* Lubbock with the same ST (ST 413) in 2016, and in the same feedlot. Evidence of gene recombination events show that serotype *S.* Mbandaka is the most likely ancestor of this new serotype Lubbock [270, 307]. This new serotype is believed to have emerged from *S.* Mbandaka by acquiring the *fliC* gene operon from *S.* Montevideo [270, 307].

Interestingly, in our phylogenetic tree analyses, *S.* Lubbock had the highest variation of SNPs (84 SNPs/135 isolates), whereas *S.* Montevideo had lowest (12 SNPs/68 isolates); this could result from a high mutation frequency that may be observed in *S.* Lubbock isolates, which may explain why the *S.* Montevideo was more conservative and less subject to change over a seven-year period.

A decade and a half earlier, Purdy et al. (2001 and 2004) isolated and characterized *Salmonella* from playas nearby the feedlots in High Plains in Texas [308, 309]. This study discovered varying serotype dynamics from the same playas of different feedlots between the winter and summer. Due to the high prevalence of coliforms and *Salmonella* found in these playas, they suggested not to use retention-pond waters to abate feedyard dust.

In contrast to these findings, Loneragan et al. (2005) measured effects of retention-pond use for dust abatement in a cohort study [310]. They used water sprinklers to direct retention-pond water at different time intervals on exposed cattle groups and kept the remaining cattle as unexposed. They observed no difference in *Salmonella* prevalence and a non-significant reduction of *E. coli* 0:157 H:7 prevalence in the exposed group when compared to the unexposed group cattle. In their study, they speculated the presence of certain virulence factors in the environment may influence survivability of bacteria. Therefore, one of the strongest hypotheses that might explain serotype selection by day, time, and location on *Salmonella* in the environment is the presence of bacteriophages [311].

Bacteriophage treatments are considered as an alternative to antibiotics in order to combat AR *Salmonella* infections and these applications also have reduced *Salmonella* prevalence in rendering facility environments [312, 313]. Xie et al. (2016) characterized both *Salmonella* and *Salmonella* bacteriophages from soil and feces in Texas feedlots and found that bacteriophages that infect one serotype may not affect others [314]. Overall, our findings suggest that regardless of the use of antibiotics, *Salmonella* dynamics fluctuate over the feeding period, and were mostly affected by the source of cattle and the pen environment of the feedlot. It seems probable that bacteriophages also can affect these

dynamics in the environment. Therefore, a bacteriophage infection targeting *S*. Mbandaka in the same research feedlot used by study of Ohta et al. (2017) and before this present study was performed may be one of the explanations of the evolutionary shift observed in the seven-year period between studies where *S*. Lubbock substituted with *S*. Mbandaka as the dominant serotype.

In the present study, *S*. Cerro appeared only at slaughter age and was otherwise absent early in the feeding period; perhaps demonstrating the unique temporal dynamics of *Salmonella*. *S*. Cerro is known to be a pathogen for at-risk cattle such as dairy cows; however, the beef cattle involved this study did not show any clinical signs of salmonellosis [59]. In contrast, *S.* Kentucky was isolated from only one origin/source of cattle in the early feeding period and it was not recovered at slaughter age. In addition, according to *Salmonella* pen and serotype distributions demonstrated in Figure 25, for the majority of the pens, a single-dominant serotype was determined to be prevalent on hide samples from animals in the same pen at slaughter age.

In our study, a single-ST was found for each serotype, which would also suggest the relatively clonal spread of *Salmonella* among the days, the sample types and within the pens, blocks, and source. This conclusion is also supported by the phylogenetic analyses illustrating the close genotypic relatedness of the isolates; that is, among serotypes that were recovered from animals in the same pen, block and origin/source were much more closely related when compared to isolates that belonged to other groups (Figure 27).

Clearly, dominant, and potentially clonal serovars found in feces, lymph nodes, and on hides could be attributed to the source (host), environmental (pen) and time-related factors. Feedlots dynamics can tremendously affect bacterial dynamics and the AR

profiles in the pathogens; including the feedlot dust, since a research article published in 2015 also found bacterial DNA and ARG genes in the particulate matter obtained from feedlot dust in the southern United States, suggesting bacteria carrying ARGs genes can disperse within and among the feedlots [162].

To summarize, we speculate that *Salmonella* serotype and AR selection in cattle may be related to pre-existing soil microbiota in the feedlots, seasonal changes, and bacteriophages in the environment, and these all may contribute to temporally dynamic selection of dominant serotypes of *Salmonella* [13, 287, 288]. None of these latter points were tested in the present study, and thus remain as future research that is needed.

6. CONCLUSION

Here, we assessed *Salmonella* population dynamics during the feeding period until slaughter following a single-dose antibiotic treatment. We found no effects of ceftiofur and tulathromycin metaphylactic use on resistant ceftriaxone or azithromycin *Salmonella* from the time of treatment until slaughter (up to 141 days later) in cattle feces, lymph nodes or hides. We identified the pen in which cattle are housed as the factor that contributed the most to *Salmonella* serotypes being shared; importantly, the dominant strain in each pen changed repeatedly over the entire feeding period. Clearly, our findings suggest that the origin/source (host), the environmental (pen) and time-related factors play an important role in the selection of the dominant serotypes and, therefore, for the selection of antibiotic resistance. The ecology of *Salmonella* within cattle populations is clearly far more complex than a proposed simple fecal-oral mode of transmission; thus, supporting the idea that origin/source and other ambient environmental factors are likely to be involved. One of the limitations of this study was in not collecting and analyzing prior environmental samples to determine the contributions of pre-existing soil microbiota in the feedlot pens and other animals (birds, pests) that had contact with cattle, feedlot equipment, and bacteriophages in the environment. Therefore, the environmental factors that influenced the temporal dynamic selection of dominant serotypes of *Salmonella* among the pens and the cattle remain unclear. Further studies are needed to evaluate the effects of bacterial, environmental and cattle-host related factors in temporal *Salmonella* serotype dynamics.

REFERENCES

1. Schultz M. 2008. Theobald Smith. Emerg Infect Dis 14:1940-42. doi:10.3201/eid1412.081188

2. Wray C, Wray A. 2000. *Salmonella* in domestic animals. CABI Pub., Wallingford, Oxon, UK ; New York, NY, USA

3. Odumeru JA, León-Velarde CG. 2012. *Salmonella* detection methods for food and food ingredients. *In* Mahmoud BSM (ed), *Salmonella* - A dangerous foodborne pathogen, ch. 17, IntechOpen, University of Guelph, Guelph, Ontario Canada. doi:10.5772/29526. Available at https://www.intechopen.com/books/salmonella-a-dangerous-foodborne-pathogen/salmonella-detection-methods-for-food-and-food-ingredients [Accessed in October 2019].

4. Crosa JH, Brenner DJ, Ewing WH, Falkow S. 1973. Molecular relationships among the Salmonelleae. J Bacteriol 115:307-15.

5. Achtman M, Wain J, Weill FX, Nair S, Zhou Z, Sangal V, Krauland MG, Hale JL, Harbottle H, Uesbeck A, Dougan G, Harrison LH, Brisse S, Group SEMS. 2012. Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica*. PLoS Pathog 8:e1002776. doi:10.1371/journal.ppat.1002776

6. Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. 2000. *Salmonella* nomenclature. J Clin Microbiol 38:2465-7.

7. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis 17:7-15. doi:10.3201/eid1701.P11101

8. CDC. 2013. Antibiotic resistance threats in the United States. Centers for Disease Control and Prevention, Atlanta, GA. Available at https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf [Accessed in October 2019].

9. CDC-NORS. 2018. National Outbreak Reporting System (NORS) dashboard. Available at https://wwwn.cdc.gov/norsdashboard/ [Accessed in October 2018].

10. HHS-IFSAC. 2017. Foodborne illness source attribution estimates for 2013 for *Salmonella*, *Escherichia coli* O157, *Listeria monocytogenes*, and *Campylobacter* using multi-year outbreak surveillance data, United States. Interagency Food Safety Analytics Collaboration. U.S. Department of Health and Human Services, CDC, FDA, USDA-FSIS, Atlanta, GA and Washington D.C. Available at https://www.cdc.gov/foodsafety/pdfs/IFSAC-2013FoodborneillnessSourceEstimates-508.pdf [Accessed in October 2019].

11. Richardson LC, Bazaco MC, Parker CC, Dewey-Mattia D, Golden N, Jones K, Klontz K, Travis C, Kufel JZ, Cole D. 2017. An updated scheme for categorizing foods implicated in foodborne disease outbreaks: A tri-agency collaboration. Foodborne Pathog Dis 14:701-10. doi:10.1089/fpd.2017.2324

12. CDC. 2013. An Atlas of *Salmonella* in the United States, 1968-2011. Centers for Disease Control and Prevention, Atlanta, GA. Available at https://www.cdc.gov/salmonella/pdf/salmonella-atlas-508c.pdf [Accessed in October 2019].

13. Laufer AS, Grass J, Holt K, Whichard JM, Griffin PM, Gould LH. 2015. Outbreaks of *Salmonella* infections attributed to beef -United States, 1973-2011. Epidemiol Infect 143:2003-13. doi:10.1017/S0950268814003112

14. Carlson JC, Hyatt DR, Ellis JW, Pipkin DR, Mangan AM, Russell M, Bolte DS, Engeman RM, DeLiberto TJ, Linz GM. 2015. Mechanisms of antimicrobial resistant *Salmonella enterica* transmission associated with starling-livestock interactions. Vet Microbiol 179:60-8. doi:10.1016/j.vetmic.2015.04.009

15. Gragg SE, Loneragan GH, Brashears MM, Arthur TM, Bosilevac JM, Kalchayanand N, Wang R, Schmidt JW, Brooks JC, Shackelford SD, Wheeler TL, Brown TR, Edrington TS, Brichta-Harhay DM. 2013. Cross-sectional study examining *Salmonella enterica* carriage in subiliac lymph nodes of cull and feedlot cattle at harvest. Foodborne Pathog Dis 10:368-74. doi:10.1089/fpd.2012.1275

16. Haneklaus AN, Harris KB, Griffin DB, Edrington TS, Lucia LM, Savell JW. 2012. *Salmonella* prevalence in bovine lymph nodes differs among feedyards. J Food Prot 75:1131-3. doi:10.4315/0362-028X.JFP-11-530

17. Brichta-Harhay DM, Arthur TM, Bosilevac JM, Kalchayanand N, Shackelford SD, Wheeler TL, Koohmaraie M. 2011. Diversity of multidrug-resistant *Salmonella enterica* strains associated with cattle at harvest in the United States. Appl Environ Microbiol 77:1783-96. doi:10.1128/AEM.01885-10

18. Rhoades JR, Duffy G, Koutsoumanis K. 2009. Prevalence and concentration of verocytotoxigenic *Escherichia coli, Salmonella enterica* and *Listeria monocytogenes* in the beef production chain: A review. Food Microbiol 26:357-76. doi:10.1016/j.fm.2008.10.012

19. Sofos JN, Kochevar SL, Bellinger GR, Buege DR, Hancock DD, Ingham SC, Morgan JB, Reagan JO, Smith GC. 1999. Sources and extent of microbiological contamination of beef carcasses in seven United States slaughtering plants. J Food Prot 62:140-5. doi:10.4315/0362-028x-62.2.140

20. Arthur TM, Brichta-Harhay DM, Bosilevac JM, Guerini MN, Kalchayanand N, Wells JE, Shackelford SD, Wheeler TL, Koohmaraie M. 2008. Prevalence and characterization of *Salmonella* in bovine lymph nodes potentially destined for use in ground beef. J Food Prot 71:1685-8. doi:10.4315/0362-028x-71.8.1685

21. DuPont HL. 2014. Acute infectious diarrhea in immunocompetent adults. N Engl J Med 370:1532-40. doi:10.1056/NEJMra1301069

22. DuPont HL. 2009. Clinical practice. Bacterial diarrhea. N Engl J Med 361:1560-9. doi:10.1056/NEJMcp0904162

23. Shane AL, Mody RK, Crump JA, Tarr PI, Steiner TS, Kotloff K, Langley JM, Wanke C, Warren CA, Cheng AC, Cantey J, Pickering LK. 2017. 2017 Infectious Diseases Society of America clinical practice guidelines for the diagnosis and management of infectious diarrhea. Clin Infect Dis 65:e45-e80. doi:10.1093/cid/cix669

24. Choi SH, Kim EY, Kim YJ. 2013. Systemic use of fluoroquinolone in children. Korean J Pediatr 56:196-201. doi:10.3345/kjp.2013.56.5.196

25. Burkhardt JE, Walterspiel JN, Schaad UB. 1997. Quinolone arthropathy in animals versus children. Clin Infect Dis 25:1196-204. doi:10.1086/516119

26. Yarrington ME, Anderson DJ, Dodds Ashley E, Jones T, Davis A, Johnson M, Lokhnygina Y, Sexton DJ, Moehring RW. 2019. Impact of FDA black box warning on fluoroquinolone and alternative antibiotic use in southeastern US hospitals. Infect Control Hosp Epidemiol doi:10.1017/ice.2019.247:1-4. doi:10.1017/ice.2019.247

27. Varma JK, Molbak K, Barrett TJ, Beebe JL, Jones TF, Rabatsky-Ehr T, Smith KE, Vugia DJ, Chang HG, Angulo FJ. 2005. Antimicrobial-resistant nontyphoidal *Salmonella* is associated with excess bloodstream infections and hospitalizations. J Infect Dis 191:554-61. doi:10.1086/427263

28. Medalla F, Hoekstra RM, Whichard JM, Barzilay EJ, Chiller TM, Joyce K, Rickert R, Krueger A, Stuart A, Griffin PM. 2013. Increase in resistance to ceftriaxone and nonsusceptibility to ciprofloxacin and decrease in multidrug resistance among *Salmonella* strains, United States, 1996-2009. Foodborne Pathog Dis 10:302-9. doi:10.1089/fpd.2012.1336

29. Gay K, Robicsek A, Strahilevitz J, Park CH, Jacoby G, Barrett TJ, Medalla F, Chiller TM, Hooper DC. 2006. Plasmid-mediated quinolone resistance in non-Typhi serotypes of *Salmonella enterica*. Clin Infect Dis 43:297-304. doi:10.1086/505397

30. Wasyl D, Hoszowski A, Zajac M. 2014. Prevalence and characterisation of quinolone resistance mechanisms in *Salmonella* spp. Vet Microbiol 171:307-14. doi:10.1016/j.vetmic.2014.01.040

31. Tadesse DA, Singh A, Zhao S, Bartholomew M, Womack N, Ayers S, Fields PI, McDermott PF. 2016. Antimicrobial resistance in *Salmonella* in the United States from 1948 to 1995. Antimicrob Agents Chemother 60:2567-71. doi:10.1128/AAC.02536-15

32. CDC-NARMS. 2018. Human Isolates Surveillance Report for 2015. National Antimicrobial Resistance Monitoring System, Centers for Disease Control and Prevention, Atlanta, GA. Available at https://www.cdc.gov/narms/reports/annual-human-isolates-report-2015.html [Accessed in October 2019].

33. Loneragan GH, Dargatz DA, Morley PS, Smith MA. 2001. Trends in mortality ratios among cattle in US feedlots. J Am Vet Med Assoc 219:1122-7. doi:10.2460/javma.2001.219.1122

34. USDA-APHIS-NAHMS. 2013. Feedlot 2011, Part IV: Health and health management on U.S. feedlots with a capacity of 1,000 or more head. United States Department of Agriculture, Animal and Plant Health Inspection Service, National Animal Health Monitoring System, Fort Collins, CO. Available at https://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot2011/Feed11_dr_PartIV_1.pdf [Accessed in October 2019].

35. USDA-ERS. 2019. Cattle & Beef Sector at a Glance U.S. Department of Agriculture, Economic Research Service. Available at https://www.ers.usda.gov/topics/animal-products/cattle-beef/sector-at-a-glance [Accessed in October 2019].

36. Ohta N, Norman KN, Norby B, Lawhon SD, Vinasco J, den Bakker H, Loneragan GH, Scott HM. 2017. Population dynamics of enteric *Salmonella* in response to antimicrobial use in beef feedlot cattle. Sci Rep 7:14310. doi:10.1038/s41598-017-14751-9

37. Kanwar N, Scott HM, Norby B, Loneragan GH, Vinasco J, Cottell JL, Chalmers G, Chengappa MM, Bai J, Boerlin P. 2014. Impact of treatment strategies on cephalosporin and tetracycline resistance gene quantities in the bovine fecal metagenome. Sci Rep 4:5100. doi:10.1038/srep05100

38. Kanwar N, Scott HM, Norby B, Loneragan GH, Vinasco J, McGowan M, Cottell JL, Chengappa MM, Bai J, Boerlin P. 2013. Effects of ceftiofur and chlortetracycline treatment strategies on antimicrobial susceptibility and on *tet*(A), *tet*(B), and *bla* CMY-2 resistance genes among *E. coli i*solated from the feces of feedlot cattle. PLoS One 8:e80575. doi:10.1371/journal.pone.0080575

39. Lowrance TC, Loneragan GH, Kunze DJ, Platt TM, Ives SE, Scott HM, Norby B, Echeverry A, Brashears MM. 2007. Changes in antimicrobial susceptibility in a population of *Escherichia coli* isolated from feedlot cattle administered ceftiofur crystalline-free acid. Am J Vet Res 68:501-7. doi:10.2460/ajvr.68.5.501

40. Agga GE, Schmidt JW, Arthur TM. 2016. Effects of in-feed chlortetracycline prophylaxis in beef cattle on animal health and antimicrobial-resistant *Escherichia coli*. Appl Environ Microbiol 82:7197-204. doi:10.1128/AEM.01928-16

41. Alali WQ, Scott HM, Norby B, Gebreyes W, Loneragan GH. 2009. Quantification of the *bla*(CMY-2) in feces from beef feedlot cattle administered three different doses of ceftiofur in a longitudinal controlled field trial. Foodborne Pathog Dis 6:917-24. doi:10.1089/fpd.2009.0271

42. Benedict KM, Gow SP, McAllister TA, Booker CW, Hannon SJ, Checkley SL, Noyes NR, Morley PS. 2015. Antimicrobial resistance in *Escherichia coli* recovered from feedlot cattle and associations with antimicrobial use. PLoS One 10:e0143995. doi:10.1371/journal.pone.0143995

43. Inglis GD, McAllister TA, Busz HW, Yanke LJ, Morck DW, Olson ME, Read RR. 2005. Effects of subtherapeutic administration of antimicrobial agents to beef cattle on the prevalence of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter hyointestinalis*. Appl Environ Microbiol 71:3872-81. doi:10.1128/AEM.71.7.3872-3881.2005

44. Alexander TW, Yanke LJ, Topp E, Olson ME, Read RR, Morck DW, McAllister TA. 2008. Effect of subtherapeutic administration of antibiotics on the prevalence of antibiotic-resistant *Escherichia coli* bacteria in feedlot cattle. Appl Environ Microbiol 74:4405-16. doi:10.1128/AEM.00489-08

45. Agga GE, Schmidt JW, Arthur TM. 2016. Antimicrobial-resistant fecal bacteria from ceftiofur-treated and nonantimicrobial-treated comingled beef cows at a cow-calf operation. Microb Drug Resist 22:598-608. doi:10.1089/mdr.2015.0259

46. Doster E, Rovira P, Noyes NR, Burgess BA, Yang X, Weinroth MD, Lakin SM, Dean CJ, Linke L, Magnuson R, Jones KI, Boucher C, Ruiz J, Belk KE, Morley PS. 2018. Investigating Effects of Tulathromycin Metaphylaxis on the Fecal Resistome and Microbiome of Commercial Feedlot Cattle Early in the Feeding Period. Front Microbiol 9:1715. doi:10.3389/fmicb.2018.01715

47. Weinroth MD, Scott HM, Norby B, Loneragan GH, Noyes NR, Rovira P, Doster E, Yang X, Woerner DR, Morley PS, Belk KE. 2018. Effects of ceftiofur and chlortetracycline on the resistomes of feedlot cattle. Appl Environ Microbiol 84:e00610-18. doi:10.1128/AEM.00610-18

48. Schmidt JW, Griffin D, Kuehn LA, Brichta-Harhay DM. 2013. Influence of therapeutic ceftiofur treatments of feedlot cattle on fecal and hide prevalences of commensal *Escherichia coli* resistant to expanded-spectrum cephalosporins, and molecular characterization of resistant isolates. Appl Environ Microbiol 79:2273-83. doi:10.1128/AEM.03592-12

49. Smith AB, Renter DG, Cernicchiaro N, Shi X, Nagaraja TG. 2016. Prevalence and quinolone susceptibilities of *Salmonella* isolated from the feces of preharvest cattle within feedlots that used a fluoroquinolone to treat bovine respiratory disease. Foodborne Pathog Dis 13:303-8. doi:10.1089/fpd.2015.2081

50. Smith AB, Renter DG, Cernicchiaro N, Shi X, Nickell JS, Keil DJ, Nagaraja TG. 2017. A randomized trial to assess the effect of fluoroquinolone metaphylaxis on the fecal prevalence and quinolone susceptibilities of *Salmonella* and

Campylobacter in feedlot cattle. Foodborne Pathog Dis 14:600-07. doi:10.1089/fpd.2017.2282

51. Holman DB, Yang W, Alexander TW. 2019. Antibiotic treatment in feedlot cattle: A longitudinal study of the effect of oxytetracycline and tulathromycin on the fecal and nasopharyngeal microbiota. Microbiome 7:86. doi:10.1186/s40168-019-0696-4

52. Apley M. 2006. Bovine respiratory disease: Pathogenesis, clinical signs, and treatment in lightweight calves. Vet Clin North Am Food Anim Pract 22:399-411. doi:10.1016/j.cvfa.2006.03.009

53. Ives SE, Richeson JT. 2015. Use of antimicrobial metaphylaxis for the control of bovine respiratory disease in high-risk cattle. Vet Clin North Am Food Anim Pract 31:341-50, v. doi:10.1016/j.cvfa.2015.05.008

54. WHO. 2019. Critically important antimicrobials for human medicine, 6th revision. Geneva: World Health Organization; 2019. Licence: CC BY-NC-SA 3.0 IGO. Available at https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf [Accessed in October 2019].

55. Wray C, Sojka WJ. 1977. Reviews of the progress of dairy science: Bovine salmonellosis. J Dairy Res 44:383-425.

56. Magossi G, Cernicchiaro N, Dritz S, Houser T, Woodworth J, Jones C, Trinetta V. 2019. Evaluation of *Salmonella* presence in selected United States feed mills. Microbiologyopen 8:e00711. doi:10.1002/mbo3.711

57. Cummings KJ, Divers TJ, McDonough PL, Warnick LD. 2009. Fecal shedding of *Salmonella* spp. among cattle admitted to a veterinary medical teaching hospital. J Am Vet Med Assoc 234:1578-85. doi:10.2460/javma.234.12.1578

58. Sanchez S, Hofacre CL, Lee MD, Maurer JJ, Doyle MP. 2002. Animal sources of salmonellosis in humans. J Am Vet Med Assoc 221:492-7. doi:10.2460/javma.2002.221.492

59. Cummings KJ, Warnick LD, Elton M, Rodriguez-Rivera LD, Siler JD, Wright EM, Grohn YT, Wiedmann M. 2010. *Salmonella enterica* serotype Cerro among dairy cattle in New York: An emerging pathogen? Foodborne Pathog Dis 7:659-65. doi:10.1089/fpd.2009.0462

60. Toth JD, Aceto HW, Rankin SC, Dou Z. 2011. Survival characteristics of *Salmonella enterica* serovar Newport in the dairy farm environment. J Dairy Sci 94:5238-46. doi:10.3168/jds.2011-4493

61. USDA-APHIS-NAHMS. 1995. *Salmonella* shedding by feedlot cattle. United States Department of Agriculture, Animal and Plant Health Inspection Service, National Animal Health Monitoring System, Fort Collins, CO. Available at https://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/cofe/COFE_is_Salmonella.pdf [Accessed in October 2019].

62. CDC. 2015. Serotypes and the importance of serotyping *Salmonella*. Centers for Disease Control and Prevention, Atlanta, GA. Available at https://www.cdc.gov/salmonella/reportspubs/salmonella-atlas/serotyping-importance.html [Accessed in October 2019].

63. Hendriksen RS, Vieira AR, Karlsmose S, Lo Fo Wong DM, Jensen AB, Wegener HC, Aarestrup FM. 2011. Global monitoring of *Salmonella* serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: Results of quality assured laboratories from 2001 to 2007. Foodborne Pathog Dis 8:887-900. doi:10.1089/fpd.2010.0787

64. Andrews WH, Jacobson A, Hammack TS. Last updated on July, 2018 Chapter 5: *Salmonella*. *In* (ed), Bacteriological Analytical Manual (BAM) ch., U.S. Food and Drug Administration, Available at https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam-chapter-5-salmonella#Isol [Accessed in September 2019].

65. Love BC, Rostagno MH. 2008. Comparison of five culture methods for *Salmonella* isolation from swine fecal samples of known infection status. J Vet Diagn Invest 20:620-4. doi:10.1177/104063870802000514

66. Manafi M. 2000. New developments in chromogenic and fluorogenic culture media. Int J Food Microbiol 60:205-18. doi:10.1016/s0168-1605(00)00312-3

67. Brichta-Harhay DM, Guerini MN, Arthur TM, Bosilevac JM, Kalchayanand N, Shackelford SD, Wheeler TL, Koohmaraie M. 2008. *Salmonella* and *Escherichia coli* O157:H7 contamination on hides and carcasses of cull cattle presented for slaughter in the United States: An evaluation of prevalence and bacterial loads by immunomagnetic separation and direct plating methods. Appl Environ Microbiol 74:6289-97. doi:10.1128/AEM.00700-08

68. Gragg SE, Loneragan GH, Nightingale KK, Brichta-Harhay DM, Ruiz H, Elder JR, Garcia LG, Miller MF, Echeverry A, Ramirez Porras RG, Brashears MM. 2013. Substantial within-animal diversity of *Salmonella* isolates from lymph nodes, feces, and hides of cattle at slaughter. Appl Environ Microbiol 79:4744-50. doi:10.1128/AEM.01020-13

69. Kunze DJ, Loneragan GH, Platt TM, Miller MF, Besser TE, Koohmaraie M, Stephens T, Brashears MM. 2008. *Salmonella enterica* burden in harvest-ready cattle populations from the Southern High Plains of the United States. Appl Environ Microbiol 74:345-51. doi:10.1128/AEM.02076-07

70. Jeffries L. 1959. Novobiocin-tetrathionate broth: A medium of improved selectivity for the isolation of Salmonellae from faeces. J Clin Pathol 12:568-71. doi:10.1136/jcp.12.6.568

71. Hoben DA, Ashton DH, Peterson AC. 1973. Some observations on the incorporation of novobiocin into Hektoen enteric agar for improved *Salmonella* isolation. Appl Microbiol 26:126-7.

72. Jensen AN, Sorensen G, Baggesen DL, Bodker R, Hoorfar J. 2003. Addition of novobiocin in pre-enrichment step can improve *Salmonella* culture protocol of modified semisolid Rappaport-Vassiliadis. J Microbiol Methods 55:249-55.

73. Pangloli P, Dje Y, Oliver SP, Mathew A, Golden DA, Taylor WJ, Draughon FA. 2003. Evaluation of methods for recovery of *Salmonella* from dairy cattle, poultry, and swine farms. J Food Prot 66:1987-95. doi:10.4315/0362-028x-66.11.1987

74. Gorski L. 2012. Selective enrichment media bias the types of *Salmonella enterica* strains isolated from mixed strain cultures and complex enrichment broths. PLoS One 7:e34722. doi:10.1371/journal.pone.0034722

75. Cox NA, Berrang ME, House SL, Medina D, Cook KL, Shariat NW. 2019. Population analyses reveal preenrichment method and selective enrichment media affect *Salmonella* serovars detected on broiler carcasses. J Food Prot 82:1688-96. doi:10.4315/0362-028X.JFP-19-166

76. Baggesen DL, Nielsen LR, Sorensen G, Bodker R, Ersboll AK. 2007. Growth inhibitory factors in bovine faeces impairs detection of *Salmonella* Dublin by conventional culture procedure. J Appl Microbiol 103:650-6. doi:10.1111/j.1365-2672.2007.03292.x

77. Kang L, Li N, Li P, Zhou Y, Gao S, Gao H, Xin W, Wang J. 2017. MALDI-TOF mass spectrometry provides high accuracy in identification of *Salmonella* at species level but is limited to type or subtype *Salmonella* serovars. Eur J Mass Spectrom (Chichester) 23:70-82. doi:10.1177/1469066717699216

78. Dieckmann R, Malorny B. 2011. Rapid screening of epidemiologically important *Salmonella enterica* subsp. enterica serovars by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 77:4136-46. doi:10.1128/AEM.02418-10

79. Bizzini A, Durussel C, Bille J, Greub G, Prod'hom G. 2010. Performance of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. J Clin Microbiol 48:1549-54. doi:10.1128/JCM.01794-09

80. Bell RL, Jarvis KG, Ottesen AR, McFarland MA, Brown EW. 2016. Recent and emerging innovations in *Salmonella* detection: a food and environmental perspective. Microb Biotechnol 9:279-92. doi:10.1111/1751-7915.12359

81. Popoff MY, Bockemuhl J, Gheesling LL. 2004. Supplement 2002 (no. 46) to the Kauffmann-White scheme. Res Microbiol 155:568-70. doi:10.1016/j.resmic.2004.04.005

82. Grimont P. A. WF-X. 2007. WHO Collaborating Centre for Reference and Research on *Salmonella* :Antigenic Formulae of the *Salmonella* Serovars. Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France. Available at https://www.pasteur.fr/sites/default/files/veng_0.pdf [Accessed in October 2019].

83. Ibrahim GM, Morin PM. 2018. *Salmonella* serotyping using whole genome sequencing. Front Microbiol 9:2993. doi:10.3389/fmicb.2018.02993

84. Yoshida CE, Kruczkiewicz P, Laing CR, Lingohr EJ, Gannon VP, Nash JH, Taboada EN. 2016. The *Salmonella* In Silico Typing Resource (SISTR): An open web-accessible tool for rapidly typing and subtyping draft *Salmonella* genome assemblies. PLoS One 11:e0147101. doi:10.1371/journal.pone.0147101

85. Zhang S, Yin Y, Jones MB, Zhang Z, Deatherage Kaiser BL, Dinsmore BA, Fitzgerald C, Fields PI, Deng X. 2015. *Salmonella* serotype determination utilizing high-throughput genome sequencing data. J Clin Microbiol 53:1685-92. doi:10.1128/JCM.00323-15

86. Deng X, Shariat N, Driebe EM, Roe CC, Tolar B, Trees E, Keim P, Zhang W, Dudley EG, Fields PI, Engelthaler DM. 2015. Comparative analysis of subtyping methods against a whole-genome-sequencing standard for *Salmonella enterica* serotype Enteritidis. J Clin Microbiol 53:212-8. doi:10.1128/JCM.02332-14

87. Yachison CA, Yoshida C, Robertson J, Nash JHE, Kruczkiewicz P, Taboada EN, Walker M, Reimer A, Christianson S, Nichani A, PulseNet Canada Steering C, Nadon C. 2017. The validation and implications of using whole genome sequencing as a replacement for traditional serotyping for a National *Salmonella* Reference Laboratory. Front Microbiol 8:1044. doi:10.3389/fmicb.2017.01044

88. Syal K, Mo M, Yu H, Iriya R, Jing W, Guodong S, Wang S, Grys TE, Haydel SE, Tao N. 2017. Current and emerging techniques for antibiotic susceptibility tests. Theranostics 7:1795-805. doi:10.7150/thno.19217

89. Flentie K, Spears BR, Chen F, Purmort NB, DaPonte K, Viveiros E, Phelan N, Krebill C, Flyer AN, Hooper DC, Smalley DL, Ferraro MJ, Vacic A, Stern E. 2019. Microplate-based surface area assay for rapid phenotypic antibiotic susceptibility testing. Sci Rep 9:237. doi:10.1038/s41598-018-35916-0

90. CLSI. 2017. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Seventh Informational Supplement. CLSI Document M100-S27, Clinical and Laboratory Standards Institute, Wayne, Pa.

91. EUCAST. 2019. European Committee on Antimicrobial Susceptibility Testing breakpoint tables for interpretation of MICs and zone diameters. EUCAST, Available at http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf [Accessed in October 2019].

92. Kahlmeter G, Giske CG, Kirn TJ, Sharp SE. 2019. Point-Counterpoint: Differences between the European Committee on Antimicrobial Susceptibility Testing and Clinical and Laboratory Standards Institute recommendations for reporting antimicrobial susceptibility results. J Clin Microbiol 57. doi:10.1128/JCM.01129-19

93. Anjum MF, Zankari E, Hasman H. 2017. Molecular methods for detection of antimicrobial resistance. Microbiol Spectr 5. doi:10.1128/microbiolspec.ARBA-0011-2017

94. Bergeron MG, Ouellette M. 1998. Preventing antibiotic resistance through rapid genotypic identification of bacteria and of their antibiotic resistance genes in the clinical microbiology laboratory. J Clin Microbiol 36:2169-72.

95. Card R, Zhang J, Das P, Cook C, Woodford N, Anjum MF. 2013. Evaluation of an expanded microarray for detecting antibiotic resistance genes in a broad range of gram-negative bacterial pathogens. Antimicrob Agents Chemother 57:458-65. doi:10.1128/AAC.01223-12

96. McDermott PF, Tyson GH, Kabera C, Chen Y, Li C, Folster JP, Ayers SL, Lam C, Tate HP, Zhao S. 2016. Whole-genome sequencing for detecting antimicrobial resistance in nontyphoidal *Salmonella*. Antimicrob Agents Chemother 60:5515-20. doi:10.1128/AAC.01030-16

97. Su M, Satola SW, Read TD. 2019. Genome-based prediction of bacterial antibiotic resistance. J Clin Microbiol 57. doi:10.1128/JCM.01405-18

98. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640-4. doi:10.1093/jac/dks261

99. McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, Bhullar K, Canova MJ, De Pascale G, Ejim L, Kalan L, King AM, Koteva K, Morar M, Mulvey MR, O'Brien JS, Pawlowski AC, Piddock LJ, Spanogiannopoulos P, Sutherland AD, Tang I, Taylor PL, Thaker M, Wang W, Yan M, Yu T, Wright GD. 2013. The comprehensive antibiotic resistance database. Antimicrob Agents Chemother 57:3348-57. doi:10.1128/AAC.00419-13

100. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, Rolain JM. 2014. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrob Agents Chemother 58:212-20. doi:10.1128/AAC.01310-13

101. Martinez JL, Baquero F. 2000. Mutation frequencies and antibiotic resistance. Antimicrob Agents Chemother 44:1771-7. doi:10.1128/aac.44.7.1771-1777.2000

102. Baym M, Lieberman TD, Kelsic ED, Chait R, Gross R, Yelin I, Kishony R. 2016. Spatiotemporal microbial evolution on antibiotic landscapes. Science 353:1147-51. doi:10.1126/science.aag0822

103. Frost LS, Leplae R, Summers AO, Toussaint A. 2005. Mobile genetic elements: the agents of open source evolution. Nat Rev Microbiol 3:722-32. doi:10.1038/nrmicro1235

104. Miriagou V, Carattoli A, Fanning S. 2006. Antimicrobial resistance islands: Resistance gene clusters in *Salmonella* chromosome and plasmids. Microbes Infect 8:1923-30. doi:10.1016/j.micinf.2005.12.027

105. Stokes HW, Gillings MR. 2011. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into gram-negative pathogens. FEMS Microbiol Rev 35:790-819. doi:10.1111/j.1574-6976.2011.00273.x

106. Siguier P, Gourbeyre E, Chandler M. 2014. Bacterial insertion sequences: Their genomic impact and diversity. FEMS Microbiol Rev 38:865-91. doi:10.1111/1574-6976.12067

107. Neuwirth C, Siebor E, Pechinot A, Duez JM, Pruneaux M, Garel F, Kazmierczak A, Labia R. 2001. Evidence of in vivo transfer of a plasmid encoding the extended-spectrum beta-lactamase TEM-24 and other resistance factors among different members of the family Enterobacteriaceae. J Clin Microbiol 39:1985-8. doi:10.1128/JCM.39.5.1985-1988.2001

108. Cottell JL, Saw HT, Webber MA, Piddock LJ. 2014. Functional genomics to identify the factors contributing to successful persistence and global spread of an antibiotic resistance plasmid. BMC Microbiol 14:168. doi:10.1186/1471-2180-14-168

109. Rychlik I, Gregorova D, Hradecka H. 2006. Distribution and function of plasmids in *Salmonella enterica*. Vet Microbiol 112:1-10. doi:10.1016/j.vetmic.2005.10.030

110. Carattoli A, Zankari E, Garcia-Fernandez A, Voldby Larsen M, Lund O, Villa L, Moller Aarestrup F, Hasman H. 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 58:3895-903. doi:10.1128/AAC.02412-14

111. Zankari E, Hasman H, Kaas RS, Seyfarth AM, Agerso Y, Lund O, Larsen MV, Aarestrup FM. 2013. Genotyping using whole-genome sequencing is a realistic alternative to surveillance based on phenotypic antimicrobial susceptibility testing. J Antimicrob Chemother 68:771-7. doi:10.1093/jac/dks496

112. Nguyen M, Long SW, McDermott PF, Olsen RJ, Olson R, Stevens RL, Tyson GH, Zhao S, Davis JJ. 2019. Using machine learning to predict antimicrobial MICs and associated genomic features for nontyphoidal *Salmonella*. J Clin Microbiol 57. doi:10.1128/JCM.01260-18

113. Fegan N, Vanderlinde P, Higgs G, Desmarchelier P. 2004. Quantification and prevalence of *Salmonella* in beef cattle presenting at slaughter. J Appl Microbiol 97:892-8. doi:10.1111/j.1365-2672.2004.02380.x

114. Kothary MH, Babu US. 2001. Infective dose of foodborne pathogens in volunteers: A review. J Food Saf 21:49-73. doi:DOI 10.1111/j.1745-4565.2001.tb00307.x

115. Malorny B, Lofstrom C, Wagner M, Kramer N, Hoorfar J. 2008. Enumeration of *Salmonella* bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. Appl Environ Microbiol 74:1299-304. doi:10.1128/AEM.02489-07

116. Moo D, O'Boyle D, Mathers W, Frost AJ. 1980. The isolation of *Salmonella* from jejunal and caecal lymph nodes of slaughtered animals. Aust Vet J 56:181-3. doi:10.1111/j.1751-0813.1980.tb05675.x

117. Brichta-Harhay DM, Arthur TM, Bosilevac JM, Guerini MN, Kalchayanand N, Koohmaraie M. 2007. Enumeration of *Salmonella* and *Escherichia coli* O157:H7 in ground beef, cattle carcass, hide and faecal samples using direct plating methods. J Appl Microbiol 103:1657-68. doi:10.1111/j.1365-2672.2007.03405.x

118. Brichta-Harhay DM, Arthur TM, Bosilevac JM, Kalchayanand N, Schmidt JW, Wang R, Shackelford SD, Loneragan GH, Wheeler TL. 2012. Microbiological analysis of bovine lymph nodes for the detection of *Salmonella enterica*. J Food Prot 75:854-8. doi:10.4315/0362-028X.JFP-11-434

119. Gilchrist JE, Campbell JE, Donnelly CB, Peeler JT, Delaney JM. 1973. Spiral plate method for bacterial determination. Appl Microbiol 25:244-52.

120. Edrington TS, Garcia Buitrago JA, Hagevoort GR, Loneragan GH, Bricta-Harhay DM, Callaway TR, Anderson RC, Nisbet DJ. 2018. Effect of waste milk pasteurization on fecal shedding of *Salmonella* in preweaned calves. J Dairy Sci 101:9266-74. doi:10.3168/jds.2018-14668

121. Edrington TS, Loneragan GH, Hill J, Genovese KJ, Brichta-Harhay DM, Farrow RL, Krueger NA, Callaway TR, Anderson RC, Nisbet DJ. 2013. Development of challenge models to evaluate the efficacy of a vaccine to reduce carriage of *Salmonella* in peripheral lymph nodes of cattle. J Food Prot 76:1259-63. doi:10.4315/0362-028X.JFP-12-319

122. Arthur TM, Bosilevac JM, Brichta-Harhay DM, Kalchayanand N, King DA, Shackelford SD, Wheeler TL, Koohmaraie M. 2008. Source tracking of *Escherichia coli* O157:H7 and *Salmonella* contamination in the lairage environment at commercial U.S. beef processing plants and identification of an effective intervention. J Food Prot 71:1752-60. doi:10.4315/0362-028x-71.9.1752

123. Burgess BA, Morley PS. 2019. Risk factors for shedding of *Salmonella enterica* among hospitalized large animals over a 10-year period in a veterinary teaching hospital. J Vet Intern Med doi:10.1111/jvim.15579. doi:10.1111/jvim.15579

124. Fossler CP, Wells SJ, Kaneene JB, Ruegg PL, Warnick LD, Bender JB, Eberly LE, Godden SM, Halbert LW. 2005. Herd-level factors associated with isolation of *Salmonella* in a multi-state study of conventional and organic dairy farms II. *Salmonella* shedding in calves. Prev Vet Med 70:279-91. doi:10.1016/j.prevetmed.2005.04.002

125. Rukambile E, Sintchenko V, Muscatello G, Kock R, Alders R. 2019. Infection, colonization and shedding of *Campylobacter* and *Salmonella* in animals and their contribution to human disease: A review. Zoonoses Public Health 66:562-78. doi:10.1111/zph.12611

126. Hutchison ML, Walters LD, Moore A, Crookes KM, Avery SM. 2004. Effect of length of time before incorporation on survival of pathogenic bacteria present in livestock wastes applied to agricultural soil. Appl Environ Microbiol 70:5111-8. doi:10.1128/AEM.70.9.5111-5118.2004

127. Miller MF, Loneragan GH, Harris DD, Adams KD, Brooks JC, Brashears MM. 2008. Environmental dust exposure as a factor contributing to an increase in *Escherichia coli* O157 and *Salmonella* populations on cattle hides in feedyards. J Food Prot 71:2078-81. doi:10.4315/0362-028x-71.10.2078

128. Bell RG. 1997. Distribution and sources of microbial contamination on beef carcasses. J Appl Microbiol 82:292-300. doi:10.1046/j.1365-2672.1997.00356.x

129. Gill CO, McGinnis JC, Bryant J. 1998. Microbial contamination of meat during the skinning of beef carcass hindquarters at three slaughtering plants. Int J Food Microbiol 42:175-84. doi:10.1016/s0168-1605(98)00074-9

130. Koohmaraie M, Scanga JA, De La Zerda MJ, Koohmaraie B, Tapay L, Beskhlebnaya V, Mai T, Greeson K, Samadpour M. 2012. Tracking the sources of *Salmonella* in ground beef produced from nonfed cattle. J Food Prot 75:1464-8. doi:10.4315/0362-028X.JFP-11-540

131. Li M, Malladi S, Hurd HS, Goldsmith TJ, Brichta-Harhay DM, Loneragan GH. 2015. *Salmonella* spp. in lymph nodes of fed and cull cattle: Relative assessment of risk to ground beef. Food Control 50:423-34. doi:10.1016/j.foodcont.2014.09.011

132. Webb HE, Brichta-Harhay DM, Brashears MM, Nightingale KK, Arthur TM, Bosilevac JM, Kalchayanand N, Schmidt JW, Wang R, Granier SA, Brown TR, Edrington TS, Shackelford SD, Wheeler TL, Loneragan GH. 2017. *Salmonella* in peripheral lymph nodes of healthy cattle at slaughter. Front Microbiol 8:2214. doi:10.3389/fmicb.2017.02214

133. Edrington TS, Loneragan GH, Genovese KJ, Hanson DL, Nisbet DJ. 2016. *Salmonella* persistence within the peripheral lymph nodes of cattle following experimental inoculation. J Food Prot 79:1032-5. doi:10.4315/0362-028X.JFP-15-325

134. Porwollik S, Genovese K, Chu W, Loneragan GH, Edrington T, McClelland M. 2018. Neutral barcoding of genomes reveals the dynamics of *Salmonella* colonization in cattle and their peripheral lymph nodes. Vet Microbiol 220:97-106. doi:10.1016/j.vetmic.2018.05.007

135. Edrington TS, Loneragan GH, Hill J, Genovese KJ, He H, Callaway TR, Anderson RC, Brichta-Harhay DM, Nisbet DJ. 2013. Development of a transdermal *Salmonella* challenge model in calves. J Food Prot 76:1255-8. doi:10.4315/0362-028X.JFP-12-317

136. Vikram A, Rovira P, Agga GE, Arthur TM, Bosilevac JM, Wheeler TL, Morley PS, Belk KE, Schmidt JW. 2017. Impact of "Raised without Antibiotics" beef cattle production practices on occurrences of antimicrobial resistance. Appl Environ Microbiol 83:e01682-17. doi:10.1128/AEM.01682-17

137. USDA-APHIS. 2014. Info sheet- *Salmonella* in U.S. Cattle Feedlots. Available at https://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot2011/Feed11_is_Salm_1.pdf [Accessed in October 2019].

138. USDA-APHIS. 2001. Info sheet- *Salmonella* in United States feedlots. United States Department of Agriculture, Animal and Plant Health Inspection Service, Fort Collins, CO. Available at https://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot99/Feedlot99_is_Salmonella.pdf [Accessed in October 2019].

139. Fluckey WM, Loneragan WG, Warner R, Brashears MM. 2007. Antimicrobial drug resistance of *Salmonella* and *Escherichia coli* isolates from cattle feces, hides, and carcasses. J Food Prot 70:551-6. doi:10.4315/0362-028x-70.3.551

140. USDA-ERS. 2019. Livestock & Meat Domestic Data-Livestock and poultry slaughter. U.S. Department of Agriculture, Economic Research Service, Washington, DC. Available at https://www.ers.usda.gov/webdocs/DataFiles/51875/SlaughterCounts.xlsx?v=3994.2 [Accessed in October 2019].

141. USDA-FSIS. 1996. Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems. Final rule. Federal register. Food Safety and Inspection Service, United States Department of Agriculture Available at https://www.federalregister.gov/documents/1996/07/25/96-17837/pathogen-reduction-hazard-analysis-and-critical-control-point-haccp-systems [Accessed in October 2019].

142. USDA-FSIS. 2016. Serotypes Profile of *Salmonella* Isolates from Meat and Poultry Products January 1998 through December 2013. Food Safety and Inspection Service, United States Department of Agriculture, Washington, D.C. Available at https://www.fsis.usda.gov/wps/wcm/connect/3866026a-582d-4f0e-a8ce-851b39c7390f/Salmonella-Serotype-Annual-2014.pdf?MOD=AJPERES [Accessed in October 2019].

143. USDA-FSIS. 2016. The Nationwide Microbiological Baseline Data Collection Program: Beef-Veal Carcass Survey August 2014 – December 2015. Food Safety and Inspection Service, Science and technology, Microbiology division, U.S. Department of Agriculture, Washinton, D.C. Available at https://www.fsis.usda.gov/wps/wcm/connect/b03963cc-0845-4cfe-b94e-2c955ee5e2ef/Beef-Veal-Carcass-Baseline-Study-Report.pdf?MOD=AJPERES [Accessed in October 2019].

144. Beach JC, Murano EA, Acuff GR. 2002. Prevalence of *Salmonella* and *Campylobacter* in beef cattle from transport to slaughter. J Food Prot 65:1687-93. doi:10.4315/0362-028x-65.11.1687

145. Beach JC, Murano EA, Acuff GR. 2002. Serotyping and antibiotic resistance profiling of *Salmonella* in feedlot and nonfeedlot beef cattle. J Food Prot 65:1694-9. doi:10.4315/0362-028x-65.11.1694

146. Carlson BA, Ruby J, Smith GC, Sofos JN, Bellinger GR, Warren-Serna W, Centrella B, Bowling RA, Belk KE. 2008. Comparison of antimicrobial efficacy of multiple beef hide decontamination strategies to reduce levels of *Escherichia coli* O157:H7 and *Salmonella*. J Food Prot 71:2223-7. doi:10.4315/0362-028x-71.11.2223

147. USDA-ERS. 2019. Livestock & Meat Domestic Data- Red meat and poultry production. U.S. Department of Agriculture, Economic Research Service, Washington, DC. Available at https://www.ers.usda.gov/data-products/livestock-meat-domestic-data/livestock-meat-domestic-data/#Livestock%20and%20poultry%20slaughter [Accessed in October 2019].

148. USDA-FSIS. 2016. Fact sheet "Ground beef and Food Safety". Food Safety and Inspection Service, Science and technology, Microbiology division, U.S. Department of Agriculture, Washinton, D.C. Available at https://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/meat-preparation/ground-beef-and-food-safety/ct_index [Accessed in October 2019].

149. Zhao S, McDermott PF, Friedman S, Abbott J, Ayers S, Glenn A, Hall-Robinson E, Hubert SK, Harbottle H, Walker RD, Chiller TM, White DG. 2006. Antimicrobial resistance and genetic relatedness among *Salmonella* from retail foods of animal origin: NARMS retail meat surveillance. Foodborne Pathog Dis 3:106-17. doi:10.1089/fpd.2006.3.106

150. CDC. 2013. National *Salmonella* Surveillance Annual Report 2013. Centers for Disease Control and Prevention, Atlanta, GA. Available at https://www.cdc.gov/nationalsurveillance/pdfs/salmonella-annual-report-2013-508c.pdf [Accessed in October 2019].

151. CDC. 2016. National Enteric Disease Surveillance: *Salmonella* Annual Report, 2016. Centers for Disease Control and Prevention, Atlanta, GA. Available at

https://www.cdc.gov/nationalsurveillance/pdfs/2016-Salmonella-report-508.pdf [Accessed in October 2019].

152. Marshall KE, Tewell M, Tecle S. 2018. Protracted outbreak of *Salmonella* Newport infections linked to ground beef: Possible role of dairy cows — 21 States, 2016–2017., Rep MMMW, doi:10.15585. Available at https://www.cdc.gov/mmwr/volumes/67/wr/mm6715a2.htm [Accessed in October 2019].

153. McLaughlin JB, Castrodale LJ, Gardner MJ, Ahmed R, Gessner BD. 2006. Outbreak of multidrug-resistant *Salmonella* Typhimurium associated with ground beef served at a school potluck. J Food Prot 69:666-70. doi:10.4315/0362-028x-69.3.666

154. CDC. 2019. Outbreak of *Salmonella* infections linked to ground beef: Final update. Centers for Disease Control and Prevention, Atlanta, GA. Available at https://www.cdc.gov/Salmonella/newport-10-18/index.html [Accessed in October 2019].

155. WHO. 2015. Estimates of the global burden of foodborne diseases: Foodborne Disease Burden Epidemiology Reference Group 2007-2015. World Health Organization, Genava. Available at https://apps.who.int/iris/bitstream/handle/10665/199350/9789241565165_eng.pdf [Accessed in October 2019].

156. WHO. 2019. Investigation of foodborne disease outbreaks. World Health Organization, Genava. Available at https://www.who.int/foodsafety/publications/foodborne_disease/Section_4.pdf [Accessed in October 2019].

157. CDC-FoodNet. 2017. FoodNet 2015 Surveillance Report (final data). Centers for Disease Control and Prevention, Atlanta, GA. Available at https://www.cdc.gov/foodnet/reports/annual-reports-2015.html [Accessed in October 2019].

158. Voetsch AC, Van Gilder TJ, Angulo FJ, Farley MM, Shallow S, Marcus R, Cieslak PR, Deneen VC, Tauxe RV, Emerging Infections Program FoodNet Working G. 2004. FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. Clin Infect Dis 38 Suppl 3:S127-34. doi:10.1086/381578

159. Hoffmann S., Bryan M., B. M. 2015. Economic Burden of Major Foodborne Illnesses Acquired in the United States. ERS, USDA. Available at https://www.ers.usda.gov/webdocs/publications/43984/52807_eib140.pdf?v=0 [Accessed in October 2019].

160. USDA-ERS. 2014. Cost of foodborne illness estimates for *Salmonella* (non-typhoidal). U.S. Department of Agriculture, Economic Research Service, Washington, DC. Available at https://www.ers.usda.gov/data-products/cost-estimates-of-foodborne-illnesses/ [Accessed in October 2019].

161. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM, International Collaboration on Enteric Disease 'Burden of Illness S. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clin Infect Dis 50:882-9. doi:10.1086/650733

162. Gordon MA. 2008. *Salmonella* infections in immunocompromised adults. J Infect 56:413-22. doi:10.1016/j.jinf.2008.03.012

163. Guerrant RL, Van Gilder T, Steiner TS, Thielman NM, Slutsker L, Tauxe RV, Hennessy T, Griffin PM, DuPont H, Sack RB, Tarr P, Neill M, Nachamkin I, Reller LB, Osterholm MT, Bennish ML, Pickering LK, Infectious Diseases Society of A. 2001. Practice guidelines for the management of infectious diarrhea. Clin Infect Dis 32:331-51. doi:10.1086/318514

164. Hohmann EL. 2001. Nontyphoidal salmonellosis. Clin Infect Dis 32:263-9. doi:10.1086/318457

165. Molbak K. 2005. Human health consequences of antimicrobial drug-resistant *Salmonella* and other foodborne pathogens. Clin Infect Dis 41:1613-20. doi:10.1086/497599

166. Underthun K, De J, Gutierrez A, Silverberg R, Schneider KR. 2018. Survival of *Salmonella* and *Escherichia coli* in two different soil types at various moisture levels and temperatures. J Food Prot 81:150-57. doi:10.4315/0362-028X.JFP-17-226

167. Espigares E, Bueno A, Espigares M, Galvez R. 2006. Isolation of *Salmonella* serotypes in wastewater and effluent: Effect of treatment and potential risk. Int J Hyg Environ Health 209:103-7. doi:10.1016/j.ijheh.2005.08.006

168. McEwen S. 2002. Uses of antimicrobials in food animals in Canada: Impact on Resistance and Human Health: Report of the Advisory Committee on Animal Uses of Antimicrobials and Impact on Resistance and Human Health. Health Canada. Available at https://www.canada.ca/en/health-canada/services/drugs-health-products/reports-publications/veterinary-drugs/uses-antimicrobials-food-animals-canada-impact-resistance-human-health-health-canada-2002.html#fig1 [Accessed in October 2019].

169. Linton AH. 1977. Antibiotic resistance: The present situation reviewed. Vet Rec 100:354-60. doi:10.1136/vr.100.17.354

170. Hoelzer K, Moreno Switt AI, Wiedmann M. 2011. Animal contact as a source of human non-typhoidal salmonellosis. Vet Res 42:34. doi:10.1186/1297-9716-42-34

171. McEachran AD, Blackwell BR, Hanson JD, Wooten KJ, Mayer GD, Cox SB, Smith PN. 2015. Antibiotics, bacteria, and antibiotic resistance genes: Aerial transport from cattle feed yards via particulate matter. Environ Health Perspect 123:337-43. doi:10.1289/ehp.1408555

172. Lefebvre SL, Reid-Smith R, Boerlin P, Weese JS. 2008. Evaluation of the risks of shedding Salmonellae and other potential pathogens by therapy dogs fed raw diets in Ontario and Alberta. Zoonoses Public Health 55:470-80. doi:10.1111/j.1863-2378.2008.01145.x

173. Jones JL, Wang L, Ceric O, Nemser SM, Rotstein DS, Jurkovic DA, Rosa Y, Byrum B, Cui J, Zhang Y, Brown CA, Burnum AL, Sanchez S, Reimschuessel R. 2019. Whole genome sequencing confirms source of pathogens associated with bacterial foodborne illness in pets fed raw pet food. J Vet Diagn Invest 31:235-40. doi:10.1177/1040638718823046

174. Li X, Bethune LA, Jia Y, Lovell RA, Proescholdt TA, Benz SA, Schell TC, Kaplan G, McChesney DG. 2012. Surveillance of *Salmonella* prevalence in animal feeds and characterization of the *Salmonella* isolates by serotyping and antimicrobial susceptibility. Foodborne Pathog Dis 9:692-8. doi:10.1089/fpd.2011.1083

175. Guo C, Hoekstra RM, Schroeder CM, Pires SM, Ong KL, Hartnett E, Naugle A, Harman J, Bennett P, Cieslak P, Scallan E, Rose B, Holt KG, Kissler B, Mbandi E, Roodsari R, Angulo FJ, Cole D. 2011. Application of Bayesian techniques to model the burden of human salmonellosis attributable to U.S. food commodities at the point of processing: Adaptation of a Danish model. Foodborne Pathog Dis 8:509-16. doi:10.1089/fpd.2010.0714

176. Parry SM, Palmer SR, Slader J, Humphrey T, South East Wales Infectious Disease Liaison G. 2002. Risk factors for *Salmonella* food poisoning in the domestic kitchen--a case control study. Epidemiol Infect 129:277-85. doi:10.1017/s0950268802007331

177. Vugia DJ, Samuel M, Farley MM, Marcus R, Shiferaw B, Shallow S, Smith K, Angulo FJ, Emerging Infections Program FoodNet Working G. 2004. Invasive *Salmonella* infections in the United States, FoodNet, 1996-1999: Incidence, serotype distribution, and outcome. Clin Infect Dis 38 Suppl 3:S149-56. doi:10.1086/381581

178. Ferri M, Ranucci E, Romagnoli P, Giaccone V. 2017. Antimicrobial resistance: A global emerging threat to public health systems. Crit Rev Food Sci Nutr 57:2857-76. doi:10.1080/10408398.2015.1077192

179. Aslam B, Wang W, Arshad MI, Khurshid M, Muzammil S, Rasool MH, Nisar MA, Alvi RF, Aslam MA, Qamar MU, Salamat MKF, Baloch Z. 2018. Antibiotic resistance: A rundown of a global crisis. Infect Drug Resist 11:1645-58. doi:10.2147/IDR.S173867

180. DuPont HL. 2016. Persistent Diarrhea: A Clinical Review. JAMA 315:2712-23. doi:10.1001/jama.2016.7833

181. Crump JA, Medalla FM, Joyce KW, Krueger AL, Hoekstra RM, Whichard JM, Barzilay EJ, Emerging Infections Program NWG. 2011. Antimicrobial resistance among invasive nontyphoidal *Salmonella enterica* isolates in the United States: National Antimicrobial Resistance Monitoring System, 1996 to 2007. Antimicrob Agents Chemother 55:1148-54. doi:10.1128/AAC.01333-10

182. Sjolund-Karlsson M, Joyce K, Blickenstaff K, Ball T, Haro J, Medalla FM, Fedorka-Cray P, Zhao S, Crump JA, Whichard JM. 2011. Antimicrobial susceptibility to azithromycin among *Salmonella enterica* isolates from the United States. Antimicrob Agents Chemother 55:3985-9. doi:10.1128/AAC.00590-11

183. CDC-NARMS. 2016. Human Isolates Surveillance Report for 2014 National Antimicrobial Resistance Monitoring System, Centers for Disease Control and Prevention, Atlanta, GA. Available at https://www.cdc.gov/narms/reports/annual-human-isolates-report-2015.html [Accessed in October 2019].

184. Magnet S, Courvalin P, Lambert T. 1999. Activation of the cryptic aac(6')-Iy aminoglycoside resistance gene of *Salmonella* by a chromosomal deletion generating a transcriptional fusion. J Bacteriol 181:6650-5.

185. Crump JA, Barrett TJ, Nelson JT, Angulo FJ. 2003. Reevaluating fluoroquinolone breakpoints for *Salmonella enterica* serotype Typhi and for non-Typhi salmonellae. Clin Infect Dis 37:75-81. doi:10.1086/375602

186. Ostrer L, Khodursky RF, Johnson JR, Hiasa H, Khodursky A. 2019. Analysis of mutational patterns in quinolone resistance-determining regions of *gyr*A and *par*C of clinical isolates. Int J Antimicrob Agents 53:318-24. doi:10.1016/j.ijantimicag.2018.12.004

187. Karp BE, Campbell D, Chen JC, Folster JP, Friedman CR. 2018. Plasmid-mediated quinolone resistance in human non-typhoidal *Salmonella* infections: An emerging public health problem in the United States. Zoonoses Public Health 65:838-49. doi:10.1111/zph.12507

188. Lin D, Chen K, Wai-Chi Chan E, Chen S. 2015. Increasing prevalence of ciprofloxacin-resistant food-borne *Salmonella* strains harboring multiple PMQR elements but not target gene mutations. Sci Rep 5:14754. doi:10.1038/srep14754

189. CLSI. 2011. Performance standards for antimicrobial susceptibility testing: Twenty first Informational supplement. CLSI document M100-S21, Clinical and Laboratory Standards Institute, Wayne, PA

190. Folster JP, Grass JE, Bicknese A, Taylor J, Friedman CR, Whichard JM. 2017. Characterization of resistance genes and plasmids from outbreaks and illness clusters caused by *Salmonella* resistant to ceftriaxone in the United States, 2011-2012. Microb Drug Resist 23:188-93. doi:10.1089/mdr.2016.0080

191. Folster JP, Pecic G, Singh A, Duval B, Rickert R, Ayers S, Abbott J, McGlinchey B, Bauer-Turpin J, Haro J, Hise K, Zhao S, Fedorka-Cray PJ, Whichard J, McDermott PF. 2012. Characterization of extended-spectrum cephalosporin-resistant *Salmonella enterica* serovar Heidelberg isolated from food animals, retail meat, and humans in the United States 2009. Foodborne Pathog Dis 9:638-45. doi:10.1089/fpd.2012.1130

192. McDermott PF, Zhao S, Tate H. 2018. Antimicrobial Resistance in Nontyphoidal *Salmonella*. Microbiol Spectr 6. doi:10.1128/microbiolspec.ARBA-0014-2017

193. Greene SK, Stuart AM, Medalla FM, Whichard JM, Hoekstra RM, Chiller TM. 2008. Distribution of multidrug-resistant human isolates of MDR-ACSSuT *Salmonella* Typhimurium and MDR-AmpC *Salmonella* Newport in the United States, 2003-2005. Foodborne Pathog Dis 5:669-80. doi:10.1089/fpd.2008.0111

194. CDC-NARMS. 2013. Human Isolates Final Report for 2011. National Antimicrobial Resistance Monitoring System, Atlanta, Georgia: U.S. Department of Health and Human Services. Available at https://www.cdc.gov/narms/pdf/2011-annual-report-narms-508c.pdf [Accessed in October 2019].

195. Guerra B, Soto S, Cal S, Mendoza MC. 2000. Antimicrobial resistance and spread of class 1 integrons among *Salmonella* serotypes. Antimicrob Agents Chemother 44:2166-9. doi:10.1128/aac.44.8.2166-2169.2000

196. Helms M, Vastrup P, Gerner-Smidt P, Molbak K. 2002. Excess mortality associated with antimicrobial drug-resistant *Salmonella* Typhimurium. Emerg Infect Dis 8:490-5. doi:10.3201/eid0805.010267

197. Varma JK, Greene KD, Ovitt J, Barrett TJ, Medalla F, Angulo FJ. 2005. Hospitalization and antimicrobial resistance in *Salmonella* outbreaks, 1984-2002. Emerg Infect Dis 11:943-6. doi:10.3201/eid1106.041231

198. Molbak K. 2004. Spread of resistant bacteria and resistance genes from animals to humans--the public health consequences. J Vet Med B Infect Dis Vet Public Health 51:364-9. doi:10.1111/j.1439-0450.2004.00788.x

199. Angulo FJ, Johnson KR, Tauxe RV, Cohen ML. 2000. Origins and consequences of antimicrobial-resistant nontyphoidal *Salmonella*: implications for the use of fluoroquinolones in food animals. Microb Drug Resist 6:77-83. doi:10.1089/mdr.2000.6.77

200. Angulo FJ, Nargund VN, Chiller TC. 2004. Evidence of an association between use of anti-microbial agents in food animals and anti-microbial resistance among bacteria isolated from humans and the human health consequences of such resistance. J Vet Med B Infect Dis Vet Public Health 51:374-9. doi:10.1111/j.1439-0450.2004.00789.x

201. Anderson AD, Nelson JM, Rossiter S, Angulo FJ. 2003. Public health consequences of use of antimicrobial agents in food animals in the United States. Microb Drug Resist 9:373-9. doi:10.1089/107662903322762815

202. Gupta A, Fontana J, Crowe C, Bolstorff B, Stout A, Van Duyne S, Hoekstra MP, Whichard JM, Barrett TJ, Angulo FJ, National Antimicrobial Resistance Monitoring System PulseNet Working G. 2003. Emergence of multidrug-resistant *Salmonella enterica* serotype Newport infections resistant to expanded-spectrum cephalosporins in the United States. J Infect Dis 188:1707-16. doi:10.1086/379668

203. Holmberg SD, Osterholm MT, Senger KA, Cohen ML. 1984. Drug-resistant *Salmonella* from animals fed antimicrobials. N Engl J Med 311:617-22. doi:10.1056/NEJM198409063111001

204. Dutil L, Irwin R, Finley R, Ng LK, Avery B, Boerlin P, Bourgault AM, Cole L, Daignault D, Desruisseau A, Demczuk W, Hoang L, Horsman GB, Ismail J, Jamieson F, Maki A, Pacagnella A, Pillai DR. 2010. Ceftiofur resistance in *Salmonella enterica* serovar Heidelberg from chicken meat and humans, Canada. Emerg Infect Dis 16:48-54. doi:10.3201/eid1601.090729

205. Marshall BM, Levy SB. 2011. Food animals and antimicrobials: Impacts on human health. Clin Microbiol Rev 24:718-33. doi:10.1128/CMR.00002-11

206. Fey PD, Safranek TJ, Rupp ME, Dunne EF, Ribot E, Iwen PC, Bradford PA, Angulo FJ, Hinrichs SH. 2000. Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle. N Engl J Med 342:1242-9. doi:10.1056/NEJM200004273421703

207. Pires SM, Duarte AS, Hald T. 2018. Source attribution and risk assessment of antimicrobial resistance. Microbiol Spectr 6. doi:10.1128/microbiolspec.ARBA-0027-2017

208. Berge AC, Dueger EL, Sischo WM. 2006. Comparison of *Salmonella enterica* serovar distribution and antibiotic resistance patterns in wastewater at municipal water treatment plants in two California cities. J Appl Microbiol 101:1309-16. doi:10.1111/j.1365-2672.2006.03031.x

209. Ghosh S, LaPara TM. 2007. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J 1:191-203. doi:10.1038/ismej.2007.31

210. van den Bogaard AE, Stobberingh EE. 1999. Contamination of animal feed by multiresistant enterococci. Lancet 354:163-4. doi:10.1016/S0140-6736(05)75297-1

211. White DG, Datta A, McDermott P, Friedman S, Qaiyumi S, Ayers S, English L, McDermott S, Wagner DD, Zhao S. 2003. Antimicrobial susceptibility and genetic relatedness of *Salmonella* serovars isolated from animal-derived dog treats in the USA. J Antimicrob Chemother 52:860-3. doi:10.1093/jac/dkg441

212. Grasselli E, Francois P, Gutacker M, Gettler B, Benagli C, Convert M, Boerlin P, Schrenzel J, Piffaretti JC. 2008. Evidence of horizontal gene transfer between human and animal commensal *Escherichia coli* strains identified by microarray. FEMS Immunol Med Microbiol 53:351-8. doi:10.1111/j.1574-695X.2008.00434.x

213. Stecher B, Denzler R, Maier L, Bernet F, Sanders MJ, Pickard DJ, Barthel M, Westendorf AM, Krogfelt KA, Walker AW, Ackermann M, Dobrindt U, Thomson NR, Hardt WD. 2012. Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. Proc Natl Acad Sci U S A 109:1269-74. doi:10.1073/pnas.1113246109

214. Winokur PL, Vonstein DL, Hoffman LJ, Uhlenhopp EK, Doern GV. 2001. Evidence for transfer of CMY-2 AmpC beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrob Agents Chemother 45:2716-22. doi:10.1128/AAC.45.10.2716-2722.2001

215. Ryan CA, Nickels MK, Hargrett-Bean NT, Potter ME, Endo T, Mayer L, Langkop CW, Gibson C, McDonald RC, Kenney RT, et al. 1987. Massive outbreak of antimicrobial-resistant salmonellosis traced to pasteurized milk. JAMA 258:3269-74.

216. Levy SB, FitzGerald GB, Macone AB. 1976. Spread of antibiotic-resistant plasmids from chicken to chicken and from chicken to man. Nature 260:40-2. doi:10.1038/260040a0

217. Committee S. 1969. Report of the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine. Her Majesty's Stationary Office, London, UK. Available at https://api.parliament.uk/historic-hansard/commons/1969/nov/20/use-of-antibiotics-in-animal-husbandry [Accessed in October 2019].

218. FDA. 2012. New Animal Drugs; Cephalosporin Drugs; Extralabel Animal Drug Use; Order of Prohibition. Final rule. Federal register. United States Food and Drug Administration, Available at https://www.federalregister.gov/documents/2012/01/06/2012-35/new-animal-

drugs-cephalosporin-drugs-extralabel-animal-drug-use-order-of-prohibition [Accessed in October, 2019].

219. FDA. 2013. #209 Guidance for Industry, The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals. Available at http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM216936.pdf. [Accessed in October 2019].

220. FDA. 2013. FDA. Guidance For Industry #213: New animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food producing animals: Recommendations for drug sponsors for voluntarily aligning product use conditions with GFI #209. Services CfVMFaDAFUSDoHaH, Available at https://www.federalregister.gov/documents/2013/12/12/2013-29697/guidance-for-industry-on-new-animal-drugs-and-new-animal-drug-combination-products-administered-in [Accessed in October 2019].

221. Klare I, Badstubner D, Konstabel C, Bohme G, Claus H, Witte W. 1999. Decreased incidence of VanA-type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. Microb Drug Resist 5:45-52. doi:10.1089/mdr.1999.5.45

222. Agerso Y, Aarestrup FM. 2013. Voluntary ban on cephalosporin use in Danish pig production has effectively reduced extended-spectrum cephalosporinase-producing *Escherichia coli* in slaughter pigs. J Antimicrob Chemother 68:569-72. doi:10.1093/jac/dks427

223. FDA. 2017. 2016 Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals. U.S. Food and Drug Administration, Silver Spring. MD. Available at https://www.fda.gov/downloads/forindustry/userfees/animaldruguserfeeactadufa/ucm588085.pdf [Accessed in October 2019].

224. FDA. 2018. 2017 Summary Report On Antimicrobials Sold or Distributed for Use in Food-Producing Animals. U.S. Food and Drug Administration, Silver Spring. MD. Available at https://www.fda.gov/media/119332/download [Accessed in October 2019].

225. FDA. 2018. Antimicrobial Animal Drug Sales and Distribution Reporting; Small Entity Compliance Guide; Availability. Notification of availability. Federal Register. United States Food and Drug Administration, Available at

https://www.federalregister.gov/documents/2018/06/29/2018-14085/antimicrobial-animal-drug-sales-and-distribution-reporting-small-entity-compliance-guide [Accessed in October 2019].

226. Grissett GP, White BJ, Larson RL. 2015. Structured literature review of responses of cattle to viral and bacterial pathogens causing bovine respiratory disease complex. J Vet Intern Med 29:770-80. doi:10.1111/jvim.12597

227. Loneragan GH, Gould DH, Mason GL, Garry FB, Yost GS, Miles DG, Hoffman BW, Mills LJ. 2001. Involvement of microbial respiratory pathogens in acute interstitial pneumonia in feedlot cattle. Am J Vet Res 62:1519-24. doi:10.2460/ajvr.2001.62.1519

228. Urban-Chmiel R, Grooms DL. 2012. Prevention and control of bovine respiratory disease. Journal of Livestock Science 3:27-36.

229. Buchanan JW, MacNeil MD, Raymond RC, McClain AR, Van Eenennaam AL. 2016. Rapid Communication: Variance component estimates for Charolais-sired fed cattle and relative economic impact of bovine respiratory disease. J Anim Sci 94:5456-60. doi:10.2527/jas.2016-1001

230. Snyder E, Credille B, Berghaus R, Giguere S. 2017. Prevalence of multi drug antimicrobial resistance in isolated from high-risk stocker cattle at arrival and two weeks after processing. J Anim Sci 95:1124-31. doi:10.2527/jas.2016.1110

231. FDA. 2005. Freedom of Information Summary for DRAXXIN® sterile solution (Tulathromycin), New Animal Drug Application (NADA) No. 141–244., Available at https://animaldrugsatfda.fda.gov/adafda/app/search/public/document/downloadFoi/789 [Accessed in October 2019].

232. FDA. 2003. Freedom of Information Summary for EXCEDE® (previosly known as NAXCEL XT®) (Ceftiofur Crystalline Free Acid Sterile Suspension), New Animal Drug Application (NADA) No.141-209.

233. Kim S, Chen J, Cheng T, Gindulyte A, He J, He S, Li Q, Shoemaker BA, Thiessen PA, Yu B, Zaslavsky L, Zhang J, Bolton EE. 2019. PubChem 2019 update: Improved access to chemical data. Nucleic Acids Res 47:D1102-D09. doi:10.1093/nar/gky1033

234. Wagner RD, Johnson SJ, Cerniglia CE, Erickson BD. 2011. Bovine intestinal bacteria inactivate and degrade ceftiofur and ceftriaxone with multiple beta-lactamases. Antimicrob Agents Chemother 55:4990-8. doi:10.1128/AAC.00008-11

235. Gomes C, Martinez-Puchol S, Palma N, Horna G, Ruiz-Roldan L, Pons MJ, Ruiz J. 2017. Macrolide resistance mechanisms in Enterobacteriaceae: Focus on azithromycin. Crit Rev Microbiol 43:1-30. doi:10.3109/1040841X.2015.1136261

236. Levent G, Schlochtermeier A, Ives SE, Norman KN, Lawhon SD, Loneragan GH, Anderson RC, Vinasco J, Scott HM. 2019. Population dynamics of *Salmonella enterica* following single dose metaphylactic antibiotic use within cohorts of beef cattle followed to slaughter. Appl Environ Microbiol doi:10.1128/AEM.01386-19.

237. Loneragan GH, Thomson DU, McCarthy RM, Webb HE, Daniels AE, Edrington TS, Nisbet DJ, Trojan SJ, Rankin SC, Brashears MM. 2012. *Salmonella* diversity and burden in cows on and culled from dairy farms in the Texas High Plains. Foodborne Pathog Dis 9:549-55. doi:10.1089/fpd.2011.1069

238. Clopper CJ, Pearson ES. 1934. The use of confidence or fiducial limits illustrated in the case of the binomial. Biometrika 26:404-13. doi:Doi 10.2307/2331986

239. Bonferroni CE. 1936. Teoria statistica delle classi e calcolo delle probabilità. Libreria internazionale Seeber, Pubblicazioni del R Istituto Superiore di Scienze Economiche e Commerciali di Firenze

240. Cock PJ, Fields CJ, Goto N, Heuer ML, Rice PM. 2010. The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Res 38:1767-71. doi:10.1093/nar/gkp1137

241. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30:2114-20. doi:10.1093/bioinformatics/btu170

242. Andrews S. 2010. FastQC: A quality control tool for high throughput sequence data. Available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc [Accessed in October 2019].

243. Ewels P, Magnusson M, Lundin S, Kaller M. 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32:3047-8. doi:10.1093/bioinformatics/btw354

244. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19:455-77. doi:10.1089/cmb.2012.0021

245. Gurevich A, Saveliev V, Vyahhi N, Tesler G. 2013. QUAST: Quality assessment tool for genome assemblies. Bioinformatics 29:1072-5. doi:10.1093/bioinformatics/btt086

246. Inouye M, Dashnow H, Raven LA, Schultz MB, Pope BJ, Tomita T, Zobel J, Holt KE. 2014. SRST2: Rapid genomic surveillance for public health and hospital microbiology labs. Genome Med 6:90. doi:10.1186/s13073-014-0090-6

247. Brettin T, Davis JJ, Disz T, Edwards RA, Gerdes S, Olsen GJ, Olson R, Overbeek R, Parrello B, Pusch GD, Shukla M, Thomason JA, 3rd, Stevens R, Vonstein V, Wattam AR, Xia F. 2015. RASTtk: A modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Sci Rep 5:8365. doi:10.1038/srep08365

248. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403-10. doi:10.1016/S0022-2836(05)80360-2

249. Ondov BD, Treangen TJ, Melsted P, Mallonee AB, Bergman NH, Koren S, Phillippy AM. 2016. Mash: Fast genome and metagenome distance estimation using MinHash. Genome Biol 17:132. doi:10.1186/s13059-016-0997-x

250. Wattam AR, Davis JJ, Assaf R, Boisvert S, Brettin T, Bun C, Conrad N, Dietrich EM, Disz T, Gabbard JL, Gerdes S, Henry CS, Kenyon RW, Machi D, Mao C, Nordberg EK, Olsen GJ, Murphy-Olson DE, Olson R, Overbeek R, Parrello B, Pusch GD, Shukla M, Vonstein V, Warren A, Xia F, Yoo H, Stevens RL. 2017. Improvements to PATRIC, the all-bacterial Bioinformatics Database and Analysis Resource Center. Nucleic Acids Res 45:D535-D42. doi:10.1093/nar/gkw1017

251. Arndt D, Marcu A, Liang Y, Wishart DS. 2017. PHAST, PHASTER and PHASTEST: Tools for finding prophage in bacterial genomes. Brief Bioinform doi:10.1093/bib/bbx121. doi:10.1093/bib/bbx121

252. Quinlan AR, Hall IM. 2010. BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics 26:841-2. doi:10.1093/bioinformatics/btq033

253. Treangen TJ, Ondov BD, Koren S, Phillippy AM. 2014. The Harvest Suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol 15:524. doi:10.1186/s13059-014-0524-x

254. Darriba D, Posada D, Kozlov AM, Stamatakis A, Morel B, Flouri T. 2019. ModelTest-NG: A new and scalable tool for the selection of DNA and protein evolutionary models. Mol Biol Evol doi:10.1093/molbev/msz189:612903. doi:10.1093/molbev/msz189

255. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. 2015. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol 32:268-74. doi:10.1093/molbev/msu300

256. Letunic I, Bork P. 2016. Interactive tree of life (iTOL) v3: An online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res 44:W242-5. doi:10.1093/nar/gkw290

257. Turner I, Garimella KV, Iqbal Z, McVean G. 2018. Integrating long-range connectivity information into de Bruijn graphs. Bioinformatics 34:2556-65. doi:10.1093/bioinformatics/bty157

258. Li H, Durbin R. 2010. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26:589-95. doi:10.1093/bioinformatics/btp698

259. Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, Handsaker RE, Lunter G, Marth GT, Sherry ST, McVean G, Durbin R, Genomes Project Analysis G. 2011. The variant call format and VCFtools. Bioinformatics 27:2156-8. doi:10.1093/bioinformatics/btr330

260. Jukes TH, Cantor CR. 1969. Evolution of protein molecules: Mammalian protein metabolism, p 21–132. *In* Munro HM (ed), ch. Academic Press, New York.

261. Felsenstein J. 1981. Evolutionary trees from DNA sequences: A maximum likelihood approach. J Mol Evol 17:368-76. doi:10.1007/bf01734359

262. Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10:512-26. doi:10.1093/oxfordjournals.molbev.a040023

263. Hasegawa M, Kishino H, Yano T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 22:160-74. doi:10.1007/bf02101694

264. Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111-20. doi:10.1007/bf01731581

265. Kimura M. 1981. Estimation of evolutionary distances between homologous nucleotide sequences. Proc Natl Acad Sci U S A 78:454-8. doi:10.1073/pnas.78.1.454

266. Posada D. 2003. Using MODELTEST and PAUP* to select a model of nucleotide substitution. Curr Protoc Bioinformatics Chapter 6:Unit 6 5. doi:10.1002/0471250953.bi0605s00

267. Tavaré S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences, in Some Mathematical Questions in Biology - DNA Sequence Analysis, p 57-86. *In* Miura RM (ed), ch. Amer Math Soc.,Providence, RI, IT.

268. Zharkikh A. 1994. Estimation of evolutionary distances between nucleotide sequences. J Mol Evol 39:315-29. doi:10.1007/bf00160155

269. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. 2017. ModelFinder: Fast model selection for accurate phylogenetic estimates. Nat Methods 14:587-89. doi:10.1038/nmeth.4285

270. Bugarel M, Cook PW, den Bakker HC, Harhay D, Nightingale KK, Loneragan GH. 2019. Complete genome sequences of four *Salmonella enterica* strains (including those of serotypes Montevideo, Mbandaka, and Lubbock) isolated from peripheral lymph nodes of healthy cattle. Microbiol Resour Announc 8:e01450-18. doi:10.1128/MRA.01450-18

271. Dohoo I, Martin S, Stryhn H. 2014. Veterinary Epidemiologic Research (2nd edition). VER Inc., Charlottetown, Prince Edward Island, Canada (2014)

272. Silvestri EE, Yund C, Taft S, Bowling CY, Chappie D, Garrahan K, Brady-Roberts E, Stone H, Nichols TL. 2017. Considerations for estimating microbial environmental data concentrations collected from a field setting. J Expo Sci Environ Epidemiol 27:141-51. doi:10.1038/jes.2016.3

273. EPA. 1992. Guidelines for exposure assessment. U.S. Environmental Protection Agency, Washington, D.C. Available at https://www.epa.gov/sites/production/files/2014-11/documents/guidelines_exp_assessment.pdf [Accessed in October 2019].

274. Levine AD, Harwood VJ, GA F. 2009. Collecting, exploring, and interpreting microbiological data associated with reclaimed water systems. Water Reuse Foundation, Alexandria, VA, USA. Available at https://www.waterboards.ca.gov/water_issues/programs/grants_loans/water_recycling/research/microbiological_data.pdf [Accessed in October 2019].

275. EPA. 2006. Data quality assessment: Statistical methods for practitioners. U.S. Environmental Protection Agency, Washington, D.C. Available at https://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=900B0D00.TXT [Accessed in October 2019].

276. Royston P. 2007. Multiple imputation of missing values: Further update of ice, with an emphasis on interval censoring. Stata Journal 7:445-64. doi:Doi 10.1177/1536867x0700700401

277. Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM. 2009. Zero-truncated and zero-inflated models for count data, p 261-93, Mixed effects models and extensions in ecology with R, ch., doi:10.1007/978-0-387-87458-6_11. Springer New York, New York, NY. doi:10.1007/978-0-387-87458-6_11.

278. Helsel DR. 2005. More than obvious: Better methods for interpreting nondetect data. Environ Sci Technol 39:419A-23A. doi:10.1021/es053368a

279. Xu L, Paterson AD, Turpin W, Xu W. 2015. Assessment and Selection of Competing Models for Zero-Inflated Microbiome Data. PLoS One 10:e0129606. doi:10.1371/journal.pone.0129606

280. Loeys T, Moerkerke B, De Smet O, Buysse A. 2012. The analysis of zero-inflated count data: Beyond zero-inflated Poisson regression. Br J Math Stat Psychol 65:163-80. doi:10.1111/j.2044-8317.2011.02031.x

281. Kassahun W, Neyens T, Molenberghs G, Faes C, Verbeke G. 2014. Marginalized multilevel hurdle and zero-inflated models for overdispersed and correlated count data with excess zeros. Stat Med 33:4402-19. doi:10.1002/sim.6237

282. Hu MC, Pavlicova M, Nunes EV. 2011. Zero-inflated and hurdle models of count data with extra zeros: Examples from an HIV-risk reduction intervention trial. Am J Drug Alcohol Abuse 37:367-75. doi:10.3109/00952990.2011.597280

283. Cragg JG. 1971. Some statistical models for limited dependent variables with application to the demand for durable goods. Econometrica (pre-1986) 39:829.

284. Yang DA, Laven RA, Heuer C, Vink WD, Chesterton RN. 2018. Farm level risk factors for bovine digital dermatitis in Taranaki, New Zealand: An analysis using a Bayesian hurdle model. Vet J 234:91-95. doi:10.1016/j.tvjl.2018.02.012

285. Brown TR, Edrington TS, Genovese KJ, Loneragan GH, Hanson DL, Nisbet DJ. 2015. Oral *Salmonella* challenge and subsequent uptake by the peripheral lymph nodes in calves. J Food Prot 78:573-8. doi:10.4315/0362-028X.JFP-14-416

286. Brown TR, Edrington TS, Loneragan GH, Hanson DL, Malin K, Ison JJ, Nisbet DJ. 2015. Investigation into possible differences in *Salmonella* prevalence in the peripheral lymph nodes of cattle derived from distinct production systems and of different breed types. J Food Prot 78:2081-4. doi:10.4315/0362-028X.JFP-15-198

287. Stephens TP, Loneragan GH, Thompson TW, Sridhara A, Branham LA, Pitchiah S, Brashears MM. 2007. Distribution of *Escherichia coli* 0157 and *Salmonella* on hide surfaces, the oral cavity, and in feces of feedlot cattle. J Food Prot 70:1346-9. doi:10.4315/0362-028x-70.6.1346

288. Schafer JL, Graham JW. 2002. Missing data: Our view of the state of the art. Psychol Methods 7:147-77.

289. Burton A, Billingham LJ, Bryan S. 2007. Cost-effectiveness in clinical trials: using multiple imputation to deal with incomplete cost data. Clin Trials 4:154-61. doi:10.1177/1740774507076914

290. Edrington TS, Hume ME, Looper ML, Schultz CL, Fitzgerald AC, Callaway TR, Genovese KJ, Bischoff KM, McReynolds JL, Anderson RC, Nisbet DJ. 2004. Variation in the faecal shedding of *Salmonella* and *E. coli* O157:H7 in lactating dairy cattle and examination of *Salmonella* genotypes using pulsed-field gel

electrophoresis. Lett Appl Microbiol 38:366-72. doi:10.1111/j.1472-765X.2004.01495.x

291. Cummings KJ, Warnick LD, Alexander KA, Cripps CJ, Grohn YT, James KL, McDonough PL, Reed KE. 2009. The duration of fecal *Salmonella* shedding following clinical disease among dairy cattle in the northeastern USA. Prev Vet Med 92:134-9. doi:10.1016/j.prevetmed.2009.07.002

292. Cull CA, Renter DG, Dewsbury DM, Noll LW, Shridhar PB, Ives SE, Nagaraja TG, Cernicchiaro N. 2017. Feedlot- and pen-level prevalence of enterohemorrhagic *Escherichia coli* in feces of commercial feedlot cattle in two major U.S. cattle feeding areas. Foodborne Pathog Dis 14:309-17. doi:10.1089/fpd.2016.2227

293. Carattoli A. 2009. Resistance plasmid families in Enterobacteriaceae. Antimicrob Agents Chemother 53:2227-38. doi:10.1128/AAC.01707-08

294. Cottell JL, Kanwar N, Castillo-Courtade L, Chalmers G, Scott HM, Norby B, Loneragan GH, Boerlin P. 2013. blaCTX-M-32 on an IncN plasmid in *Escherichia coli* from beef cattle in the United States. Antimicrob Agents Chemother 57:1096-7. doi:10.1128/AAC.01750-12

295. Garcia-Fernandez A, Villa L, Moodley A, Hasman H, Miriagou V, Guardabassi L, Carattoli A. 2011. Multilocus sequence typing of IncN plasmids. J Antimicrob Chemother 66:1987-91. doi:10.1093/jac/dkr225

296. Kahan FM, Kahan JS, Cassidy PJ, Kropp H. 1974. The mechanism of action of fosfomycin (phosphonomycin). Ann N Y Acad Sci 235:364-86. doi:10.1111/j.1749-6632.1974.tb43277.x

297. Arca P, Rico M, Brana AF, Villar CJ, Hardisson C, Suarez JE. 1988. Formation of an adduct between fosfomycin and glutathione: a new mechanism of antibiotic resistance in bacteria. Antimicrob Agents Chemother 32:1552-6. doi:10.1128/aac.32.10.1552

298. Silver LL. 2017. Fosfomycin: Mechanism and Resistance. Cold Spring Harb Perspect Med 7. doi:10.1101/cshperspect.a025262

299. Guo Q, Tomich AD, McElheny CL, Cooper VS, Stoesser N, Wang M, Sluis-Cremer N, Doi Y. 2016. Glutathione-S-transferase FosA6 of *Klebsiella*

pneumoniae origin conferring fosfomycin resistance in ESBL-producing *Escherichia coli*. J Antimicrob Chemother 71:2460-5. doi:10.1093/jac/dkw177

300. Rehman MA, Yin X, Persaud-Lachhman MG, Diarra MS. 2017. First detection of a fosfomycin resistance gene, *fosA*7, in *Salmonella enterica* serovar Heidelberg isolated from broiler chickens. Antimicrob Agents Chemother 61. doi:10.1128/AAC.00410-17

301. Tyson GH, Li C, Ayers S, McDermott PF, Zhao S. 2016. Using whole-genome sequencing to determine appropriate streptomycin epidemiological cutoffs for *Salmonella* and *Escherichia coli*. FEMS Microbiol Lett 363. doi:10.1093/femsle/fnw009

302. Kaldhone PR, Carlton A, Aljahdali N, Khajanchi BK, Sanad YM, Han J, Deck J, Ricke SC, Foley SL. 2019. Evaluation of incompatibility group I1 (IncI1) plasmid-containing *Salmonella enterica* and assessment of the plasmids in bacteriocin production and biofilm development. Front Vet Sci 6:298. doi:10.3389/fvets.2019.00298

303. Salipante SJ, Hall BG. 2003. Determining the limits of the evolutionary potential of an antibiotic resistance gene. Mol Biol Evol 20:653-9. doi:10.1093/molbev/msg074

304. Leon IM, Lawhon SD, Norman KN, Threadgill DS, Ohta N, Vinasco J, Scott HM. 2018. Serotype diversity and antimicrobial resistance among *Salmonella enterica* isolates from patients at an equine referral hospital. Appl Environ Microbiol 84. doi:10.1128/AEM.02829-17

305. Tsang J. 2017. Bacterial plasmid addiction systems and their implications for antibiotic drug development. Postdoc journal : a journal of postdoctoral research and postdoctoral affairs 5:3-9.

306. Smith H, Bossers A, Harders F, Wu G, Woodford N, Schwarz S, Guerra B, Rodriguez I, van Essen-Zandbergen A, Brouwer M, Mevius D. 2015. Characterization of epidemic IncI1-Igamma plasmids harboring ambler class A and C genes in *Escherichia coli* and *Salmonella enterica* from animals and humans. Antimicrob Agents Chemother 59:5357-65. doi:10.1128/AAC.05006-14

307. Bugarel M, den Bakker HC, Nightingale KK, Brichta-Harhay DM, Edrington TS, Loneragan GH. 2015. Two draft genome sequences of a new serovar of *Salmonella*

enterica, serovar Lubbock. Genome Announc 3:e00215-15. doi:10.1128/genomeA.00215-15

308. Purdy CW, Straus DC, Clark RN. 2004. Diversity of *Salmonella* serovars in feedyard and nonfeedyard playas of the Southern High Plains in the summer and winter. Am J Vet Res 65:40-4. doi:10.2460/ajvr.2004.65.40

309. Purdy CW, Straus DC, Parker DB, Williams BP, Clark RN. 2001. Water quality in cattle feedyard playas in winter and summer. Am J Vet Res 62:1402-7. doi:10.2460/ajvr.2001.62.1402

310. Loneragan GH, Brashears MM. 2005. Effects of using retention-pond water for dust abatement on performance of feedlot steers and carriage of *Escherichia coli* O157 and *Salmonella* spp. J Am Vet Med Assoc 226:1378-83. doi:10.2460/javma.2005.226.1378

311. Switt AI, den Bakker HC, Vongkamjan K, Hoelzer K, Warnick LD, Cummings KJ, Wiedmann M. 2013. *Salmonella* bacteriophage diversity reflects host diversity on dairy farms. Food Microbiol 36:275-85. doi:10.1016/j.fm.2013.06.014

312. Capparelli R, Nocerino N, Iannaccone M, Ercolini D, Parlato M, Chiara M, Iannelli D. 2010. Bacteriophage therapy of *Salmonella enterica*: A fresh appraisal of bacteriophage therapy. J Infect Dis 201:52-61. doi:10.1086/648478

313. Gong C, Jiang X, Wang J. 2017. Application of bacteriophages to reduce *Salmonella* contamination on workers' boots in rendering-processing environment. Poult Sci 96:3700-08. doi:10.3382/ps/pex070

314. Xie Y, Savell JW, Arnold AN, Gehring KB, Gill JJ, Taylor TM. 2016. Prevalence and characterization of *Salmonella enterica* and *Salmonella* bacteriophages recovered from beef cattle feedlots in South Texas. J Food Prot 79:1332-40. doi:10.4315/0362-028X.JFP-15-526