EVALUATION OF ANIMAL AND MICROBIAL FACTORS AFFECTING PREVALENCE OF SHIGA TOXIN PRODUCING E. coli (STEC) AND CEFOTAXIME RESISTANCE

By

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To the Scientific Community: thanks for unravelling nature especially microbes
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<td>ARM&lt;sub&gt;s&lt;/sub&gt;</td>
<td>Antibiotic resistant microorganisms</td>
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<td>BLA</td>
<td>β- lactamase</td>
</tr>
<tr>
<td>BRU</td>
<td>beef research unit</td>
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<tr>
<td>CDC</td>
<td>centers for disease control and prevention</td>
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<tr>
<td>CEFR</td>
<td>cefotaxime resistant</td>
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<tr>
<td>CEFS</td>
<td>cefotaxime susceptible</td>
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<tr>
<td>CFU</td>
<td>colony forming unit</td>
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<td>CM</td>
<td>chitosan microparticles</td>
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<td>CT-SMAC</td>
<td>MacConkey sorbitol agar with cefixime and potassium tellurite</td>
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<td>EAEC</td>
<td>enteroaggregative <em>E. coli</em></td>
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<td>EHEC</td>
<td>enterohemorrhagic <em>E. coli</em></td>
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<tr>
<td>EIEC</td>
<td>enteroinvasive <em>E. coli</em></td>
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<tr>
<td>EPEC</td>
<td>enteropathogenic <em>E. coli</em></td>
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<td>ESBLs</td>
<td>extended spectrum β-lactamases</td>
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<td>FDA</td>
<td>food and drug administration</td>
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<tr>
<td>Gb3</td>
<td>globotriaosylceramide</td>
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<tr>
<td>GIT</td>
<td>gastrointestinal tract</td>
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<td>GRAS</td>
<td>generally recognized as safe</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HC</td>
<td>hemorrhagic colitis</td>
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<td>HUS</td>
<td>hemolytic uremic syndrome</td>
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<tr>
<td>IACUC</td>
<td>institutional animal care and use committee</td>
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<tr>
<td>IUPEC</td>
<td>intrauterine pathogenic <em>E. coli</em></td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>NFREC</td>
<td>North Florida Research and Education Center</td>
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<td>NGS</td>
<td>next generation sequencing</td>
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<td>NMEC</td>
<td>neonatal meningitis <em>E. coli</em></td>
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<tr>
<td>OmpA</td>
<td>outer membrane protein A</td>
</tr>
<tr>
<td>OTU</td>
<td>operational taxonomic unit</td>
</tr>
<tr>
<td>PCoA</td>
<td>principal coordinates analysis</td>
</tr>
<tr>
<td>RAJ</td>
<td>recto-anal junction</td>
</tr>
<tr>
<td>SRP</td>
<td>siderophore receptor and porin</td>
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<tr>
<td>STEC</td>
<td>Shiga toxin-producing <em>E. coli</em></td>
</tr>
<tr>
<td>STX</td>
<td>Shiga toxin</td>
</tr>
<tr>
<td>T3SS</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>UPEC</td>
<td>Uropathogenic <em>E. coli</em></td>
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<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
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Various factors that affect shedding of pathogenic bacteria by beef cattle are poorly understood. Some of these pathogens like Shiga toxin-producing Escherichia coli (STEC) and the antibiotic resistance present huge challenges to human health. Understanding the animal and microbial factors that affect shedding of pathogenic bacteria is essential to develop effective mitigation strategies. The results from this study indicate that animal age is a significant factor influencing the prevalence of STEC in beef cattle and the young beef calves have high level of cefotaxime resistance even in the absence of any antibiotic exposure. This study utilized the culture-independent analysis of gut microflora to determine the effect of microflora on STEC shedding and cefotaxime resistance dynamics. As calf age increases, the diversity in gut microflora increases which reduces the STEC shedding by calves. The high prevalence of cefotaxime resistance in young beef calves indicates that there is a role of environment in the acquisition and spread of antibiotic resistance. Environmental stressors like high
temperature, rainfall and crowding increased the prevalence of cefotaxime resistance in beef cattle.
CHAPTER 1
INTRODUCTION

The main hurdle in food industry especially meat production is the shedding of pathogenic bacteria and antibiotic resistance in food animals. Bacteria are part of normal gut microflora and live in a mutual or symbiotic relationship with the host [1]. One of such bacteria is *Escherichia coli*. Majority of the *E. coli* serotypes are beneficial bacteria being a part of normal microflora but some *E. coli* serotypes are harmful as they contain virulence factors and produce Shiga toxins [2]. Shiga toxin producing *Escherichia coli* (STEC) cause about 269,000 cases of illness (3,700 hospitalizations and 30 deaths) in the United States every year [3]. Cattle are primary reservoirs of STEC and can transmit STEC to humans mainly through food consumption, water, and animal contact [4]. A multilayer of factors including bacterial, environmental and animal characteristics can influence the STEC shedding [5,6].

To control outbreaks of STEC, understanding risk factors that increase the prevalence and shedding of STEC in cattle is essential. There is a knowledge gap about these factors especially animal factors that affect the prevalence of STEC in cattle. In Chapter 3, animal age was evaluated as one of the factors responsible for affecting STEC shedding. We used a multi-breed calf population to determine the effect of breed group and sex on STEC shedding. Chapter 4 reveals the effect of animal factors and microflora on the dynamics of STEC in a cohort of beef calves from birth until one year of age. Animal factors like breed and sex of the calf were not significantly associated with the STEC shedding.
The other problem commonly associated with microflora of food animals is the antibiotic resistance. Antimicrobial resistance causes more than 23000 deaths and costs $55 billion in overall societal costs in the US [7]. Few reports have blamed the animal producers for the rise in antibiotic resistance in human hospitals [8,9], but there is a knowledge gap about the role of animal factors in the dynamics of antibiotic resistance. Cefotaxime is a third generation cephalosporin and has never been used as prophylactic agent in beef cattle. This study rationalized that antibiotic resistance develops in nature. In Chapter 5, naturally occurring cefotaxime resistant bacteria were isolated from cattle in several farms. These cattle had no history of exposure to cefotaxime. We hypothesized that animal factors are not the major impacts on cefotaxime resistance but the environmental factors are responsible for the origin of cefotaxime resistance. To understand the role of animal and microbial factors in the cefotaxime resistance, a group of beef calves was followed for first year of life. To evaluate our hypothesis, we conducted metagenomic analysis of the fecal microbiome and collected environmental data of the geographical location of the farm. In Chapter 6, we evaluate the animal, microbial and environmental factors that affect cefotaxime resistance.
CHAPTER 2
LITERATURE REVIEW

Pathogenic *Escherichia coli* and Antibiotic Resistance in Cattle

*Escherichia coli* (*E. coli*) is a Gram negative facultative anaerobic bacterium that lives in the gastrointestinal tract (GIT) of humans and animals [10]. Most *E. coli* serotypes are part of the normal microflora [11] and they prevent the colonization of pathogens via the production of bacteriocins or competitive exclusion [2]. The pathogenic *E. coli* isolates use many virulence strategies and characteristic mechanisms of attaching to and exploiting host cells by adhesion to host cells, manipulating signaling pathways, evasion of host immune responses and efficient colonization [2,12]. Cattle are primary reservoirs of some of the pathogenic *E. coli* like EHEC and therefore, they can spread infections to humans mainly through beef, water, or animal contact [13]. Pathogenic *E. coli* are categorized as pathovars based on specific virulence factors like adhesins, invasins, haemolysins, toxins, effacement factors, cytotoxic necrotic factors and capsules [14]. The most commonly reported pathovars are enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC). The first six pathovars are called as diarrheagenic *E. coli* while as UPEC and NMEC are extra intestinal *E. coli* [2].

Enterohemorrhagic *E. coli* (EHEC) was first recognized in 1982 as a causative agent of hemorrhagic colitis, non-bloody diarrhea and hemolytic uremic syndrome (HUS) in humans [15]. EHEC is highly infectious attaching and effacing (A/E) pathogens
that colonize the large intestine in humans causing severe gastroenteritis [2]. The *E. coli* O157:H7 serotype is the most important EHEC pathogen in North America, the United Kingdom and Japan, but several other non-O157 serogroups, called as Big Six (O26, O45, O103, O111, O121 and O145), can also cause disease and outbreaks in many countries including the US [10]. The main virulence factor of EHEC is the phage-encoded Shiga toxin (also called as verocytotoxin) and *E. coli* serotypes that produce Shiga toxin are called as Shiga toxin producing *E. coli* (STEC) [16,17].

**Shiga Toxin-producing *Escherichia coli***

Shiga toxin producing *E. coli* (STEC) are reported to have acquired the toxin genes from *Shigella sp* through a gene transfer event [10]. Several bacteria like *Shigella dysenteriae, Escherichia coli, Citrobacter freundii, Aeromononas hydrophila, Aeromononas caviae* and *Enterobacter cloacae* have been reported to produce Shiga toxins but among them Shiga toxin producing *E. coli* is the most important pathogen [18,19]. Shiga toxin and Shiga-like toxins belong to a large family of bacterial toxins that kill cells by first binding to the cell surface, get endocytosed and then enzymatically active part of toxin enters the cytosol where it inhibits protein synthesis and kills the host cell [20].

Shiga toxins belong to the AB family of protein toxins with one enzymatically active part (A) and one cell surface binding part (B). The Shiga toxins are AB₅ subunit toxins with one enzymatically active A subunit and 5 identical copies of the B subunit [21]. The B subunit pentamer is a doughnut-shaped structure composed of the individual subunits arranged symmetrically around a central helix–lined pore with A subunit over one face and the receptor-binding domains of the B pentamer over the
opposite face [22]. There are two main variants of Shiga toxin, Stx1 and Stx2. Shiga toxin 1 (Stx1) produced by *Escherichia coli* is almost identical to Shiga toxin produced by *Shigella dysenteriae* whiles as Stx2 and its variants have sequence differences, cytotoxic and immunological properties different than Stx1 [19]. Also, Stx2 is more prevalent among the STEC serotypes [23] and has a greater cytotoxic effect on human glomerular endothelial cells than Stx1 and predisposes human patients to hemolytic uremic syndrome [24].

STEC are zoonotic pathogens often associated with food- or waterborne illness and cause diarrhea, hemorrhagic colitis and the hemolytic-uremic syndrome (HUS) in humans [25,26]. Because of the absence of secretory mechanism for Shiga toxin, the treatment of EHEC infections is complicated as toxin will be released through lambdoid phage-mediated lysis in response to DNA damage and the SOS response [27]. STEC have been isolated from cattle and other ruminant species including deer and camel [28-30]. Cattle are considered the primary reservoir for STEC and multiple STEC serogroups have been isolated from cattle [31-34]. Over 380 STEC serotypes have been identified but only few serotypes like enterohemorrhagic *E. coli* (EHEC) produce the majority of human diseases [35,36]. Paneth cells in the human intestinal mucosa and kidney epithelial cells contains the Shiga toxin receptors globotriaosylceramides (Gb3s) [37] but these receptors are absent in cattle leading to asymptomatic colonization of EHEC in cattle [38].
Antimicrobial Resistance and Extended Spectrum β-Lactamase (ESBL) Producers in Cattle

Another big problem associated with food animals including beef cattle is the antimicrobial resistance (AR) which is defined as “the resistance of a microorganism to an antimicrobial drug to which it was previously sensitive so that the standard treatments become ineffective and infections persist and may spread to others” [39]. Showing no signs of moderation, the number of resistant organisms and types of resistance has increased over the last several decades [40]. Although there have been debates regarding the direct associations between antimicrobial use in farm animals and the increase of antimicrobial resistant bacteria in humans [41], there is undeniable rise in the overall number of antibiotic resistant microorganisms isolated from animals as well as human hospitals (ARMs) [42].

One of the major threats to human medicine causing extended hospital stays and economic losses is the development of resistance against cephalosporins which are the β-lactam antibiotics used therapeutically in human medicine [39]. During the recent years, there has been an increase in the detection of ESBL-producing bacteria in companion animals and livestock [43,44] and have been identified in clinical isolates of human patients [45,46]. Presence of plasmid encoded \( \text{blaCMY-2} \) enzyme in several zoonotic pathogens has been reviewed [47] and it has been suggested that the increased prevalence of \( \text{blaCMY-2} \) producers in the USA is associated with the use of ceftiofur, a 3rd generation cephalosporin, licensed in 1988 and approved for veterinary use in many countries [48,49]. A significant number of ESBL-producing bacteria are
observed in food animals, suggesting that animals may serve as reservoirs or sources of ESBL-producing bacteria [48,50].

Cefotaxime (iminomethoxy aminothiazolyl cephalosporin) is a 3rd generation cephalosporin antibiotic used in human medicine for the treatment of bacterial infections and surgical care [51,52]. The third-generation cephalosporins like cefotaxime (Figure 2-1) have an aminothiazolyl substitution at the R\textsuperscript{1} position, which increases affinity to penicillin binding proteins (PBPs), thereby enhancing activity against Gram negative bacteria. Substitution at the R\textsuperscript{1} site results in increased resistance to bacterial β-lactamases, whereas substitution at R\textsuperscript{2} can prolong half-life of the antibiotic [53]. Cefotaxime has a broad spectrum of activity against Gram positive and Gram negative bacteria and is eliminated by liver and kidney with elimination half-time (t1/2) ranging between 1.5 and 2.5 hours after the therapeutic administration in humans [54]. Like other antibiotics, several studies reported the emergence of resistance to the broad-spectrum cephalosporins in pathogenic bacteria like *Pseudomonas* spp., *Serratia* spp., and *Morganella* spp. in humans [55] and a recent study in China reported 5.5 and 8.6% of cefotaxime resistance levels in *Salmonella* spp. isolates from chickens and pigs was respectively [56]. The authors also identified ciprofloxacin and cefotaxime co-resistant *S. enterica* serovar Indiana isolates from chicken and pigs which is a major risk to public health [56]. Third generation cephalosporins are degraded by *blaCTX-M* producing bacteria which have been isolated from cattle and other food animals in several countries including the US, Asia, Europe and South America [44,47,57-62]. It has been reported that *E. coli* is the dominant worldwide producer of ESBL but, other Gram
negative bacteria such as *K. pneumonia*, *K. oxytoca*, *Proteus* spp., and *Acinetobacter* spp. have also been identified as ESBL producers [43]. ESBLs are usually plasmid mediated and these plasmids are transferable among *Enterobacteriaceae*. Also, various ESBL-producing isolates are known for displaying co-resistance to sulfonamides, aminoglycosides, and tetracycline’s [63]. Consequently, antimicrobial resistance genes accumulate on plasmids, resulting in multidrug resistance [64]. Most of the ESBL producers are multi-drug resistant as shown in a recent study in which out of 91 ESBL-producing isolates, 76, 59, 43, and 31 were resistant to tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid and aminoglycosides, respectively [65]. Furthermore, resistance to chloramphenicol and ciprofloxacin was found in 22% and 19.8% of the ESBL producing isolates, respectively [65]. ESBL-producing bacterial isolates studied in Japan were found resistant to almost all generations of cephalosporins, but remained susceptible to carbapenems [66].

Over the years, the rate of detection of ESBL-producing bacteria from infections (mainly *E. coli*) has increased [43]. With the emergence of ESBLs spread among food animals well accepted, the United States Food and Drug Administration’s (FDA) Center for Veterinary Medicine (CVM) issued, on January 6, 2012, an order prohibiting the preventative uses of the cephalosporin class of antimicrobial drugs in food-producing animals [67].

**Cattle as Source of STEC and Antibiotic Resistance**

Majority of Shiga toxin-producing *E. coli* (STEC) strains are being carried asymptptomatically by adult cattle which serve as the reservoirs of the STEC and do not have vascular receptors for Shiga toxins, so there is no systemic vascular damage in
adult cattle [68,69]. Some strains of EHEC pathotype cause diarrhea in young calves up to 2 months of age [70]. The preferred receptor for Shiga toxin Stx1 and Stx2 is globotriaosylceramides (Gb3) and the preferred receptor for Stx2e (a variant of Stx2) is globotetraosylceramide (Gb4) and these receptors have been shown in humans, rabbits, and pigs which develop Shiga toxin mediated vascular damage [68,69].

Reported prevalence in cattle varies greatly according to the country and to the detection method (PCR-based methods always give much higher results). Animal prevalence varies from 0.1 to 63% and herd prevalence between 0.3 and 87% [70]. Some of the studies that have reported the prevalence of STEC in cattle, especially beef cattle from various countries around the world are summarized in Table 2-1. After the first STEC O157 outbreak in 1982, a majority of human infections have been ascribed to the contaminated animal products especially beef [16,34,71,72], and it has been proposed that a reduction in fecal shedding by cattle may significantly reduce human infections [73]. Microbial factors that affect transmission of STEC and its associated illnesses have been studied but several factors effect on the colonization and prevalence of STEC in beef cattle, and thus reduction of STEC at the pre-harvest level is challenging [74,75].

Food animals like beef cattle are exposed to antibiotics to treat an identified illness; to prevent illness in advance; for growth promotion or to increase feed conversion, growth rate or yield [76]. The prevalence and persistence of antimicrobial resistance genes in fecal deposits from cattle fed sub-therapeutic levels of antimicrobial growth promoters suggest that animal husbandry practices may influence the extent of
resistance genes being released into the environment [77]. Although antibiotics have been used for growth promotion since the 1940s in many countries [78], growth promoters or sub therapeutical doses of antibiotics used in animal feed may lead to the selection of antibiotic resistant populations in the gastrointestinal tract and reach the environment through feces [79]. A causal association was identified between the use of avoparcin, a glycopeptide antibiotic used as a growth promoter, and the occurrence of vancomycin-resistant *Enterococcus faecium* (VREF) on farms [80]. The use of tetracycline both oral and injectable was associated with the prevalence of antimicrobial resistance (AR) in fecal *Escherichia coli* from feedlot cattle [81]. A similar trend of increased resistance was shown with other antibiotics including sulfamethoxazole [82].

Bovine feces also serve as resistant bacteria reservoirs for an extended period of time, as target resistance genes were quantifiable even up to day 175, indicating the importance of animal husbandry practices to reduce AR on farms [77]. The frequency of cleaning the pen floor in the animals’ housing is an important husbandry tactic to prevent the spread of resistant bacteria [83].

**Role of Cattle in Transmission of STEC and Antimicrobial Resistance**

Since several factors modulate the colonization and persistence of STEC in cattle, the outbreaks of STEC continue despite a great deal of research focuses on improving food safety. Likewise, the ability of commensal or non-pathogenic bacteria to transfer resistance genes to human pathogens is a tremendous threat to public health as it eliminates options for antibiotic treatment against bacterial infections [84].
Role of Animals in Transmission of STEC

Shiga toxin producing *E. coli* (STEC) were first implicated as etiologic agents in foodborne gastrointestinal illness in 1982, when *E. coli* O157:H7 was isolated from stool samples of human patients as well as from a beef patty from a suspected lot of meat in the states of Oregon and Michigan [85]. Feces and the hides of cattle are important sources of contamination during processing, and there is a positive correlation between pre-harvest fecal and hide prevalence of *E. coli* O157:H7 and carcass contamination [86]. Therefore, reducing the prevalence of STEC in cattle at the pre-harvest level could potentially reduce carcass contamination and has recently been highlighted as an intervention point [87,88]. STEC are estimated to cause more than 265,000 illnesses each year in the United States, with more than 3,600 hospitalizations and 30 deaths [89]. STEC have a low infectious dose, therefore, person to person transmission is common and in rural areas contact with infected animals and contaminated water predisposes to the infection [90,91]. In a mathematical model it was shown that *E. coli* O157 shedding at high concentrations was significantly associated with the presence of the *stx*2 variant, indicating that human infections occur because of high cattle shedding levels associated with Stx2 genotype and super shedding of *E. coli* O157 by cattle increases risk of human infections [73].

Several studies have isolated the STEC strains from the wild animals and birds and results indicate that the wildlife animals play an important role for carriage and transmission of STEC to farm animals and thereby to humans [92-95]. In a study involving wildlife, 701 fecal samples were collected from roe deer, wild boar and foxes and results indicated that 28.5% of the wildlife-carried samples were Shiga toxin
positive by PCR detection method [93]. The results indicated 81.5% of the STEC strains carried by wild animals were assigned to seropathotypes associated with human disease [93]. In another study, 243 fecal samples were collected from red deer (206 samples), roe deer (20 samples), fallow deer (6 samples) and mouflon (11 samples) and STEC strains were detected in 58 (23.9%) of the animals sampled [94]. In a study in Spain, the fecal samples from wild boars were positive for *E. coli* O157:H7 (3.3%) and non-O157 STEC (5.2%) and wildlife carried STEC strains resembled to five were identical to the ones detected in human hospital [95]. Deer have been suggested to play a role as transmitters of EHEC O157 strains to cattle by fecal contamination of farmland [96]. Wild migrating birds have been identified as STEC excretors and participate in the spread of EHEC O157 and other STEC strains over long distances [97].

**Role of Animals in Spread of Antimicrobial Resistance**

Resistance may be transferred to other animals or humans through the movement of animals between farms, through the food chain, or by animal handlers [98]. Antibiotic resistant pathogenic *E. coli* from chicken abattoirs were shown to belong to the same clonal group as human pathogens indicating that they may have originated from a recent common ancestor and spread to multiple hosts [99]. Fecal deposits can also be reservoirs for antibiotic resistant bacteria for months after defecation and contaminate waste waters and spread resistance to indigenous soils [100].

Animal gastrointestinal tract is an ideal place to promote resistance proliferation because horizontal gene transfer especially among *Enterobacteriaceae* members occurs in niches with high bacterial densities resulting in increased cell-to-cell contact and therefore plasmid conjugation [42]. Resistant microbes can disseminate into the
environment and transmission from animals to humans can occur with relative ease [101]. Many fast-spreading β-lactamase genes are now endemic in certain parts of the world including CTX-M ESBLs in European, Asian, and South American countries [63]. ESBLs are more predominant than originally predicted, however, the rise of ESBL-producing microorganisms on farms is yet to be fully understood [63]. A study reported that among 177 ESBL-producing *E. coli* isolates from companion animals, six CTX-M-15-positive animal strains exhibited a PFGE pattern of >85% similarity to the human strain, which shows the features of the human pandemic clone [102]. The treatment with amoxicillin, ceftiofur, or cefquinome resulted in the selection of CTX-M-producing *E. coli* in the intestinal flora of pigs [103]. In addition, co-selection is one mechanism where other resistant genes carried on the same mobile genetic element select for an entire class of antibiotics [104]. Plasmids carrying ESBL genes also encode for resistance to other antimicrobials including aminoglycosides, trimethoprim, tetracyclines, and fluoroquinolones, where the use of any one of these drug classes will kill sensitive bacteria and may select for ESBL-producing *Enterobacteriaceae* [105]. A study observed the shift in a hospital setting from *blaCTX-M-9*, the dominant enzyme since its first isolation in 1989, to the *blaCTX-M-1* and CTX-M-14 being the most prevalent enzymes. However, the rise of ESBL-producing microorganisms on farms is yet to be fully understood [63]. It is suggested that the inappropriate use of antibiotics in livestock and companion animals poses a great risk to the human population but ESBL-producing microorganisms originated from human clinical hospitals can disseminated to farm and companion animals [102].
In addition to the use of antibiotics for sub-therapeutic purposes, increasing lines of evidence identify selective pressures occurring naturally [101]. Natural reservoirs of resistant genes in bacteria exist in humans, animals, and the environment [101,106]. A study using whole genome sequencing in Scotland during a Salmonella Typhimurium epidemic in humans and animals reported that antibiotic resistance genes were maintained separately in human and animal populations and there was limited transmission in either direction [107]. Many environmental factors like the frequency, duration, and intensity of rainfall; slope; vegetative cover; soil type; organic-matter content; temperature; available nutrients; and the presence of toxic materials affect the development and spread of resistance [101]. The importance of interaction between environmental factors like geography, the population density, the size of the treatment area, and the number of bacteria surviving the treatment and antibiotic resistance has been previously reviewed [108].

**Types of STEC and Antibiotic Resistant Microorganism Shed by Cattle**

*E. coli* are classified based on the somatic (O) and flagellar (H) antigens, regarded as the gold standard for classification of *E. coli* for taxonomic and epidemiological purposes [109]. STEC is a broad group of pathogens [91] and almost ~250 different O serogroups of *E. coli* have been shown to produce Shiga toxin and more than 100 serotypes of STEC have been associated with human disease [110,111].

**STEC O157:H7**

One of the most frequently isolated STEC strain from clinics in North America is the serotype O157:H7 causing hemorrhagic colitis and hemolytic-uremic syndrome (HUS) in humans and remains the major food-borne pathogen of concern for the beef
industry [91,112,113]. It has been reported that that *E. coli* O157:H7 strains cause two-thirds of the human EHEC infections in the United States, with the remaining cases attributed to the non-O157 STEC serotypes although this estimation may be biased toward O157 because several clinical laboratories only screen for *E. coli* O157:H7, leaving non-O157 STEC diseases under-reported [113,114]. *Escherichia coli* O157:H7 infection occurs by contaminated beef (carcass contamination by hides and feces), water or direct contact with animals and a study reported that vaccination of cattle could potentially reduce the human infections [73,86,87].

**Non-O157 STEC**

In addition to *E. coli* O157:H7, several non-O157 STEC are isolated from clinical cases and prevalence of non-O157 STEC infections among patients with STEC infections has been summarized in Table 2-2. Non-O157 STEC are also responsible for sporadic illness and outbreaks of bloody diarrhea and HUS in many parts of the world including the US [115-118]. The major serogroups associated with severe disease outbreaks are O26, O103, O111, O121, O125 and O145, commonly referred to as ‘Big Six’ serogroups, which in some countries are reported to cause more disease outbreaks than O157 [110,119-121]. After the STEC outbreak in 1982 in USA, the, the CDC has confirmed 940 non-O157 STEC isolates that had been submitted by 42 state public health laboratories between 1983 and 2002. Although the virulence of *E. coli* O157:H7 is well described, during the last two decades the capacity of the heterogeneous family of non-O157 STEC to induce human disease has been discussed in detail [110,115,121].
Antibiotic Resistant Microorganisms and ESBL Producers

An antibiotic is defined as a substance produced by or a semisynthetic substance derived from a microorganism and able in dilute solution to inhibit or kill another microorganism [122]. There are more than 15 classes of antibiotics based on their mode of action on the bacteria and one of the important antibiotic groups is cephalosporins which act on the bacterial cell wall [123]. Cephalosporins are β-lactam antibiotics exerting bactericidal action on bacteria by inhibiting the cell wall synthesis [124]. Cephalosporins are one of the major antibiotics used worldwide and contribute 14% of total dispensed antibiotics in the US [125]. The cephalosporins are classified into five generations (1<sup>st</sup>- 5<sup>th</sup>) depending on the modification of the basic cephalosporin structure which gives respective antibacterial, pharmacokinetic and pharmacodynamic properties [53].

Bacteria can acquire resistance by several mechanisms like, interfering with the stereospecific requirements necessary for binding of the drug to its target site, destroy or alter the conformational integrity of the drug, or prevent the drug from attaining an effective concentration at its site of action [126]. Mutations often modify the binding sites of antibiotics and the result is antimicrobial resistance [127]. An additional strategy that bacteria have adapted is the inactivation of antibiotics [128]. Some bacteria produce acetyltransferase that covalently modifies antibiotics, such as aminoglycosides, rendering them ineffective [129]. There are various bacteria that produce enzymes known as β-lactamases that attack and break the amide bond in the β-lactam ring, which is the key structure of penicillin, cephalosporin, and their derivatives [130]. Few
bacterial genera have evolved membrane transporters that can pump antibiotics directly outside the cells to lower the concentration within their cytoplasm [101,131].

In 1983, a new group of enzymes described as ESBLs [64] were identified in resistant nosocomial isolates of *Klebsiella* spp. β-lactam antibiotics are widely used in humans and animals for therapeutic purposes all over the world [132]. Some bacteria are resistant to β-lactam antibiotics because they produce β-lactamase enzymes which hydrolyze the β-lactam ring, the primary structure of these antibiotics [127]. ESBLs are able to hydrolyze extended-spectrum cephalosporins, including cefotaxime, ceftriaxone, ceftazidime, cefepime and monobactams [133]. Currently, more than 600 ESBL variants are known, including *bla*TEM, *bla*SHV and *bla*CTX types, with many more expected to be identified in the future [101]. The name TEM is derived from the patient Temoniera from whom the resistant bacteria were isolated, SHV is sulphhydryl variable owing to the biochemical properties of this β-lactamase enzyme, while as CTX was named because of its resistance to cefotaxime [134]. Previously, 3rd generation cephalosporins were considered a critical breakthrough in treating β-lactamase-mediated resistance, but several recent studies have reported resistance against this group of antibiotics in animals and human hospitals [43,60,63,103]. ESBL-expressing pathogens have proven to be difficult to treat, having resistance to the majority of β-lactam antibiotics used in veterinary and human medicine [135,136].

**Public Health and Economic Importance of STEC and Antibiotic Resistance**

Cattle are reservoirs of STEC and although direct person-to-person spread can occur, people are usually infected by either consuming contaminated food and water or contact with livestock feces in the environment, increasing the burden of food borne
illnesses [73]. It has been reported that approximately 48 million new cases of food-related illness, resulting in 3,000 deaths and 128,000 hospitalizations, occur in the United States annually [89]. In another study which used basic and enhanced cost-of-foodborne-illness models, the cost of foodborne illness was $51.0 billion in annual health-related costs in the basic model and $77.7 billion in the enhanced model [137]. The difference between the two models is that the enhanced model includes a measure for lost quality of life. The study reported that cost per case of foodborne illness caused by STEC O157:H7 is up to $10,048 and the cost per case of foodborne illness caused by STEC non-O157 is up to $1,366 [137]. The overall economic cost of foodborne illness caused by STEC O157:H7 is 607 million USD while as, the overall economic cost of foodborne illness by STEC non-O157 is 101 million USD [137]. It has been predicted that a typical vaccine that reduces high shedding to produce a 50% reduction in STEC shedding frequency in cattle may result in 83% reduction in human cases [73] but mass vaccination of cattle in the developed and developing world depends on the costs and benefits of vaccination.

Antimicrobial resistance is one of the fundamental challenges facing the public health and causes more than 23,000 deaths and costs $55 billion in overall societal costs in the US [7]. Antibiotic resistance can be acquired by inappropriate use of antimicrobials in animals as well as in human hospitals which allows bacteria a lot more opportunities to gain resistance against previously effective drugs [63]. The problem of antibiotic resistance is complicated by the fact that previously susceptible bacteria become resistant and difficult to treat as shown by the multi-drug resistant \textit{E. coli} O104:
H4 outbreak in Germany [138] and evolution of methicillin resistant *Staphylococcus aureus* (MRSA) into a first-class multidrug-resistant pathogen [84]. Both these pathogens have acquired resistance in very short course of time and pose severe public health risks. Another major hurdle in prevention of antibiotic resistance is the natural resistome and development of resistance even in the absence of human interference [101]. Soil samples were shown to contain antibiotic resistance genes similar to those found in clinics but the rate of direct and horizontal transmission from soil microbes to the human pathogens is not significantly higher and calls for further studies to understand the dynamics [106,139].

**Control Strategies Against STEC and Antibiotic Resistance**

The effective intervention for the control of STEC infections relies heavily on good hygiene practices by food producers and personal hygiene including hand washing by consumers [73]. Reducing occurrence in cattle is one route to control but current intervention and control practices fail to completely eliminate STEC in general and STEC O157 in particular, from cattle and the food supply [140]. Total elimination of antibiotic resistance is impossible because it is a natural phenomenon. However, effective strategies may avoid the development of new types of resistance [60].

**Changing Diet to Reduce STEC Shedding by Cattle**

Since cattle are the main reservoirs of STEC, several intervention strategies have been reported to reduce the shedding and pre-harvest contamination of beef. These methods include altering diets, adding probiotics to feed, spraying cattle with bacteriophage, and vaccination of the cattle [73,141]. The animal diet like forage plants which contain phenolic acids has been shown to affect the colonization and decrease
shedding of pathogenic bacteria including *E. coli* O157:H7 [142]. Cattle diets containing grains with lower ruminal starch degradation are associated with lower prevalence of *E. coli* O157:H7 [143]. Increasing hindgut fermentation of starch and consequent acid production will create inhospitable conditions for *E. coli* O157:H7. Short-chain fatty acids (acetic, propionic and butyric) have been shown to suppress and inhibit growth of *E. coli* O157:H7 [144].

**Chitosan Microparticle (CM) Reduces STEC Shedding by Cattle**

Chitosan is a hydrophilic biopolymer obtained by N-deacetylation of chitin and can be applied as an antimicrobial agent [145]. Chitosan microparticles (CM), derived from chitosan, have been shown to kill pathogenic bacteria and reduce *E. coli* O157:H7 shedding in cattle, indicating potential use as an alternative antimicrobial agent [146].

The problem of treating STEC infections in humans is that antibiotic therapy leads to the induction of Shiga toxin production by the bacteria, therefore, CM can be a potential candidate to decrease *E. coli* O157:H7 shedding because CM does not induce bacteriophage or Shiga-toxins in *E. coli* O157:H7 [146]. In one of the studies involving cattle, a significant reduction in the carriage and shedding of STEC O157 was observed when cattle were fed CM-supplemented feed [147].

**Bacteriophage and Colicin Treatment for Reducing STEC in Cattle**

Bacteriophage can be used to reduce *E. coli* O157:H7 in cattle hides and naturally phage-infected ruminants have been shown to be more resistant to *E. coli* O157:H7 colonization [148]. The use of probiotic bacteria like lactic acid bacteria to inhibit *E. coli* O157:H7 in cattle have been reported to reduce *E. coli* O157:H7 fecal shedding or prevalence in cattle [149,150]. Colicins are antimicrobial proteins produced...
by some *E. coli* strains that inhibit other bacteria. Colicin E7 is a type of colicin protein targeted against *E. coli* O157:H7 which when supplemented with feed, reduce the fecal shedding of *E. coli* O157:H7 [151]. Studies *in vivo* found that a sea weed called as Tasco-14 feeding reduces fecal and hide prevalence of *E. coli* O157 in cattle. Also, feeding orange peel and citrus pellets (a 50/50 mixture) at levels up to 10% DM, reduced artificially inoculated populations of *E. coli* O157:H7 [152].

**Vaccination of Cattle Against STEC**

Vaccines have proven to be effective intervention strategies in reducing the shedding of STEC by the cattle and thereby reducing human infections. Results from vaccine trials indicate that frequency, duration and intensity of STEC O157 shedding by cattle is significantly decreased [153,154]. In a study using mathematical models of transmission the effect of vaccination on shedding frequency and concentration in cattle and its impact on human cases was simulated and results indicate that eliminating just the 12% highest shedding densities produces a 50% drop in the frequency of shedding in cattle but an 83% drop in human cases [73]. Vaccination of cattle with proteins secreted by *E. coli* O157:H7 significantly reduced the numbers of bacteria shed in feces, the numbers of animals that shed, and the duration of shedding in an experimental challenge model [140].

There are two types of vaccines for use in cattle, Siderophore receptor and porin protein (SRP) based vaccine and the other vaccine which targets the type III secreted proteins (T3SS) of *E. coli* O157 [155]. The siderophore receptor and porin (SRP)–based vaccine uses purified SRP proteins as antigenic components to induce an immune
response in cattle while as in the second type of vaccine, T3SS components were used as to develop vaccine and block colonization or reduce shedding [156].

**SRP–based vaccine**

The results show that prevalence of *E. coli* O157:H7 among the vaccinated animals (2.91%) was 49.7% lower than that among the controls (1.47%). In their second trial, on day 98, however, *E. coli* O157:H7 was 84.7% less likely to be recovered from feces from cattle administered the three-dose regimen of the vaccine compared to control animals [153,157]

**T3SS components based vaccine**

Immunization with IM H7+ T3S antigens (IM-H7-T3S), IM T3S antigens alone (IM-T3S) or H7 + T3S antigens resulted in the colonization of 29%, 50% and 37.5% of animals, respectively. Mean bacterial counts in the control group were higher and persisted for longer duration than the vaccinated group. Following vaccination, serum and rectal mucosal IgG antibody titers to all antigens increased significantly [154].

Pre-harvest meta-analysis evaluating the efficacy of these two commercial vaccines combined (SRP and type III secreted proteins) demonstrated good vaccine efficacy [155,156]. The vaccines are fully licensed in Canada and have restricted licenses permit in the United States, however, farmers have shown little interest in vaccinating their cattle in both countries [73].

**Control of Antibiotic Resistance by Banning Sub-therapeutic Use**

Several nations have implemented policies banning the use of antibiotics in livestock other than to treat disease. The South Korean Ministry for Food, Agriculture, Forestry and Fisheries (MAFF) banned the use of antibiotic growth promotors in animal
feed in July, 2011; it is still too early to determine the effects of the ban. In 2006 the European Union prohibited the use of antibiotics as growth promoters in livestock feed in order to protect human and animal health from the emergence, spread, and transfer of antibiotic resistant bacteria [60]. Determining the real effects in human populations when growth promoters are banned in food animals is difficult to assess at this time, but preliminary findings are promising.

In Germany there was a significant decrease of glycopeptide-resistant *E. faecium* in humans from 13% to 4% only four years after the implementation of the feed additive prohibition policy. Many countries, including Italy and the Netherlands, adopted the same program with avoparcin and had similar results in pigs, humans, and broiler hens [60]. However, there are other conditions that potentially cause the persistence of antibiotic-resistant *E. faecium* in the environment. In Denmark, after a five-year ban of avoparcin was enforced, Vancomycin-resistant *E. faecium* was still found in broilers and pigs [158]. In Norway, the ban produced the opposite effect, where resistant commensal *Enterococci*, able to proliferate, negatively affected animal health [159]. After Denmark banned the use of avoparcin in 1995, the use of virginiamycin in broilers and tylosin in pigs increased. One year after the policy took effect, the glycopeptide-resistant *E. faecium* remained at 20% because of the use of tylosin [126,158]. In Sweden, immediately after the 1986 ban on animal growth promoters, there was a rise in bacterial infections in poultry and pigs [41]. After ten years of the ban, the total amount of antibiotics used in animals decreased by 55% and antibiotics in veterinary prescriptions decreased by 33% [41]. In European countries such as Russia and
Belarus, the use of penicillins, cephalosporins, macrolides, quinolones, and carbapenems are less than aminoglycosides and chloramphenicol while in Poland, Slovakia, and Hungary, they use more recently discovered antibiotics [41,160].

Strategies for Mitigating Antibiotic Resistance

The use of medically important antimicrobial drugs in food-producing animals should be limited to those uses that are considered necessary for assuring animal health and require veterinary oversight or consultation. The following strategies or approaches can be adopted to minimize the development and spread of antibiotic resistance:

1. Use antibiotics only when etiology is confirmed or the bacterial infection is strongly suspected. Also, in-vitro sensitivity testing and pharmacokinetic data should be used to increase efficacy of treatment.

2. Select narrow-spectrum agents and avoid prolonged and unnecessary use of antibiotics.

3. Monitor development of resistance by means of well-designed surveillance schemes.

By implementing a multi-pronged strategy, we will emerge out of the antibiotic crisis today and forestall future epidemics of resistant infections. Appropriate use of antibiotics will delay and in many cases prevent the emergence of the resistance [161-163].

To reduce the number and spread of antibiotic resistant microbes, the use of antibiotics should be rejected when risks of irreparable harm are comparable with those that we tolerate everyday [164]. One solution to the problem can be the regulated and prudent use of antibiotics and the development of drugs that self-destruct after treatment. The investigation of novel non-antibiotic approaches for the prevention of and
protection against infectious diseases should be encouraged. Adequate attention should be focused on the use of antibacterial vaccines, phage therapy, immuno-stimulants, adjuvants, anti-virulence therapies, probiotics and combinations of these treatments [165].
<table>
<thead>
<tr>
<th>Country</th>
<th>Year</th>
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<th>Prevalence of STEC (%)</th>
<th>Diagnostic method</th>
<th>Sample</th>
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<td>Isolation</td>
<td>Rectal swabs</td>
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</tr>
<tr>
<td>USA</td>
<td>2001-02</td>
<td>Apr-Feb</td>
<td>33.3</td>
<td>PCR</td>
<td>Fecal</td>
<td>[173]</td>
</tr>
<tr>
<td>USA</td>
<td>2002-03</td>
<td>Sep-Jan</td>
<td>20.7</td>
<td>Isolation</td>
<td>Fecal</td>
<td>[174]</td>
</tr>
</tbody>
</table>
Table 2-2. Prevalence of non-O157 STEC infection among patients with STEC infection

<table>
<thead>
<tr>
<th>Country</th>
<th>non-O157 STEC serogroups</th>
<th>Prevalence of infections (%)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>O8, O26, O103, O113, O121, O145, O174, ONT</td>
<td>40</td>
<td>[175]</td>
</tr>
<tr>
<td>Australia</td>
<td>O6, O26, O48, O98, O111</td>
<td>69</td>
<td>[176]</td>
</tr>
<tr>
<td>Canada</td>
<td>O2, O26, O91, O103, O111, O113, O145</td>
<td>20</td>
<td>[177-180]</td>
</tr>
<tr>
<td>Chile</td>
<td>O26, O111</td>
<td>63</td>
<td>[181]</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>O1, O5, O26, O55, O111</td>
<td>57</td>
<td>[182,183]</td>
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<tr>
<td></td>
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<td>75</td>
<td>[184,185]</td>
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<tr>
<td>Denmark</td>
<td>OR, O15, O26, O103, O145, O174</td>
<td>53</td>
<td>[186]</td>
</tr>
<tr>
<td>Finland</td>
<td>OR, O15, O26, O103, O145, O174</td>
<td>53</td>
<td>[186]</td>
</tr>
<tr>
<td>France</td>
<td>O91, O103, O113, OX3</td>
<td>33</td>
<td>[187-189]</td>
</tr>
<tr>
<td></td>
<td>O26, O55, O76, O91, O103, O111, O113, O118, O128, O145, O146, ONT, OR</td>
<td>44</td>
<td>[115,118,190,191]</td>
</tr>
<tr>
<td>Germany</td>
<td>O118, O128, O145, O146, ONT, OR</td>
<td>34</td>
<td>[117,192]</td>
</tr>
<tr>
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<td>34</td>
<td>[117,192]</td>
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<td>Japan</td>
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<td>19</td>
<td>[193]</td>
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<td>New Zealand</td>
<td>O22, O26, ONT, OR</td>
<td>100</td>
<td>[194]</td>
</tr>
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<td>Spain</td>
<td>O26, O98, O118, O145, O150</td>
<td>78</td>
<td>[116,191]</td>
</tr>
<tr>
<td>Sweden</td>
<td>O8, O117, O121, OR, ON</td>
<td>33</td>
<td>[195]</td>
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<tr>
<td>United Kingdom</td>
<td>O26, O55, O145</td>
<td>28</td>
<td>[196]</td>
</tr>
<tr>
<td>United States</td>
<td>O26, O45, O103, O104, O111, O119, O121, O145, OR</td>
<td>44</td>
<td>[120,197,198]</td>
</tr>
</tbody>
</table>
Figure 2-1. Chemical structure of cefotaxime.
ASSOCIATION BETWEEN ANIMAL AGE AND THE PREVALENCE OF SHIGA TOXIN-PRODUCING *Escherichia coli* IN A COHORT OF BEEF CATTLE

Even with advancements in pre- and post-harvest food safety, Shiga toxin-producing *Escherichia coli* (STEC) still present challenges to human health. Since cattle are the primary reservoir for STEC, lowering the prevalence of this pathogen in farm animals may reduce STEC outbreaks in humans. However, because many of the factors that modulate the colonization and persistence of STEC in cattle remain unknown, reducing STEC in this host is challenging. In this study, we evaluated a cohort of beef cattle one to eleven years of age to better understand the effect of animal age on the prevalence of STEC. During the first year of sample collection, heifers had significantly lower STEC prevalence than cows (37.5% vs. 70%). In the second year of sample collection, STEC prevalence peaked in cows that were two years of age and tended to decrease as animals became older. In addition, by studying a subset of the animals in both years, we observed an increase in STEC prevalence from 40.6% to 53.1% in heifers, whereas cows had a net decrease in STEC prevalence from 71.4% to 61.9%. The results from this study indicate that animal age is a significant factor that influences the prevalence of STEC in cattle. These findings have clear implications for the development of on-farm mitigation strategies by targeting animals with the highest risk of colonization and the highest rate of shedding; it could be possible to reduce pathogen transmission among cattle and prevent zoonotic or foodborne transmission to humans.
Background

Even though progress has been made to reduce the incidence of diseases caused by foodborne pathogens, they remain of great economic and public health concern. The Centers for Disease Control and Prevention (CDC) has estimated that pathogenic Shiga-toxin producing *Escherichia coli* (STEC) cause about 269,000 cases of illness (including ca. 3,700 hospitalizations and 30 deaths) in the United States every year [89]. Because of the severity of illness and potential to cause outbreaks, STEC infections are designated as a notifiable infectious disease, which is required to be reported to the government by the National Notifiable Disease Surveillance System [3]. Among STEC strains, *E. coli* O157:H7 is the most well-known and can cause hemorrhagic colitis, bloody diarrhea, and hemolytic uremic syndrome (HUS) in humans [10]. Non-O157 STEC serogroups, such as O26, O45, O103, O111, O121, and O145, commonly called the “Big Six” serogroups, accounted for about 71% of non-O157 STEC isolates between 1983 and 2002, and have been also associated with human disease outbreaks in United States [110]. As such, the Big Six non-O157 STEC have been declared as food adulterants by the U.S. Department of Agriculture, Food Safety and Inspection Service [199].

Cattle are considered the primary reservoir for STEC with multiple STEC serogroups isolated from both dairy and beef cattle [31-33,200,201]. Estimates of the prevalence of STEC in herds of cattle vary widely from 4.6% to 55.9% [31,202,203]. A multilayer of factors modulates the colonization and persistence of STEC in cattle. These include bacterial factors that affect the colonization of the recto-anal junction (RAJ), which likely allows the persistence and shedding of this pathogen for weeks or
months [6,204]. Environmental factors, such as precipitation, temperature, soil characteristics, and presence of wild animals and insects are also believed to modulate the presence of STEC in the environment [205,206]. It has been recently shown that animal factors, including genetic breeds and physiological differences may affect the prevalence of *E. coli* O157 in cattle [207].

Since several factors modulate the colonization and persistence of STEC in cattle, controlling the prevalence of STEC in cattle to reduce outbreaks of this pathogen in humans is challenging. It may explain why outbreaks of STEC continue despite a great deal of research focusing on improving food safety. Reducing the prevalence of STEC in cattle at the pre-harvest level has been recently highlighted as an intervention point [87,88,147], and it has been suggested that lowering the STEC prevalence on farms may reduce the total number of *E. coli* O157:H7 outbreaks in humans [73]. Several studies have reported that diet and management changes may affect the prevalence of STEC, especially in *E. coli* O157:H7 [208-212]. For example, cattle on sorghum-based diets had high prevalence of *E. coli* O157:H7 and fecal shedding of *E. coli* O157:H7 was associated with the inclusion of distillers grain in the feed [210,211]. In a study with beef calves, weaning increased fecal shedding of *E. coli* O157 from 16.6% to 38.3% and a corn silage-based high moisture diet showed increased *E. coli* O157:H7 prevalence compared to a peanut/soy hull-based dry feed during weaning [212], suggesting the prevalence of *E. coli* O157 is associated with animal management and diet. However, information at the pre-harvest level, including animal factors and management practices that may modulate the non-O157 STEC prevalence is limited.
Recently we have shown that animal physiological differences contribute to the prevalence of *E. coli* O157 in cattle [207], which lead us to hypothesize that animal age may also play a role in the prevalence of this pathogen. To better understand the effect of cattle age on the prevalence of STEC, a herd of beef cattle, one to eleven years of age, was monitored for two years to determine the prevalence of multiple STEC genotypes over time. The findings from this study indicate that animal age is a critical factor influencing the prevalence of STEC, with heifers having lower prevalence than cows. Furthermore, by sampling a cohort of the animals in both years, we were able to investigate the dynamics of STEC transmission among cattle and to determine that the rate at which animals acquire and lose STEC colonization is also affected by age. These findings could be used to improve livestock management practices at the pre-harvest level to reduce the prevalence in cattle, resulting in reduction of the number of STEC entering the food chain in order to prevent zoonotic transmission to humans.

**Materials and Methods**

**Ethics Statement**

Standard practices of animal care and use were applied to animals used in this project. Research protocols, including permission for animal care facility in the University of Florida-North Florida Research and Education Center (NFREC, Marianna, Florida, USA), were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC Protocol #: 201308027).

**Animal Management and Sample Collection**

Fecal samples were collected with cotton-tipped swab (Fisher Scientific, USA) to scrape off colonized cells from recto-anal junction (RAJ) of cattle that were housed in
the University of Florida-NFREC with free access to pasture. The NFREC farm has a loose system of housing with average stocking density equal to 2 acres (0.8 Ha) per cow. Typically cows are bred from mid-March to mid-June (approximately 90 days) and calving takes place from late December to late March. Thus, at the time of sampling, bulls were already removed (in mid-June) and calves were weaned and moved to another pasture prior to the 2011 collection. Only heifers and cows were included in the study. Animals had free access to the watering points in the pasture and in the pen. The general health condition of animals was monitored by veterinarians working on the farm and any gastrointestinal disease or symptom was ruled out before collecting the swab sample. Samples were taken from 341 cattle over two years, including heifers at least one year of age and cows at least two years old with at least one live birth. For sample size calculations for year 1 (2011), our numbers were based on previous publications with similar objectives [114,173,213-215]. However in the second year (2012) we decided to sample the entire population available to strengthen our statistical power. Since the incidence of STEC infections in humans increases in summer months [110] and a causal relationship has been suggested by previous studies between STEC shedding in animals and infections in humans [216,217], samples were collected in the summer. The first year sample (n=90) was collected in August 2011 that included 40 heifers and 50 cows. The second year sample (n=251) was collected in July 2012 and consisted only of cows, including 32 animals from the first year that had since calved. A sub-sample of cows (n=74) was sampled both years and used to ascertain the
incidence of STEC in different age groups. All RAJ fecal swab samples were transported on ice and processed the same day using the following protocol.

**Identification of Shiga Toxin-producing Escherichia coli**

A combination of culture-based and nucleic acid-based methods for the detection and enumeration of Shiga toxin-producing *Escherichia coli* (STEC) were used. We used MacConkey agar (Becton Dickinson Company, MD, USA) to culture Gram-negative enteric bacteria in fecal samples. Approximately one gram of fecal sample was resuspended with 2 ml of Tryptic Soy Broth (TSB) and then diluted by a ten-fold serial dilution method (up to 10-6). After serial dilution, 100 μl of each diluted sample was plated on MacConkey agar. Plates were incubated at 37°C for 24 hours followed by the enumeration of enteric bacteria. From the resulting plates, ten lactose fermenting, pink colonies were randomly picked and were further purified. The purified isolates were subjected to colony PCR for the detection of *stx*1 and *stx*2 genes. Colonies carrying *stx* gene were further analyzed using CHROMagar *E. coli* (CHROMagar, France), and blue colonies were scored as STEC. Over 90% of the tested colonies were STEC. The multiplex PCR was carried out using two sets of primers [218]: KCP11/KCP12 (655 bp amplicon) for *stx*1 and KCP13/KCP14 (477 bp amplicon) for *stx*2. Primer sequences were as follows: KCP11 (5'-TGTCGACATAGTGGACACCTCA-3'), KCP12 (5'-TGCGCACTGAGAAGAAGAGA-3'), KCP13 (5'-CCATGACAACGGACAGCTGTT-3'), and KCP14 (5'-TGTCGCCAGTTATCTGACATTC-3'). The PCR reaction conditions were 95°C for 5min; 30 cycles of 95°C for 30 seconds, 55°C for 35 seconds, and 72°C for 90 seconds; with a final extension at 72°C for 7 min. The amplified products were resolved on 1% agarose gel stained with ethidium bromide and visualized with a UV gel doc.
system (Bio-Rad, USA). For interpretation of PCR results, \textit{E. coli} O157:H7 (EDL933) and DH5α were used as positive and negative controls, respectively. Strains were sub-grouped depending on the \textit{stx} genotype. Strains with amplification of only one \textit{stx} gene were classified as \textit{stx}1 (\textit{stx}1 positive) or \textit{stx}2 (\textit{stx}2 positive), while strains with amplification of both \textit{stx} genes were classified as \textit{stx}1/\textit{stx}2 (\textit{stx}1 and \textit{stx}2 positive). STEC was used to describe strains carrying any type of \textit{stx} genes. The concentration of STEC in the RAJ swab samples were calculated by multiplying the total number of blue colonies on CHROMagar with the fraction of \textit{stx} positive colonies following multiplex PCR.

To further confirm the absence of STEC in the RAJ samples, we enriched \textit{stx} negative samples scored by the culture/PCR-based detection method above. One milliliter of neat samples (1/3 dilution of the RAJ fecal swab sample) were enriched at 37°C for 12 hours in TSB. DNA was then extracted by boiling from the collected bacterial culture for 10 minutes followed by centrifugation at 12,000 x g for 5 min to remove cell debris. Extracted DNA was used as template for multiplex PCR with \textit{stx}1 and \textit{stx}2 primers before determination of the genotype as described above.

**Statistical Analyses of the Prevalence and Concentration of STEC**

Statistical analysis of the microbiological findings with animal factors was conducted using STATA software package (STATA® MP 11.2, StataCorp, College Station, Texas, USA). Simple logistic regression of the presence of STEC, \textit{stx}1, \textit{stx}2, and \textit{stx}1/\textit{stx}2 was used to assess the effect of the age and parity of the cattle and the likelihood of being colonized with STEC. The concentration of STEC and \textit{Enterobacteriaceae} isolated from samples was log transformed before regression was
used to assess the effects of animal age on the concentration of STEC, \textit{stx}1, \textit{stx}2, and \textit{stx}1/\textit{stx}2 present in the sample. For the cattle that were sampled in both years, McNemar’s test was used to determine if the marginal prevalence of STEC, \textit{stx}1, \textit{stx}2, and \textit{stx}1/\textit{stx}2 significantly increased or decreased between years one and two of the study. The proportion of cattle that became colonized, stopped shedding, or remained colonized or not colonized between years was stratified by age to determine if cattle age influenced the likelihood of STEC colonization over time. In all cases an alpha value (\(\alpha\)) of 0.05 was used to determine statistical significance; p-value less than 0.05 and less than 0.001 are denoted in the figures by a superscript double and triple asterisk, respectively. Values approaching significance were denoted by a single asterisk.

\textbf{Results}

\textbf{Heifers had Lower Prevalence of STEC Compared to Older Cows}

In the samples collected during the first year (\(n=90\)), the prevalence of STEC in cattle was 55.6\%. Among STEC genotypes, \textit{stx}2 (33.3\%) and \textit{stx}1/\textit{stx}2 (30.0\%) were the most dominant while only 5.6\% of cattle carried STEC with \textit{stx}1. Multiple genotypes of STEC isolates were detected from 11.1\% of the animals (Figure 3-1A). To test if animal age affects the prevalence of STEC, we categorized the animals into two groups: heifers (at least one year of age with no previous live births) and cows (at least two years old with at least one calf). Heifers (\(n=40\)) had a significantly lower prevalence of STEC than cows (\(n=50\)) (37.5\% vs. 70\%), demonstrating that animal factors may modulate STEC prevalence. The proportion of animals harboring non-STECEnterobacteriaceae was not significantly different (84\% vs. 97.5\%), indicating that the significant difference with the STEC prevalence is STEC specific. The prevalence of
STEC carrying \textit{stx1/stx2} (7.5\% vs. 48\%) was significantly lower in heifers (p-value < 0.001), whereas the differences in \textit{stx1} or \textit{stx2} genotypes (2.5\% vs. 8\% or 30\% vs. 36\%) were not significantly different when compared to cows (Figure 3-1A). The likelihood of STEC colonization in heifers compared to cows was 74\% lower for any type of STEC (95\% CI OR: 0.107-0.620), 92\% lower for \textit{stx1/stx2} (95\% CI OR: 0.024-0.323), and 89\% lower for multiple genotypes (95\% CI OR: 0.014-0.965).

The average number of STEC shed was lower in heifers, but when colonized with STEC, heifers were associated with a 1.11 log increase in concentration of STEC compared to cows (Figure 3-1B, p-value = 0.039). Heifers also had over a 2 log increase in concentration of \textit{Enterobacteriaceae} (95\% CI OR: 1.50 - 2.66) when compared to cows, which was significant at any level (p-value < 0.001).

**Two-year Old Cows had the Highest Prevalence of STEC**

The prevalence of STEC in the second year of sample collection (n=251, all cows) was 54.6\%, which was almost identical to the first year prevalence. However, the prevalence of each STEC genotype was different from the first year with 41\% of cattle positive for \textit{stx2}, 14.7\% of cattle positive for \textit{stx1/stx2}, 7.2\% of cattle positive for \textit{stx1}, and multiple genotypes of STEC isolated from 8\% of the cattle. To determine the effect of parity, we studied the correlation between the prevalence of STEC and number of previous live births. As shown in Figure 3-2A, uniparous cows had significantly higher (p-value = 0.001) prevalence of \textit{stx2} compared to older cows (58.7\% vs. 35.1\%), were more than twice as likely to carry \textit{stx2} (OR = 2.63, 95\% CI OR: 1.46-4.72), and also more than twice as likely to be colonized by multiple STEC genotypes (OR=2.13, 95\% CI OR: 0.83-5.49). After a becoming multiparous (at least two previous births), no
significant differences were observed in the prevalence of STEC or the likelihood of being colonized by STEC carrying stx1, stx2, or stx1/stx2. After the first live birth, there was no significant difference in the concentration of STEC among cows (data not shown).

However, since it is common practice to only retain animals that calf every year, parity and age were highly correlated. To further demonstrate this relationship, we plotted cattle age (range 2 to 12 years) and the parity of the cattle (number of previous live births). As shown in Figure 3-2B, the relationship between cattle age and parity in the cattle sampled during the second year of the study was found to be linear ($R^2 = 0.9025$), with approximately 85% of the cattle having a calf every year. Because both age and parity were directly related in this cohort, it was impossible to determine if the increase in prevalence in two-year old cows is due to aging through the first years of life or if the first pregnancy is responsible for the increase in STEC prevalence. Furthermore, because of the known seasonality of STEC shedding [110,217], future studies should include longitudinal sampling from a cohort of cattle to determine the seasonal effects on the prevalence of STEC by age group.

**Dynamics of STEC Prevalence Among Animals Studied in Both Years**

A subset of cattle (n=74) was sampled in both years to determine if the likelihood of colonization by STEC increased during the transition from heifer to cow, or was different for cows as they continued to age. The prevalence of STEC carrying stx1, stx2, and stx1/stx2 are presented in Table 3-1 for the cattle in the subset (n=74), and for heifers and cows separately. In this sub-sample, the overall prevalence of STEC in the herd remained the same between years at 58%, resulting from an increase in the
prevalence of stx2 from 36.5% to 48.7% (p-value = 0.128), a significant decrease in the prevalence of stx1/stx2 from 31.1% to 17.6% (p-value = 0.049), and no net change in the prevalence of cattle colonized by stx1. Among heifers, the prevalence of STEC increased from 40.6% to 53.1%, which supports our previous observation (Figure 3-2A) that heifers have higher rates of colonization when they become uniparous cows at two years of age. This increase was driven primarily by STEC carrying stx2, which increased from 31.3% to 46.9%. In contrast, cows had a reduction in the prevalence of STEC from 71.4% to 61.9% and had a lower likelihood of being colonized the second year. This was driven primarily by a 28.6% decrease in the prevalence of STEC carrying stx1/stx2 in cows between years, with cows being more than 70% less likely to be colonized by STEC the following year (p-value = 0.007).

The stx2 Genotype is Dominant in Cows in the Entire Cohort

Of cattle sampled in years one or two of this study (n=341), over half (54.8%) were found to harbor STEC. Among animals colonized by STEC, stx2 was the dominant genotype responsible for 70% of all colonization, followed by 34.2% and 12.3% stx1/stx2 and stx1, respectively. Heifers had significantly lower prevalence of STEC compared to cows (37.5% vs 57.14%; p-value = 0.021), which translated into a 55% reduction in the likelihood of being colonized by STEC (OR=0.45; 95% CI: 0.228, 0.888). Compared to cows greater than three years of age, those that were less than three years of age had a higher rate of colonization by stx2 (55.2% vs. 36.6%; p-value = 0.011) and were more than twice as likely to be colonized by stx2 (OR: 2.13; 95% CI: 1.19-3.8); however did not show significantly increases in prevalence of STEC (Figure 3). Above the age of three, cows did not have significantly different rates of STEC
colonization. This trend was further illustrated after the stratification of the prevalence of STEC carrying \textit{stx}1, \textit{stx}2, and \textit{stx}1/\textit{stx}2 by animal age (Figure 3-3). The resulting pattern of STEC prevalence indicates that most animals are likely exposed to STEC after their first or during their second year of life. After this period of peak prevalence, colonization of cattle by STEC begins to stabilize.

**Discussion**

Our findings provide insight into the prevalence of STEC in cattle that could be used to mitigate transmission to humans by lowering this pathogen at the pre-harvest level. In this study, we demonstrated that animal age is related to the prevalence of STEC in cattle. Heifers less than two years old without any previous live births had significantly lower STEC prevalence compared to cows that had previously given birth. We showed that STEC shedding is less prevalent in heifers compared to cows (Figure 3-1A). It is possible that multiple physiological factors could be responsible for the differences in prevalence observed in this study. These include the absence of pregnancy and/or lactation stress in animals that have yet to calve, changes in the microbial ecology of the gut, and differences in the function of animal immune systems. In a previous study, it was shown that older cows with a parity of \(\geq 4\) had greater odds of STEC prevalence compared to cows with a parity of \(<4\) [71], suggesting that parity influences the prevalence of STEC in cattle. However, we only observed a significant effect of parity on the likelihood of being colonized by STEC between heifers and cows, with no increased likelihood after first birth regardless of the subsequent number of pregnancies. Since this study included a herd of female cattle where over 85% gave birth
to a calf every year, the association between age and STEC prevalence was almost identical to that between parity and STEC prevalence (Figure 3-2B). Due to the difficulties of obtaining cows greater than two years old without previous live births, because the cow/calf operations remove cows that fail to calve by two years by culling it was particularly challenging to distinguish if the peak of STEC prevalence in the second year was mediated by first live birth or physiological maturity at the age of two.

Microbial ecology of the gut itself could be another possible explanation for why heifers had lower rates of STEC. Animals obtain indigenous microflora through grazing and contact with soil microbes that becomes more diverse and better adapted to ecological changes in older animals [219]. It has been reported that increased microbial diversity may inhibit pathogen proliferation and can lead to lower rates of colonization in animals [72,220,221] and persistence in manure [222,223]. Therefore, it is likely that older animals have more diverse microflora in the gastrointestinal tract and STEC may be outcompeted by normal microflora. However, further analysis of microflora diversity and STEC prevalence is necessary to understand this potential relationship.

Another speculation that may explain the dynamics of STEC prevalence in cows is that changes in the immune system affect the prevalence of STEC and the risk of STEC colonization. The presence of maternally acquired antibodies to STEC in the first year of life has been demonstrated to confer temporary resistance to STEC infection. It has been shown that maternal immunity may persist until weaning and maternal immunity is critical for STEC prevalence. After the disappearance of maternal antibodies, calves shed high concentrations of STEC for several months [224]. In the
current study, we determined that the prevalence and risk of shedding is greatest in cattle two years of age, after which the prevalence of STEC decreases significantly and continues to fluctuate bi-annually. This pattern of infection, recovery, and then re-infection could indicate that immunity develops as a result STEC infection in naïve cattle exposed during the first years of life and that the host immune response persists for a limited interval (i.e. approximately two years). If this is the case, a cycle of periodic infection and recovery would lead to a relatively constant prevalence in older animals, which is in agreement with the observations from this study. A similar pattern has been reported in humans, where the highest prevalence of infections were observed in the youngest age groups corresponding to a lack of Shiga toxin specific antibodies, with more frequent detection of antibodies after the first decade of life that are associated with a period of acquired immunity, after which they decrease and lead to re-infection [225]. Future studies should combine microbiological findings with the presence of antibodies to further investigate if this process is similar in cattle.

In summary, we have shown that animal age is a critical factor for the prevalence of STEC in cattle. These findings have clear implications for the development of on-farm mitigation strategies. By segregating animals with the highest risk of infection and highest rates of shedding, it could be possible to lower the concentrations of STEC at the pre-harvest level and prevent transmission from cattle to humans. This information could also be used to develop dynamic transmission models that use different rates of colonization with respect to age to simulate the prevalence in herds over time and evaluate the efficacy of potential interventions. Since these findings indicate when
animals become two years old they are more likely to be colonized and are more likely to transmit STEC to other animals through increased shedding, further understanding of factors that increases the STEC prevalence in this stage of development will be critically important to provide interventions to reduce STEC levels in animals. This could reduce the prevalence in older animals and prevent the harvesting of cattle that are more likely to harbor high concentrations of STEC and result in lower rates of transmission to humans through contaminated beef.
Table 3-1. Prevalence of Shiga toxin-producing *E. coli* in cattle in two years

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Year1</th>
<th>Year2</th>
<th>Diff.</th>
<th>p-value&lt;sup&gt;e&lt;/sup&gt;</th>
<th>OR&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Gained</th>
<th>Lost</th>
<th>Remained</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>58.1</td>
<td>58.1</td>
<td>0.0</td>
<td>1.0</td>
<td>1.00</td>
<td>20.3</td>
<td>20.3</td>
<td>37.8</td>
<td>21.6</td>
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<td>(n=74)</td>
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<td></td>
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</tr>
<tr>
<td>stx1</td>
<td>6.8</td>
<td>6.8</td>
<td>0.0</td>
<td>1.0</td>
<td>1.00</td>
<td>5.4</td>
<td>5.4</td>
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</tr>
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<td><strong>Year1</strong></td>
<td><strong>Year2</strong></td>
<td><strong>Diff.</strong></td>
<td><strong>p-value&lt;sup&gt;e&lt;/sup&gt;</strong></td>
<td><strong>OR&lt;sup&gt;d&lt;/sup&gt;</strong></td>
<td><strong>Gained</strong></td>
<td><strong>Lost</strong></td>
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<td><strong>Year1</strong></td>
<td><strong>Year2</strong></td>
<td><strong>Diff.</strong></td>
<td><strong>p-value&lt;sup&gt;e&lt;/sup&gt;</strong></td>
<td><strong>OR&lt;sup&gt;d&lt;/sup&gt;</strong></td>
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<td>19.0</td>
<td>-28.6</td>
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<td>9.5</td>
<td>38.1</td>
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<sup>a</sup>Prevalence of STEC determined by screening of fecal sample using Polymerase Chain Reaction (PCR)

<sup>b</sup>Percentage of cattle that gained (-)/(+) or lost (+)/(-) colonization by STEC; remain colonized (+)/(+) or uncolonized (-)/(-)

<sup>c</sup>Statistical significance of the change in STEC prevalence between years given by McNemar’s test; ** Denotes statistical significance at α = 0.05, *** denotes statistical significance at α = 0.01

<sup>d</sup>Likelihood of becoming colonized with STEC between years one and two

<sup>e</sup>Heifers are cattle aged 1 or 2 years old in the first study year with no previous live births

<sup>f</sup>Cows are cattle aged more than 2 years of age in the first study year with at least one previous live birth
Figure 3-1. Prevalence and concentration of STEC in a cohort of beef cattle. (A) The prevalence of STEC and *Enterobacteriaceae* (p-value < 0.05 **, p-value < 0.001 ***). (B) Concentration of STEC and *Enterobacteriaceae* isolated from heifers and cows.
Figure 3-2. Prevalence of STEC among uniparous and multiparous cattle. (A) The prevalence of STEC isolated from cows in the second year of the study (n=251). (B) The relationship between the cattle age (from 2 to 12 years) and number of previous live births (from 1 to 10 births ($R^2 = 0.90$)).
Figure 3-3. Prevalence of STEC isolated from all cattle sampled. All genotypes combined (black dotted), \textit{stx1} (blue line), \textit{stx2} (red line), \textit{stx1/stx2} (purple line), as well as cows colonized with multiple genotypes (orange line).
CHAPTER 4
COLONIZATION OF BEEF CATTLE BY SHIGA TOXIN-PRODUCING *Escherichia coli*
DURING THE FIRST YEAR OF LIFE: A COHORT STUDY

Each year Shiga toxin-producing *Escherichia coli* (STEC) are responsible for 2.8 million acute illnesses around the world and > 250,000 cases in the US. Lowering the prevalence of this pathogen in animal reservoirs has the potential to reduce STEC outbreaks in humans by controlling its entrance into the food chain. However, factors that modulate the colonization and persistence of STEC in beef cattle remain largely unidentified. This study evaluated if animal physiological factors such as age, breed, sex, and weight gain influenced the shedding of STEC in beef cattle. A cohort of beef calves (n=260) from a multi-breed beef calf population was sampled every three months after birth to measure prevalence and concentration of STEC during the first year of life. Metagenomic analysis was also used to understand the association between the STEC colonization and the composition of gut microflora. This study identified that beef calves were more likely to shed STEC during the first 6 months and that STEC shedding decreased as the animal matured. Animal breed group, sex of the calf, and average weight gain were not significantly associated with STEC colonization. The metagenomic analysis revealed for the first time that STEC colonization was correlated with a lower diversity of gut microflora, which increases as the cattle matured. Given these findings, intervention strategies that segregate younger animals, more likely to be colonized by STEC from older animals that are ready to be harvested, could be investigated as a method to reduce zoonotic transmission of STEC from cattle to humans.
Background

Infections from Shiga toxin-producing *Escherichia coli* (STEC) are responsible for 2.8 million acute illnesses around the world [226] and are estimated to cause more than 265,000 illnesses annually in the United States [89]. Among STEC serotypes, *E. coli* O157:H7 is the most well-known and causes illnesses such as diarrhea, hemorrhagic colitis, thrombocytopenic purpura and hemolytic uremic syndrome [110]. Cattle are asymptomatic carriers of STEC and have been suggested to be the primary reservoirs of human illness, where transmission can occur through several routes including contaminated food, fecal-oral contamination, and direct contact with animals [227,228].

Since the majority of human infections by STEC likely originate from contaminated animal products, especially beef [34], it has been proposed that a reduction in fecal shedding by cattle may significantly reduce human infections [73,229]. Thus, the reduction of STEC at the pre-harvest level represents an opportunity to control the exposure to contaminated animal products and reduce transmission to humans [230]. However, many of the factors that affect the colonization dynamics of STEC in beef cattle remain unidentified, which makes the reduction of STEC at the pre-harvest level challenging.

In a recent study [231], animal factors such as age and parity were significantly associated with STEC shedding, with the highest prevalence in cattle two years of age and the lowest prevalence in heifers. However, since the sample population only included female cattle older than one year of age, the colonization dynamics of STEC during the first year of life were unable to be examined. Likewise, with the inclusion of only female cattle of a single breed, the previously reported findings that animal breed...
and sex could affect the prevalence of *E. coli* O157:H7 in cattle were also unable to be corroborated [207]. To further investigate age, sex, and breed as potential factors for the colonization of STEC in beef cattle during the first year of life, a prospective cohort of new-born beef calves was followed for one year.

Additionally, we hypothesized that the differences in STEC colonization between heifers and cattle could be the result of differences in the gut microflora. Though adhesion factors and virulence profiles that affect colonization of STEC among cattle have been studied in detail [74,75], little attention has been given to the role of the diversity of bovine microflora. Metagenomic sequencing is a rapidly emerging as a tool for analysis of complex samples (fecal, rumen and soil) that defy conventional techniques and are able to identify microbes present at low concentrations [232]. Using metagenomic analysis, we hope to elucidate the role of microflora in STEC colonization by comparison of the composition of the microflora as the calves matured during the first year of life.

Our results indicated that animal age was associated with STEC shedding while breed, sex, or weight gain were not statistically significant factors in the likelihood of colonization by STEC during the first year of life. We found higher STEC prevalence and lower diversity of bovine microflora at early ages along with fundamental differences in the microflora composition between cattle that were colonized by STEC compared to those that were not.
Materials and Methods

Ethics Statement

Standard practices of animal care and use were applied to animals used in this project. The research protocols used in this study were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC Protocol #: 201308027).

Animal Management and Sample Collection

The study was conducted over a period of two years on a cohort of beef calves from a multi-breed beef calf population derived from Brahman and Angus cattle. Calves were housed at the Beef Research Unit of University of Florida (Gainesville, Florida USA). This farm has a loose system of housing with average stocking density equal to 1.5 acres (0.6 Ha) per cow. Typically cows are bred from March to May and calving takes place from late December to February. In this study calves were followed from birth through their first year of life with samples collected four times, spaced evenly three months intervals. Calves were kept with their dams until weaning, which took place in August (7-9 months of age) during the third sampling period. Calves were classified into six breed groups: breed group 1 (80 to 100% Angus and 0 to 20% Brahman), breed group 2 (60 to 79% Angus and 21 to 40 % Brahman), breed group 3 or "Brangus" (62.5% Angus and 37.5% Brahman), breed group 4 (40 to 59% Angus and 41 to 60% Brahman), breed group 5 (20 to 39% Angus and 61 to 80% Brahman), breed group 6 (0 to 19% Angus and 81 to 100% Brahman). Body weights were recorded at birth and at the time each fecal sample was collected.
**Fecal Sample Collection**

Fecal samples were collected in March (n=259), June (n=263), August (n=261) and December (n=193), representing the time at which calves were 1-3, 4-6, 7-9, and 10-12 months of age, respectively. Due to culling of calves for reasons unrelated to this study, only 193 calves were available for the December sample. The sampling scheme resulted in 188 animals that had four matched samples, which were used to assess colonization dynamics of STEC. Calves were categorized into three groups based on sex including bulls, steers (castrated males) and heifers. Fecal samples were collected from the recto-anal junction (RAJ) of calves using sterile cotton swabs, placed in a sterile 15 mL conical tube and all samples were transported on ice and processed the same day using the protocol described below.

**Identification of Shiga Toxin-producing *Escherichia coli***

This study utilized a combination of culture-based and nucleic acid-based methods for the detection and enumeration of Shiga toxin-producing *Escherichia coli* (STEC) from the fecal samples. Fecal swabs were resuspended with 2 mL of Tryptic Soy Broth (TSB) broth and spread plated onto MacConkey agar (Becton Dickinson Company, MD, USA) after 10-fold serial dilutions (up to 106). Plates were incubated at 37°C and examined after 24 hours for the enumeration of bacterial colonies. From the resulting plates, 20 pink colonies were selected randomly and purified on CHROMagar E. coli (CHROMagar, Paris, France). Ten blue colonies were subjected to colony PCR for the detection of stx1 and stx2 genes. A multiplex PCR was carried out using two sets of primers: KCP 11/12 for stx1 (655 bp amplicon) and KCP13/14 (477 bp amplicon) for stx2 gene. The primer sequence is as follows; KCP11 (5’-
TGTCGCA TAGTGGAACCTCA-3'), KCP12 (5'-TGCGCAGTGAAGAAGAGA-3'), KCP13 (5'-CCATGACACGGACAGCAGTT-3') and KCP14 (5'-TGTCGCCAGTTATCTGACATTC-3') [218]. The PCR reaction conditions were 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds; with a final extension at 72°C for 7 minutes. The amplified products were resolved by electrophoreses using a 1% agarose gel stained with ethidium bromide and visualized with a UV gel doc system (Bio-Rad, USA). For interpretation of PCR results, *E. coli O157:H7* (EDL933) and DH5α were used as positive and negative controls, respectively. Strains were sub-grouped depending on the toxin genotype. STEC positive was used to describe the strains carrying either or both Shiga-toxin genotypes and were further classified as stx1 or stx2, while strains with both genotypes were designated as stx1/stx2 positive.

**Statistical Analyses**

Simple logistic regression was used to determine if differences in herd prevalence were significantly different by cattle age as well as the association between the presence of STEC, stx1, stx2, and stx1/stx2 and the animal breed group (groups 1-6), and sex (bull, steer, or heifer), for the four sampling periods. Physical development was evaluated by the change in animal weight (kg) between sampling periods, which was used to determine animals that fell under the 25th, between the 25th and 75th, and above the 75th percentiles in weight gain. For the animals with four consecutive samples collected (n=188), the results of the microbiological testing between two sampling dates was used to determine the proportion of cattle that were not previously colonized and did not become colonized (−/−), were not previously colonized and
became colonized (-/+), those that remained colonized (+/+), and those animals that were previously colonized and are no longer colonized (+/-). Significant increases and decreases in prevalence between any two consecutive sampling periods were determined by using McNemar's test for matched pairs. All statistical analyses were conducted using STATA software package (STATA® MP 11.2, StataCorp, College Station, Texas, USA) with a significance threshold of $\alpha < 0.05$.

**Metagenomic DNA Extraction and Pyrosequencing**

To understand the association of microflora with animal age and its effect on the STEC dynamics, metagenomic analyses of the fecal samples from male and female calves from all six breed groups was conducted using 454 pyrosequencing (Macrogen, South Korea). The samples were collected at four different times during the first year of life. The DNA was extracted from the fecal samples with PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer’s protocol. Library was prepared using PCR products according to the GS FLX plus library prep guide. Libraries were quantified using Picogreen assay (VICTOR 3). The emPCR, corresponding to clonal amplification of the purified library, was carried out using the GS-FLX plus emPCR Kit (454 Life Sciences). Briefly, libraries were immobilized onto DNA capture beads. The library-beads obtained were added to a mixture of amplification mix and oil and vigorously shaken on a Tissue Lyser II (Qiagen) to create "micro-reactors" containing both amplification mix and a single bead. Emulsion was dispensed into a 96-well plate and the PCR amplification program was run according to the manufacturer's recommendations. Twenty monogram aliquot of each sample DNA was used for a 50 ul PCR reaction. The 16S universal primers 27F (5’-
GAGTTTGATCMTGGCTCAG-3'), 518R (5'-WTTACCGCGGCTGCTGG-3') were used for amplifying of 16S rRNA genes. FastStart High Fidelity PCR System (Roche) was used for PCR under the following conditions: 94°C for 3 minutes followed by 35 cycles of 94°C for 15 seconds; 55°C for 45 seconds and 72°C for 1 minute; and a final elongation step at 72°C for 8 minutes. After the PCR reaction, the products were purified using AMPure beads (Beckman coulter) and sequenced using the following method by Macrogen Ltd. (Seoul, Korea). Following PCR amplification, the emulsion was chemically broken and the beads carrying the amplified DNA library were recovered and washed by filtration. Positive beads were purified using the biotinylated primer A (complementary to adaptor A), which binds to streptavidin-coated magnetic beads. The DNA library beads were then separated from the magnetic beads by melting the double-stranded amplification products, leaving a population of bead-bound single-stranded template DNA fragments. The sequencing primer was then annealed to the amplified single-stranded DNA. Lastly, beads carrying amplified single-stranded DNA were counted with a Particle Counter (Beckman Coulter). Sequencing was performed on a Genome Sequencer FLX plus (454 Life Sciences), and each sample was loaded in 1 region of a 70 mm-75 mm PicoTiter plate (454 Life Sciences) fitted with a 8-lane gasket.

**Selection of 16S rRNAs and Taxonomic Assignment**

Using the basic local alignment search tool (BLASTN), all the sequence reads were compared to Silva rRNA database. Sequence reads which had sequence similarity with less than 0.01 E-value were admitted as partial 16S rRNA sequences. Non-16S rRNA sequence reads comprised less than 1% of all reads. Taxonomic assignment of
the sequenced read was carried out using NCBI Taxonomy Databases. The five most similar sequences for each sequence read were found by their bit scores and E-value from the BLAST program. Needleman-Wunsch global alignment algorithm was used to find the optimum alignment of two sequences along their entire length. A pairwise global alignment was performed on selected candidate hits to identify the best aligned hit. The taxonomy of the sequence with the highest similarity was assigned to the sequence read. By the similarity, we assigned the taxonomy down to these taxonomical hierarchies; species with more than 97% similarity, genus 94%, family 90%, order 85%, class 80%, and phylum 75%.

**Operational Taxonomic Unit (OTU) Analysis for Community Richness**

CD-HIT-OTU software was used for clustering. Mothur software was used for analyzing microbial communities and Shannon-Weaver diversity index and Simpson index were used for species diversity. Statistical analysis of the metagenomic data for the microflora diversity and richness within the calves for different ages was analyzed using SAS (Version 9.4) and R program (R Development Core Team, 2010 #228) was used to generate heat maps for the various OTUs present in the fecal samples. Mean proportions and 95% CIs were used to describe the changes in proportions of the 16S rDNA reads assigned to different OTUs present in the fecal samples. Mean proportions of the bacterial taxa (OTUs) within the study groups were analyzed using a generalized linear mixed model in SAS (version 9.4, SAS Institute Inc., Cary, NC, USA).
Results

High Proportion of Young Beef Calves were Colonized with STEC

The herd prevalence of STEC in young calves 1-3 months old was 60.3%, which was significantly higher compared to older calves (P < 0.001). As the calves matured, the prevalence decreased to 39.5% at 4-6 months of age, 20.3% at 7-9 months of age, and 20.7% in 12 month old calves (Figure 4-1A). The prevalence of stx2 genotype was higher compared to stx1 and stx1/stx2 genotypes in all the four sampling times, except in 7-9 months old calves, where it was lower but not significantly different than stx1 (7.4 vs 9.0%). The prevalence of stx1/stx2 genotype was the lowest in all samplings except for the 12 month old calves. In contrast to the steady decline of animals colonized with the stx1 genotype, the shedding of the stx2 genotype began to increase at the age of 12 months, however only in female cattle (7.4 vs 14.9%).

The relationship between natural gut microflora on the STEC prevalence was shown by plotting the concentration of Enterobacteriaceae and STEC (log CFU) during the four sampling periods (Figure 4-1B). The concentration (log CFU) of Enterobacteriaceae and STEC shed by the calves was found to be normally distributed (Shapiro-Wilk normality test, P < 0.0001), allowing us to carry out further analysis on the concentration data. Calves shed the highest concentration of STEC in early age (1-3 month period) and as they matured, the colonization became less prevalent and the concentrations shed were lower. The concentration of Enterobacteriaceae shed remained the same among various age groups (Figure 4-1B). Reduced STEC prevalence with increased calf age was confirmed in the second year of sample collection wherein, the overall STEC prevalence at 9-12 months of age was 20.3% and
20.7%, respectively. Similarly, a higher proportion of the calves were shedding stx2 genotype compared to the stx1 or stx1/stx2 genotypes at 9 and 12 months of age in second year of sampling.

**Association Between Animal Factors and STEC Prevalence**

To understand if animal sex affects the prevalence of STEC, the animals were stratified by sex. Calves in all three groups (bull, heifer, steer) had higher prevalence of STEC shedding at 1-3 months of age and the prevalence of STEC generally decreased as they matured (Figure 4-2A). No significant association was found between calf sex and any STEC genotype. However, heifers had significantly higher rates of STEC shedding at 12 months of age (OR= 2.11, P = 0.004) compared to their male counterparts, which actually increased from 6 months of age from 15.9 to 30.2 % (Figure 4-2A). There was no significant difference in the concentration of *Enterobacteriaceae* or STEC (of those animals that were positive) shed by the males and females, but the concentration of *Enterobacteriaceae* was higher than STEC (Figure 4-2B). The prevalence of stx1 decreased with the age of calves irrespective of the sex (Figure 4-2C). The prevalence of stx2 genotype was higher (43.1 %) in young calves of 1-3 months of age and decreased with the age of the calf except in females, which actually increased at 10-12 months of age (Figure 4-2D). The prevalence of stx1/stx2 genotype was very low in all age groups of the calves and the concentration was not associated with the sex of calf (Figure 4-2E).

To understand if animal genetics affects the prevalence of STEC, the animals were stratified by breed groups. All breed groups showed a general trend of decreasing STEC prevalence from higher rates of colonization at 1-3 months of age to very low
levels at one year age (Figure 4-3A). Overall breed was not significantly associated with STEC colonization, however there were some differences observed between breeds. No significant differences in the concentration of Enterobacteriaceae or STEC shed by the different breed groups were identified, even though the concentration of Enterobacteriaceae was higher than STEC (Figure 4-3B). There was a higher prevalence of stx1 genotype in breed group 1 at 1-3 months of age while the prevalence remained low and was not significantly different among breed groups as the calves matured (Figure 4-3C). The prevalence of stx2 genotype was higher in breed group 6 in 1-3 month old calves (P < 0.05), while the 4-6 month old calves of breed group 1 had a higher prevalence of stx2 (Figure 4-3D). The prevalence of stx2 genotype decreased in all breed groups as the calves matured (Figure 4-3D). At each sampling point there was a very low prevalence of the stx1/stx2 genotype that was not significantly different among the breed groups (Figure 4-3E).

Cattle growth was also examined as a potential factor for STEC colonization. The average cattle weights show a steady linear growth pattern during the first year of life which inversely correlates with STEC prevalence in the herd (Figure 4-4A). After further stratification of the animal growth rates into < 25th, 25th to 75th and > 75th percentiles, animals that gained more weight between sampling periods had no significant differences in the likelihood of becoming colonized with STEC, but the STEC prevalence was lower in animals that gained more weight (75th percentile) (Figure 4-4B). There was also no significant association between breed group, sex of the calf, and
average weight gain with the STEC shedding in the second year of sampling (data not shown).

**Colonization Dynamics of STEC in Calves**

Assuming that the calves were not colonized at birth, 61.2% of the animals had become colonized by the first sampling period at 1-3 months of age, followed by a net decrease of 23.9% driven primarily by a large proportion of previously colonized animals no longer shedding stx1 (27.7%) and stx2 (33.5%). Though 16.5% of cattle became colonized between 3-6 months of age, after the peak prevalence observed at 3 months age, the number of animals that were becoming colonized was much lower (Table 4-1). Furthermore, the number of animals which remained uncolonized after 3 months of age increased from 23.4% at 6 months of age to 63.8% in one year old calves. Similarly, those animals colonized at 3 months of age, 21.8% of them remained colonized at 6 months, followed by a marked decrease to only 7.4 and 4.3% remaining colonized at 9 and 12 months of age, respectively. This high rate of colonization followed by a period of recovery from colonization leads to a relatively lower herd prevalence of STEC at the age of one year, where no net gain in animals becoming newly colonized was observed. These dynamics are shown graphically in Figure 4-5, where after an initially high rate of colonization by STEC, a high proportion of the animals become no longer colonized and remain STEC negative. After the majority of cattle were no longer colonized by STEC, a much smaller proportion of the animals fluctuate between being colonized and not colonized, resulting in a stabilization of herd prevalence by the end of the first year of life.
**Metagenomic Analysis Revealed a More Diverse Microflora in Older Cattle**

In order to determine the role of fecal microflora in STEC dynamics, DNA collected from the fecal samples of calves was analyzed using 454 pyrosequencing. Shannon index has been used to measure microbial diversity and the relative abundance of Firmicutes (F) and Bacteroidetes (B) have been used to measure overall animal health and production traits. It has been shown that the higher F: B ratio is positively associated with milk production and animal health. In this study, we identified that the Shannon diversity index was higher in fecal samples from older animals (P = 0.0074) (Figure 4-6A) and increased linearly with the F: B ratio (Figure 4-6B). Samples collected from calves not colonized by STEC had a higher Shannon index (P = 0.073) (Figure 4-6C), which decreased as the concentration of STEC increased (Figure 4-6D). Calves shedding stx1 or stx1/stx2 genotypes had a higher percentage of the phylum Bacteroidetes (P = 0.011) and class Bacteroidia (P = 0.0704). Some of the bacterial taxonomic units such as Proteobacteria, Gammaproteobacteria, Sphingobacteriales, Enterobacteriales, were more prevalent in STEC positive samples (P<0.05), while the STEC negative samples had a higher proportion containing Erysipelotrichi, Xanthomonadales, Bacillales, Neisseriales (P<0.05) (Figure 4-7) (Table 4-2). Microflora diversity increased with age (Figure 4-8A) and was linearly correlated with F: B ratio (Figure 4-8B) and microflora showed difference among four ages (Figure 4-8C). We also observed differences in the abundance of various Operational Taxonomic Units (OTUs) between male and female calves (Table 4-3, Figure 4-9A), indicating that microflora might be related to animal sex. Only few of the OTUs were different among breed groups (Figure 4-9B), with Breed 1 having higher Firmicutes (P= 0.0786),
Tenericutes ($P=0.0343$) and Lentisphaerae ($P = 0.0021$) (Table 4-4). The OTUs that were identified at 1-3 months of age were significantly different ($P<0.05$) from the OTUs identified at 10-12 months of age (Table 4-5, Figure 4-8C). For example, older calves had higher Spirochaetes ($P = 0.0039$) while the younger calves had higher Desulfovibrionales ($P = 0.00132$) and Proteobacteria ($P = 0.0003$).

In the fecal samples analyzed in this study the F: B ratio was higher in older calves (Figure 4-8A) and lower in STEC positive animals, with respect to both the prevalence ($P = 0.043$), and the concentration (log CFU) of STEC ($P = 0.0468$). STEC negative samples had a higher F: B ratio (Figure 4-8B) which increased linearly, but not significantly with the concentration of Enterobacteriaceae ($P = 0.83$). The Shannon index increased linearly with F: B ratio ($P = 0.246$) (Figure 4-6B) and was higher, but not statistically significant in STEC negative samples ($P= 0.073$). No significant differences were found in the Shannon index among different breed groups or sex categories of the calves.

**Discussion**

By following a cohort of beef calves born in the same calving period from a single farm, we were able to gain novel insights into the dynamics of STEC colonization. Utilization of metagenomic analyses also allowed for the first time an investigation of the role of gut microflora on the dynamics of STEC colonization.

Though in the previous study [231] heifers were identified to have a lower prevalence of STEC compared to older cattle, further analysis during the first year of life revealed that the highest rates of STEC shedding were observed during the first 6 months of calf life. In agreement with the previous study, by the end of the first year of
life only approximately 20% of cattle were colonized by STEC. Interestingly, the major
STEC genotype isolated from 12 months old calves was stx2. Two stx genes encode
Shiga toxins which are stx1AB and stx2AB, and it has been reported that Stx2 is
associated with more severe human infections [23,233]. The predominance of stx2
genotype in older calves is noteworthy, although the overall STEC prevalence declined
when animals aged, because Stx2 is 1000 times more toxic than Stx1 as shown
previously in animal models [234,235]. In addition, recent Shiga toxin producing E. coli
O157:H7 outbreak strains have been found to carry two copies of the stx2AB gene
cluster instead of one of the stx1AB and one of the stx2AB clusters each, making them
more virulent than the isolates carrying only the stx1AB gene cluster, due to lack of
competition for GB3 binding [23,233]. Taken together, although the prevalence of STEC
was lower in older animals, the calves were carrying more pathogenic STEC,
suggesting further STEC genotyping is necessary for proper risk assessment.

Genome wide analysis has shown that STEC O157 can be grouped into three
main phylogenetic lineages, called lineages I, II and I/II. Lineage I isolates originate from
human clinical and bovine sources, while a majority of lineage II isolates have bovine
origins. Lineage I/II include strains isolated from human infections and hyper-virulent
strains that include a multistate spinach outbreak [229,236,237]. These three STEC
O157 lineages carry a genetic signature in which each lineage encodes unique stx
genes; stx1 and stx2a are more frequently identified in lineage I; whereas, stx1 and
stx2c are detected in lineage II and stx2a and stx2c are predominantly identified from
lineage II [236]. In this study, we have evaluated the dynamic prevalence of variant
STEC subtypes of stx1, stx2, and/or stx1/2 during animal aging. Further analysis to differentiate stx2 subtypes could provide clues to assign STEC isolates into the three lineages that may help predict potential burden of the isolates in cattle.

In this study we investigated other physiological factors such as breed, sex and average weight gain for their role in STEC shedding, but were unable to find any significant relationships between these factors and STEC colonization. However, the metagenomic analyses indicated that older calves have a more diverse microflora that was associated with a reduced prevalence of STEC as the calves matured. The correlation between increased diversity of microflora and STEC prevalence was confirmed in a second year of sample collection using a separate group of animals. The results from the second year of sampling also indicated that there was no significant association between breed, sex or weight gain of animal and STEC prevalence.

The low concentration of normal Enterobacteriaceae microflora coupled with higher concentrations of STEC in the early stages of life may be explained by the lower concentration and diversity of commensal microflora in the gut of the young calves, which increases with age [220,221]. The microflora could have also been affected by the diet of the animals [238], which changes as the animal matures, weans, and begins grazing in the pasture. Thus, the animal diet might be one mechanism by which the microflora and the colonization of STEC are associated with the age of the animals. In the current study, it was also observed that the Shannon index increased as the calves mature and was associated with a lower STEC prevalence in older calves. No significant differences in the Shannon index were observed between breed groups or
between male and female calves. Previously, a higher F: B ratio was associated with a more diversified microflora, which leads to better health and production traits of cows [239,240]. Since the results of this study indicated that the Shannon index was linearly correlated with the F: B ratio, our hypothesis that older calves have more diverse microflora which reduces STEC colonization was supported by the metagenomics analyses.

The prevalence of STEC has been suggested to increase markedly after weaning (16.6% to 38.3%) and remain elevated during the pre-conditioning period in high moisture fed calves due to stresses caused by diet change [212]. Similar results were also reported in dairy calves where the shedding of STEC and E. coli O157 increased soon after weaning [208]. However, in this study, evaluated with beef calves that were weaned around 6-8 months of age onto a pasture with free access to water, no significant increase in the STEC shedding was identified after weaning in both years. This might be the result of the access to grazing on loose pasture that reduces animal density and prevents transmission of STEC from other animals, which could have led to a reduction in the shedding of STEC by weaned calves. Another possible reason for the increased prevalence during the first sampling period was the waning of maternal antibodies and subsequent colonization, followed by the development of the calf’s own antibodies to STEC. In dairy calves, it has been reported that colostrum antibodies against stx1 and stx2 reach peak titers within first 24 hours of life and stx1 antibody can be detected up to four weeks [224]. However, since antibodies were not investigated in
this study, the effect of maternal or acquired immunity was not able to be determined and could be a focus of future research.
<table>
<thead>
<tr>
<th>Animal Factors</th>
<th>1 to 3</th>
<th>4 to 6</th>
<th>7 to 9</th>
<th>10 to 12</th>
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<tr>
<td>Sex</td>
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<td></td>
</tr>
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<td>Bull</td>
<td>68</td>
<td>59.7</td>
<td>67</td>
<td>37.3</td>
</tr>
<tr>
<td>Steer</td>
<td>60</td>
<td>59.3</td>
<td>64</td>
<td>45.3</td>
</tr>
<tr>
<td>Heifer</td>
<td>131</td>
<td>61.1</td>
<td>132</td>
<td>37.9</td>
</tr>
<tr>
<td>Group 1</td>
<td>42</td>
<td>68.3</td>
<td>42</td>
<td>47.6</td>
</tr>
<tr>
<td>Genetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Group 2</td>
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<td>41</td>
<td>39.0</td>
</tr>
<tr>
<td>Breed</td>
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<td></td>
<td></td>
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<tr>
<td>Group 3</td>
<td>30</td>
<td>53.3</td>
<td>30</td>
<td>33.3</td>
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<tr>
<td>Group 4</td>
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<td>38.9</td>
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<td>Group 5</td>
<td>40</td>
<td>57.5</td>
<td>41</td>
<td>48.8</td>
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<td>Group 6</td>
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<td>75.0</td>
<td>37</td>
<td>27.0</td>
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<tr>
<td>Weight</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>&lt; 25%</td>
<td>61</td>
<td>67.2</td>
<td>65</td>
<td>47.7</td>
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<tr>
<td>Gain</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>25 to 75%</td>
<td>131</td>
<td>58.0</td>
<td>123</td>
<td>39.8</td>
</tr>
<tr>
<td>&gt;75%</td>
<td>67</td>
<td>56.7</td>
<td>75</td>
<td>32.0</td>
</tr>
<tr>
<td>Total</td>
<td>259</td>
<td>60.3</td>
<td>263</td>
<td>39.5</td>
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Table 4-2. STEC genotype prevalence and dynamics in calves

<table>
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<tr>
<th>Sample</th>
<th>Genotype</th>
<th>Prevalence</th>
<th>Diff.</th>
<th>P value OR</th>
<th>Gained (-)/(+)</th>
<th>lost (-)/(-)</th>
<th>remained (+)/(+)</th>
<th>remained (-)/(-)</th>
</tr>
</thead>
<tbody>
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<td>Sample I</td>
<td>STEC</td>
<td>61.2</td>
<td>61.2</td>
<td>-</td>
<td>61.2</td>
<td>-</td>
<td>-</td>
<td>38.8</td>
</tr>
<tr>
<td>1 to 3</td>
<td>stx1</td>
<td>32.4</td>
<td>32.4</td>
<td>-</td>
<td>32.4</td>
<td>-</td>
<td>-</td>
<td>67.6</td>
</tr>
<tr>
<td>Months</td>
<td>stx2</td>
<td>43.1</td>
<td>43.1</td>
<td>-</td>
<td>43.1</td>
<td>-</td>
<td>-</td>
<td>56.9</td>
</tr>
<tr>
<td>March</td>
<td>stx1/2</td>
<td>6.9</td>
<td>6.9</td>
<td>-</td>
<td>6.9</td>
<td>-</td>
<td>-</td>
<td>93.1</td>
</tr>
<tr>
<td>Sample II</td>
<td>STEC</td>
<td>37.2</td>
<td>-23.9</td>
<td>0.000</td>
<td>0.39</td>
<td>15.4</td>
<td>39.4</td>
<td>21.8</td>
</tr>
<tr>
<td>4 to 6</td>
<td>stx1</td>
<td>14.4</td>
<td>-18.1</td>
<td>0.000</td>
<td>0.35</td>
<td>9.6</td>
<td>27.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Months</td>
<td>stx2</td>
<td>26.1</td>
<td>-17.0</td>
<td>0.001</td>
<td>0.49</td>
<td>16.5</td>
<td>33.5</td>
<td>9.6</td>
</tr>
<tr>
<td>June</td>
<td>stx1/2</td>
<td>0.5</td>
<td>-6.4</td>
<td>0.001</td>
<td>0.08</td>
<td>0.5</td>
<td>6.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Sample III</td>
<td>STEC</td>
<td>20.2</td>
<td>-17.0</td>
<td>0.000</td>
<td>0.43</td>
<td>12.8</td>
<td>29.8</td>
<td>7.4</td>
</tr>
<tr>
<td>7 to 9</td>
<td>stx1</td>
<td>9.0</td>
<td>-5.3</td>
<td>0.105</td>
<td>0.58</td>
<td>7.4</td>
<td>12.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Months</td>
<td>stx2</td>
<td>7.4</td>
<td>-18.6</td>
<td>0.000</td>
<td>0.24</td>
<td>5.9</td>
<td>24.5</td>
<td>1.6</td>
</tr>
<tr>
<td>August</td>
<td>stx1/2</td>
<td>3.7</td>
<td>3.2</td>
<td>0.033</td>
<td>7.00</td>
<td>3.7</td>
<td>0.5</td>
<td>0.0</td>
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<tr>
<td>Sample IV</td>
<td>STEC</td>
<td>20.2</td>
<td>0.0</td>
<td>1.000</td>
<td>1.00</td>
<td>16.0</td>
<td>16.0</td>
<td>4.3</td>
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<tr>
<td>10 to 12</td>
<td>stx1</td>
<td>1.6</td>
<td>-7.4</td>
<td>0.002</td>
<td>0.18</td>
<td>1.6</td>
<td>9.0</td>
<td>0.0</td>
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<td>Months</td>
<td>stx2</td>
<td>14.9</td>
<td>7.4</td>
<td>0.027</td>
<td>2.08</td>
<td>14.4</td>
<td>6.9</td>
<td>0.5</td>
</tr>
<tr>
<td>December</td>
<td>stx1/2</td>
<td>3.7</td>
<td>0.0</td>
<td>1.000</td>
<td>1.00</td>
<td>3.7</td>
<td>3.7</td>
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85
Table 4-3. Metagenomic analysis of microflora of STEC positive and negative animals

<table>
<thead>
<tr>
<th>Non-STEC OTUs</th>
<th>p-value</th>
<th>STEC OTUs</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>c- Erysipelotrichi</td>
<td>0.0471</td>
<td>p- Proteobacteria</td>
<td>0.0452</td>
</tr>
<tr>
<td>o- Xanthomonadales</td>
<td>0.0232</td>
<td>c- Gammaproteobacteria</td>
<td>0.0214</td>
</tr>
<tr>
<td>o- Neisseriales</td>
<td>0.0101</td>
<td>o- Sphingobacteriales</td>
<td>0.0188</td>
</tr>
<tr>
<td>o- Bacillales</td>
<td>0.0143</td>
<td>o- Enterobacteriales</td>
<td>0.022</td>
</tr>
<tr>
<td>o- Erysipelotrichales</td>
<td>0.0469</td>
<td>f- <em>Enterobacteriaceae</em></td>
<td>0.0332</td>
</tr>
<tr>
<td>f- Erysipelotrichaceae</td>
<td>0.0469</td>
<td>g- Blautia</td>
<td>0.0102</td>
</tr>
</tbody>
</table>
Table 4-4. Metagenomic analysis of microflora of calves from different age groups

<table>
<thead>
<tr>
<th>Abundant OTUs</th>
<th>1-3 months</th>
<th>p- value</th>
<th>4-6 months</th>
<th>p- value</th>
<th>7-9 months</th>
<th>p- value</th>
<th>10-12 months</th>
<th>p- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>o- Desulfovibrionales</td>
<td>0.0132</td>
<td></td>
<td>f- Proteobacteria</td>
<td>0.0003</td>
<td>f- Lachnospiraceae</td>
<td>0.0064</td>
<td>p- Spirochaetes</td>
<td>0.0039</td>
</tr>
<tr>
<td>o- Entomoplasmatales</td>
<td>0.0072</td>
<td></td>
<td>o- Sphinobacteriales</td>
<td>0.028</td>
<td>g- Dermatophilus</td>
<td>0.005</td>
<td>p- Actinobacteria</td>
<td>0.0799</td>
</tr>
<tr>
<td>o- Pasteurellales</td>
<td>0.0155</td>
<td></td>
<td>o- Enterobacteriales</td>
<td>0.0208</td>
<td>g- Sutterella</td>
<td>0.0062</td>
<td>c- Spirochaetes</td>
<td>0.0039</td>
</tr>
<tr>
<td>o- Sphinobacteriales</td>
<td>0.028</td>
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<td>f- Paenibacillaceae</td>
<td>0.0057</td>
<td>g- Gemella</td>
<td>0.0081</td>
<td>c- Erysipelotrichi</td>
<td>0.003</td>
</tr>
<tr>
<td>o- Enterobacteriales</td>
<td>0.0208</td>
<td></td>
<td>f- Pasteurellaceae</td>
<td>0.0064</td>
<td>g- Desulfuvibrio</td>
<td>0.0148</td>
<td>o- Spirochaetales</td>
<td>0.0038</td>
</tr>
<tr>
<td>f- Spiroplasmataceae</td>
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<td></td>
<td>f- Lachnospiraceae</td>
<td>0.0064</td>
<td></td>
<td></td>
<td>o- Anaeroplasmatales</td>
<td>0.0028</td>
</tr>
<tr>
<td>f- Desulfovibrionaceae</td>
<td>0.0134</td>
<td></td>
<td>g- Gemella</td>
<td>0.0081</td>
<td></td>
<td></td>
<td>o- Erysipelotrichales</td>
<td>0.003</td>
</tr>
<tr>
<td>f- Paenibacillaceae</td>
<td>0.0057</td>
<td></td>
<td>g- Paenibacillus</td>
<td>0.0065</td>
<td></td>
<td></td>
<td>f- Peptococcaceae</td>
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<td>f- Pasteurellaceae</td>
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<td>f- Dermatophilaceae</td>
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<td>f- Acetobacteraceae</td>
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<tr>
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<td>f- Spirochaetaceae</td>
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<td>f- Anaeroplasmataceae</td>
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<tr>
<td>g- Spiroplasma</td>
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<td></td>
<td></td>
<td></td>
<td>f- Peptostreptococcaceae</td>
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<td>g- Butyricimonas</td>
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<td>g- Hespellia</td>
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<td>g- Turicibacter</td>
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<td>g- Treponema</td>
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<tr>
<td>g- Paenibacillus</td>
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<td>g- Blautia</td>
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<td>g- Anaeroplasma</td>
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</table>

OTUs = Operational Taxonomical Units; p = Phylum; c = Class; o = Order; f = Family; g = Genus
Figure 4-1. STEC prevalence and conc. (Log cfu/swab) with calf age (A) The prevalence of STEC and various genotypes at different ages in beef calves. (B) The concentration (LogCFU/swab) of the STEC and normal flora (Enterobacteriaceae) with the calf age.
Figure 4-2. STEC prevalence and sex of the calf. (A) Overall prevalence of STEC. (B) Concentration of STEC and Enterobacteriaceae. (C) Prevalence of Stx1 genotype. (D) Prevalence of Stx2 genotype. (E) Prevalence of Stx1/Stx2 genotype in different sexes of calves
Figure 4-3. STEC genotypes with animal breed group of the calf (A) Overall prevalence of STEC. (B) Concentration of STEC and Enterobacteriaceae. (C) Prevalence of Stx1 genotype. (D) Prevalence of Stx2 genotype. (E) Prevalence of Stx1/Stx2 genotype in different breeds of calves
Figure 4-4. STEC dynamics with weight gain. A) Average body weight and STEC prevalence. B) Body weight gain and STEC prevalence (%)
Figure 4-5. STEC dynamics in the first year of life of beef calves
Figure 4-6. Microflora diversity and STEC prevalence. (A) Association of Shannon index with age. (B) Association of Shannon index with F: B ratio. (C) Association of Shannon index with Enterobacteriaceae. (D) Association of Shannon index with STEC concentration.
Figure 4-7. Microflora and STEC animals
Figure 4-8. Microflora diversity and STEC prevalence (A) Difference in F:B ratio with age. (B) Positive association between concentration of *Enterobacteriaceae* and F: B ratio (C) Heat map showing microflora diversity in different age groups
Figure 4-9. Heat map of microflora diversity in animals. (A) Difference in microflora between male and female calves. (B) Difference in microflora among six breed groups.
CHAPTER 5
IDENTIFICATION OF NATURALLY OCCURRING CEFOTAXIME RESISTANT MICROORGANISMS IN FARM ANIMALS

We isolated cefotaxime (a third-generation cephalosporin) resistant microorganisms from cattle, which have never been exposed to antibiotics. The prevalence of cefotaxime resistant Enterobacteriaceae bacteria in cattle was varying among farms, ranging from 5.2% to 100%. The bacterial isolates were resistant to high concentrations of cefotaxime and showed multi drug resistance against ten different antibiotics. Analysis of gene sequences revealed that most of the isolates are commensal environmental bacteria. The resistance is not confined to few genera of bacteria but is prevalent in the wide range of genera. Several extended spectrum β-lactamase producers, identical to clinical isolates, were detected by multiplex PCR. The findings of this study suggest that antibiotic resistance develops in nature and may be transmitted to the food animals from the environment. The basic mechanism of development of antibiotic remains yet to be understood.

Background

Antibiotic resistance causes more than 23000 deaths and $55bn in US (overall societal costs) and resistance to several classes of antibiotics has been reported from hospitals in more than 100 countries around the world [39]. Natural bacterial resistome plays a vital role in evolution and spread of antibiotic resistance [241,242] and acquisition of resistance is independent of antibiotic usage [243,244]. The conjugative transfer of resistance genes between bacteria in the absence of antibiotic selection has already been shown in turkey poults [245]. Previous studies have reported the presence of antibiotic resistance in food animals, but the origin of resistance remains unknown.
Also, the mechanisms by which bacteria acquire antibiotic resistance are poorly understood [241,248,249]. The US is one of the intensive users of antibiotics in the developed world and cephalosporins contribute 14% of total dispensed antibiotics in the US [125,250]. Cefotaxime is a third generation cephalosporin widely used to treat infections and surgical care [251,252]. But resistance to antibiotics including cephalosporins has been reported from hospitals in more than 100 countries around the world [39]. The resistance is usually mediated by production of extended spectrum β-lactamase (ESBL) enzymes by bacteria [253]. Previous studies have reported the presence of ESBL producers in food animals, but the origin of resistance remains unknown and animals in those studies had history of antibiotic exposure [61,246,247,253-255]. Present study describes the prevalence of cefotaxime resistance in cattle farms with no history of prophylactic antibiotic use and identifies the factors that influence antibiotic resistance. Knowledge about the factors that influence the dynamics of resistance can be useful in controlling antibiotic resistance [50,256,257].

To understand the factors that affect the prevalence of cefotaxime resistance in animals we sampled cattle directly at the farms. Cefotaxime is not used as a prophylactic agent in animals. We found that the microflora in farm animals is resistant to cefotaxime even if the animals were never exposed to prophylactic antibiotics. Members of the Enterobacteriaceae family carry and transfer resistance genes against third-generation cephalosporins at high rate [258-261]. Percentage of resistance varied among different farms. We also found that animals at each farm have characteristic predominant microflora. Sequencing and identification of these resistant isolates
showed that most of them are commensals in nature and the bacteria have resistance genes even if the antibiotic was never used on these farms. This study provides insight to the understanding of prevalence of cefotaxime resistance in food animals. Further study about the farm and animal factors is needed to better understand the whole dynamics of antibiotic resistance.

**Method and Materials**

**Fecal Swab Samples were Collected from 1365 Animals**

Swab samples were collected directly from animals. We selected 11 different herds across the North and Central Florida (Figure 5-1A) with different rearing systems, herd density, management policy and stage of production. One swab sample from each animal was collected at the recto-anal junction of the animal and a total of 1365 samples were collected for the prevalence study. The samples were collected with a sterile cotton swab (Fisher®) and placed in a sterile 15mL centrifuge tube (Falcon®). The samples were transported on ice and were processed same day.

**Samples were Plated on Media Containing 4µg/ml of Cefotaxime**

Samples were serially diluted (up to 10-4) with Luria Bertanii (LB) broth and then spread on to Tryptic Soy Agar and MacConkey agar (Becton Dickinson Company, MD USA) both containing cefotaxime @ 4µg/ml. Plates were incubated at 37ºC and examined after 24 hours for the enumeration of bacterial colonies.

**Cefotaxime Resistance was Characterized Both Phenotypically and Genetically**

Resistance to cefotaxime is acquired due to the production of extended spectrum β- lactamase which was detected by streaking the cefotaxime positive isolates on ChromAgar ESBL®. The production of colored colonies confirms the ESBL producers
For the genetic characterization, the DNA of cefotaxime resistant isolates was used as a template for the multiplex PCR to amplify nine ESBL genes using primer set shown in (Table 1) [264]. The PCR conditions were 95°C for 5 min for initial denaturation, 95°C for 30 seconds, 55°C for 35 seconds, 72°C for 90 seconds (30 cycles) and a final extension at 72°C for 7 min. The amplified products were resolved on 1% agarose gel stained with ethidium bromide and visualized with a UV gel doc system (Bio-Rad, USA). For the sequencing of the ESBL genes (blaTEM and blaCTX-M), the PCR product was amplified and eluted using QIAEX II Gel Extraction Kit (Qiagen Inc, Germany). The eluted product was sent for sequencing to Cancer and Genetics Research Center (CGRC) at University of Florida, Gainesville FL, USA. The raw unedited gene sequences were analyzed using Mega5® and Bio Edit® programs.

**Bacterial Isolates were Tested for Multidrug Resistance Using Antibiotic Discs**

The isolates were tested against 10 antimicrobials by the standard Kirby Bauer disk diffusion method following the Clinical and Laboratory Standards Institute guidelines [265]. Mueller Hinton agar was used to carry out the antibiogram of the resistant isolates. The following antimicrobial discs were used Ampicillin (A; 10µg), Cefotaxime (X; 30 µg), Ceftazidime (Z; 30 µg), Ceftriaxone (R; 30 µg), Chloramphenicol (C; 30 µg), Ciprofloxacin (I; 5 µg), Gentamicin (G; 10 µg), Nalidixic acid (N; 30 µg), Streptomycin (S; 10 µg), Tetracycline (T; 30 µg) (BD Sensi-Disc Inc.).

**Extent of Resistance against Cefotaxime was Determined by Minimum Inhibitory Concentration (MIC) Test**

Minimum inhibitory concentration of cefotaxime was determined using CLSI methodology [265]. The control strains used for this study were obtained from American
Type Culture Collection and included *Escherichia coli* (ATCC 35401), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853).

**Identification of Cefotaxime Resistant Bacteria**

Resistant bacterial colonies on MacConkey plus cefotaxime agar were purified and genomic DNA was extracted using Qiagen® DNA mini kit. Then 16Sr RNA gene was amplified using primers, KCP 576 and KCP577. The PCR products were eluted with QIAquick PCR Purification Kit from Qiagen® and sent for sequencing to Cancer and Genetics Research Center (CGRC) at UF. The raw unedited gene sequences were analyzed using Mega5® and Bio Edit® programs.

**Results**

**Cefotaxime Resistant Bacteria are Widely Prevalent in Cattle**

All of the 1365 samples showed growth on the Tryptic Soy Agar containing cefotaxime. We used TSA media plates to obtain the maximum number of bacteria as there is no inhibitory ingredient in TSA media and it allows growth of almost all the cultivable bacteria. MacConkey is a selective and differential media and supports growth of *Enterobacteriaceae* members. Members of the family *Enterobacteriaceae* are known to be most common bacteria encountered in hospitals and they have the highest capacity to spread resistance by virtue of their plasmids. For further experiments we used only MacConkey positive resistant isolates. Cefotaxime resistance ranged from 6% (herd # 5) to 100% (herd # 7) (Figure 5-1B). The results indicate that even though the cephalosporins are not used for prophylactic treatment in these farms, the cefotaxime resistance is widely prevalent and the resistance has developed irrespective of the anthropogenic selection pressure. The variation in the prevalence levels of
Cefotaxime resistance at different farms indicate that there are some unknown microbial and environmental factors that determine the prevalence and spread of resistance.

**The Isolates are Resistant to Clinical Therapeutic Concentrations of Cefotaxime**

We tested bacterial isolates for minimum inhibitory concentration of cefotaxime following CLSI methodology. The bacterial isolates from clinical settings are classified as resistant if they grow ≥ 64µg/ml of cefotaxime [265] but for environmental bacteria the MIC is 20µg/ml for any antibiotic [241,266]. As shown in Figure 5-2A, most of the bacterial isolates tested in this study have MIC more than 20µg/ml, confirming that they are intrinsically resistant to the therapeutic treatment of cefotaxime in the clinics. This is of public health importance because if this cefotaxime resistance and the associated genes reach to clinics or clinical isolates, it would be difficult to treat the bacterial infections leading to severe health and economic losses.

**Identification by Sequencing the Isolates Revealed an Extensive Bacterial Diversity in Cattle**

The homology search of 16S rRNA gene sequences using NCBI database showed that these cefotaxime resistant isolates are commensal, soil, plant and environmental bacteria (Figure 5-2B). These results indicate that our isolates have developed resistance independently in nature and maybe without anthropogenic selection pressure. Some of these bacteria like *Agrobacterium tumefaciens* are plant bacteria while others like *Acinetobacter sp.*, and *Kuthria sp.* have recently been shown to resist antibiotic treatments in human hospitals [267-269]. Adaptation of bacterial genera to a cattle farms and bacterial diversity among farms suggests that the
resistance to cefotaxime is not confined to a few genera or only one herd but is widely prevalent.

**The Cefotaxime Resistant Isolates Grew on ChromAgar ESBL and Contain ESBL Genes Previously Reported from Humans**

For the phenotypic conformation of ESBL production, the cefotaxime resistant isolates were streaked on ChromAgar ESBL® media and we found that 40%, 26% and 34% of isolates produce purple, metallic blue and yellow color, respectively, indicating these isolates are ESBL producers belonging to different bacterial genera (Table 2).

We tested a representative subset of isolates for the presence of nine ESBL genes commonly reported in human isolates. We were able to amplify *bla* TEM and *bla* CTX-M (in all of the isolates, 100%), *bla* CMY consensus (22%), *bla* SHV (13%), *bla* OXA-2 (39%) and *bla* VEB consensus (30%) genes in our cefotaxime resistant isolates (Figure 5-3A). None of the farm isolates amplified *bla* ACC like, *bla* OXA-1 or *bla* DHA consensus genes. Presence of multiple genes in the same isolate was evident as we found that 70% and 34% of our isolates were carrying more than two or three ESBL genes, respectively. The presence of several resistance genes might increase the possibility of transmission to other isolates including human pathogens.

**Genotypic Characterization of Cefotaxime Resistance**

The PCR characterization showed that all of our isolates were carrying *bla* TEM and *bla* CTX-M genes. To find the genetic nature of this resistance, we sequenced both these genes in all of our isolates. The gene sequences were analyzed by the Jukes and Cantor model with uniform substitution rates for the *bla* TEM sequences and Jukes and Cantor model with Gamma distribution for the *bla* CTX-M sequences. Neighbor-joining
phylogenetic trees were drawn to determine the genetic relatedness among the genes from various isolates (Figure 5-3B, 5-3C). Nucleotide Blast search and sequence analysis of *bla*TEM genes indicated high similarity with the previously reported *bla*TEM from clinical cases submitted to NCBI website. Nucleotide Blast search of our CTXM positive isolates showed that our isolates were carrying genes which resembled previously reported *bla*CTXM-15 gene and *bla*CTXM-1 gene. These results indicate that cefotaxime resistance in our isolates is genetically related to clinical isolates thereby pose severe public health risks.

**Multidrug Resistance was Confirmed by Antibiotic Sensitivity Testing**

Antibiotic sensitivity testing of the cefotaxime resistant isolates showed that these isolates are resistant to several ß-lactam antibiotics including cephalosporins as well as to other classes of antimicrobials (Figure 5-4). The resistance against Streptomycin, Nalidixic acid, Gentamicin and tetracycline resistance was found in 60%, 26%, 30% and 39% isolates respectively. Our study mainly focused on resistance to cefotaxime because it is most widely used in clinics but from the data we obtained, it is evident that these isolates are having resistance against other antibiotics as well. Presence of multidrug resistant members of *Enterobacteriaceae* is an alarming health risk for the clinics because these bacteria can get to the clinics or they can transfer the resistance to pathogens easily.

**Discussion**

This study reports the presence of cefotaxime resistance in food producing animals. Cefotaxime is a third-generation cephalosporin and is classified as Critically Important antimicrobial in human medicine by World Health Organization [132]. High
Levels of antibiotic resistance are found in all regions of the world preventing successful treatment of bacterial infections in human and veterinary medicine [39,270]. The presence of resistance genes has been reported from humans [135,264,271] animals [98,245] as well as from isolated environments like glaciers [272], cave microbiome [266] deep terrestrial subsurface [273] and deep sea [274].

Previous studies have reported that the Enterobacteriaceae members harbor resistance genes against third-generation cephalosporins and have the highest capacity to transfer the resistance to other bacteria [45,259-261,275]. We used MacConkey agar which supports the growth of most of the members of family Enterobacteriaceae and other Gram negative bacteria (GNB). In our study the cattle were never exposed to any prophylactic or growth promoting antibiotics and the animals had never been treated with cefotaxime. But we were able to find the animals shedding the cefotaxime resistant bacteria. The variation in percentage of cefotaxime resistant animals indicates that resistance has developed at these farms independent of each other. All of the farms have loose housing type management of animals except the herd #7 which is intensive feedlot unit. Percentage of resistance increased as the density of animals increased on farms indicating that environmental or managerial factors might influence the spread of resistance.

High level of Minimum Inhibitory Concentrations (MIC) of cefotaxime in farm isolates is important in clinics because if these resistance genes reach to clinical isolates, it will be impossible to treat infections caused by pathogens harboring such genes [45]. We found very high MIC of cefotaxime in our isolates and the MIC levels
were comparable to the clinical strain isolated from human hospital (KCJ 1409) (Figure 5-2A), indicating that the cefotaxime resistance is intrinsic among the farm isolates and may lead to treatment failures if such resistance is transferred to the human pathogens. ESBL producers show colored colonies on ChromAgar ESBL®. We streaked our farm isolates on ChromAgar ESBL® and found 40%, 26% and 34% of isolates produce purple, metallic blue and yellow color indicating the presence of ESBL genes in cefotaxime resistant isolates. Members of *Enterobacteriaceae* have been implicated in multi-drug resistant infections in clinics [264,276,277]. These bacteria resist antibiotics by inactivating, modifying or increasing the efflux of antibiotics or producing β-lactamase enzymes [135,249,253,275]. Multiplex PCR results indicate that our farm isolates are harboring several different types of β-lactamase genes and it is plausible that in favorable conditions these isolates can transfer such genes to clinical pathogens. Results from sequencing of the resistance genes (*bla*TEM and *bla*CTX-M) indicated that there is high genetic similarity among these and genes from clinical pathogens, which is in accordance with a previous report [106]. This increases the chances of resistant infections if such genes reach to hospitals. The distribution of bacterial flora varies among different farms and presence of resistance in several bacterial genera increases the chance of resistance spillover to clinical pathogens [278]. The presence of diverse bacterial flora in cattle suggests that there is a huge pool of resistant bacteria in food animals. The better understanding of this natural antibiotic resistance at pre harvest stage is important for safe and quality food animal production will be helpful to develop a mitigation strategy against this problem [258,271,279,280]. This study shows that the
development of antibiotic resistance is a natural phenomenon and animal and managemental factors influence the acquisition and distribution of cefotaxime resistance in food animals. Our results are in accordance with the previous reports which showed the presence of antibiotic resistance genes and hitherto undiscovered mechanisms of resistance in bacterial population without exposure to antibiotics [266,272-274]. The genetic characterization of these resistant isolates will give us better understanding of the phenomenon of resistance development. Further study of animal and farm factors is also essential to understand whole scenario of resistance development.
<table>
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<tr>
<th>Target genes</th>
<th>Primer name</th>
<th>Sequences</th>
<th>Size (bp)</th>
</tr>
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<td>KCP 550</td>
<td>ATGAGTATTCAAAT TCT CG</td>
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</tr>
<tr>
<td>blaTEM-R</td>
<td>KCP 551</td>
<td>CCAATGCTTTAACAG TGA GG</td>
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</tr>
<tr>
<td>blaSHV-F</td>
<td>KCP 552</td>
<td>TTGCCTGTTATATTACTACCTG</td>
<td>854 bp</td>
</tr>
<tr>
<td>blaSHV-R</td>
<td>KCP 553</td>
<td>TTAGCGTTGCCAGTGYTCG</td>
<td></td>
</tr>
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<td>blaCMY-F</td>
<td>KCP 556</td>
<td>ATGATGAAAAAAATCGTTATGC</td>
<td>1200 bp</td>
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<tr>
<td>blaCMY-R</td>
<td>KCP 557</td>
<td>TTGCAGCTTTTCAGAATGCCG</td>
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<tr>
<td>blaOXA-1-F</td>
<td>KCP 558</td>
<td>ATGAAAAACAAATACATCAACTCAGTCAACTTCCG</td>
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<td>blaACC-like-F</td>
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<tr>
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<td>16Sr RNA-R</td>
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Table 5-2. Antibiotic Sensitivity testing and ChromAgar ESBL characterization

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<th>Isolate</th>
<th>AST Profile</th>
<th>ChromAgar ESBL growth</th>
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<tr>
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<td><em>Wautersiella falsenii</em></td>
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<tr>
<td>2117</td>
<td><em>Pseudomonas mendocina</em></td>
<td>ACX</td>
<td>Yellow</td>
</tr>
<tr>
<td>2470</td>
<td><em>Kuthria gibsonii</em></td>
<td>AXZC</td>
<td>Deep purple</td>
</tr>
<tr>
<td>3301</td>
<td><em>Escherichia coli</em></td>
<td>XGS</td>
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</tr>
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</tr>
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<td><em>Achromobacter xylosoxidans</em></td>
<td>AXZRCGST</td>
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<td>3427</td>
<td><em>Escherichia coli</em></td>
<td>AXZRST</td>
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</table>

Antibiotic Symbols: Ampicillin (A), Cefotaxime (X), Ceftazidime (Z), Ceftriaxone (R), Chloramphenicol (C), Ciprofloxacin (I), Gentamicin (G), Nalidixic acid (N), Streptomycin (S), Tetracycline (T).
Figure 5-1. Prevalence of cefotaxime resistance (CefR) (A) Number of farms (B) Prevalence of CefR in the farms.
Figure 5-2. Characterization of resistant isolates (A) Minimum Inhibitory Concentration testing of the resistant isolates. (B) Phylogenetic tree of the 16 S rRNA gene sequence of CefR isolates.
Figure 5-3. Characterization of resistance genes (A) Prevalence of multi-drug resistance genes (B) Phylogenetic tree of blaTEM gene (C) Phylogenetic tree of blaCTX gene from the cefotaxime resistant isolates
Figure 5-4. Multidrug- resistance profile of isolates
The third-generation cephalosporins are used extensively in human medicine and to some extent in veterinary medicine therapeutically. We evaluated several factors that affect the dynamics of cefotaxime (a third-generation cephalosporin) resistance in beef calves with no history of antibiotic exposure and followed the calves for one year. Four samples were collected around 3 months apart from a multi-breed beef calf population derived from Brahman and Angus cattle. This study utilized a combination of culture-based and nucleic acid-based methods for the detection and enumeration of cefotaxime resistant bacteria from the fecal samples. Fecal swabs were collected, processed and inoculated in culture media containing the cefotaxime at the same concentration at which it is used in clinical cases. Metagenomic analysis was carried on the DNA extracted from fecal samples at four sampling points. Prevalence data were analyzed by logistic regression methods using STATA software while genomic and meta-genomic data were analyzed using previously described protocols and software. Although the beef calves here were never exposed to any of the cephalosporin antibiotics, they had a high prevalence of cefotaxime resistance (CefR). The cefotaxime resistance was 61.07%, 50.7%, 68.57%, and 6.25% in March, June, August, and December sampling, respectively. Cefotaxime resistance was not significantly associated with animal factors like breed, sex, castration, or weight gain. Commensal bacteria were shown to contain the \( \text{bla}_{\text{CTXM}} \) gene and microflora of resistant animals was shown to be different from that of susceptible calves. The effect of climate could be evident by the lowest prevalence of cefotaxime resistance in the December sampling,
which could indicate that antibiotic use in animals is not the only factor that influences antibiotic resistance.

Background

Cephalosporins contribute 14% of total dispensed antibiotics in the US [125] and cefotaxime is a third generation cephalosporin used in human and veterinary medicine for the treatment of bacterial infections and surgical care [51,52]. Bacteria resist cephalosporins by the production of β-lactamase enzymes including extended spectrum β-lactamases (ESBL) [44]. Previous studies have isolated ESBL producers from companion and food animals but the prevalence and dynamics of cefotaxime resistance in beef cattle remains unknown [255,278,281]. We found cefotaxime resistance in beef calves that had no history of antibiotic exposure from birth to one year age. We selected beef calves for this study because more than 25 billion pounds of beef is consumed in the US annually [282] and several reports of multi-drug resistant bacterial infections have been associated with beef [283]. Also, resistance levels in food production animals are positively correlated with levels in human patients [284]. Spread of antibiotic resistance among humans, animals and environment has been shown [257,285], but dynamics of resistance in beef cattle is poorly understood. In this study we used a herd of multi-breed calves (from Angus to Brahman) to determine the influence of various factors like breed group, sex, sampling time, microflora and climate on the dynamics of resistance and genes responsible for cefotaxime resistance.

Antibiotic resistance genes have been isolated from environment [139] and commensal bacteria including Enterobacteriaceae members like E. coli have the highest capacity to spread resistance genes among bacteria [98,286]. The commensal bacteria
in animals serve as reservoirs of antibiotic resistance genes which under favorable conditions can be transferred to pathogenic bacteria [280]. It has been suggested that cephalosporin resistance genes are mainly disseminated in animals and humans via distinct plasmids [8], but several other factors including the animal and environmental factors haven’t been discussed and their effect on antibiotic resistance remains unknown. In this study we identified multidrug resistance in commensal bacteria of beef calves and observed the role of soil and environment in the dynamics of cefotaxime resistance in beef calves.

The association of the microflora with the antibiotic resistance has been studied by sequence-based metagenomics in diverse environments, including cattle manure [287,288]. Next gen sequencing technology has been utilized to study the dissemination of the cephalosporin resistance in humans and animals [8]. Recently, metagenomic analysis has been used to study the gastro intestinal microbial diversity in dairy and beef cattle [240,289] and to understand the functional genomics of rumen microbiome [290]. We used metagenomic analysis on fecal and soil samples collected at four different sampling times to determine the association between commensal microflora and cefotaxime resistance in beef calves.

For an effective pre-harvest control strategy we need to understand the dynamics and transmission of antibiotic resistance before the beef calves reach the feedlot [291]. The objective of the present study was to determine the prevalence and understand the influence of breed group, sex and climate on cefotaxime resistance in beef calves. The calves had never been exposed to any prophylactic antibiotic but still were shedding
cefotaxime resistant bacteria. Breed group was associated with cefotaxime resistance in the calves of 1-3 months of age. Prevalence of cefotaxime resistance was not different between male and female calves. We observed high resistance in hot months compared to December sampling. Metagenomic analysis of the fecal and soil samples indicates that the microflora of animals is different at different sampling times and affects cefotaxime resistance.

**Method and Materials**

**Ethics Statement**

Standard practices of animal care and use were applied to animals used in this study. The research protocols used in this study were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC Protocol #: 201308027).

**Animal Management and Sample Collection**

We used calves that were born and raised at the Beef Research Unit of University of Florida (Gainesville, Florida USA in North Central Florida) (Figure 6-1A). This farm has a loose system of housing with average stocking density equal to 1.5 acres (0.6 Ha) per cow. We used a cohort of beef calves from a multi-breed population derived from Brahman and Angus cattle for one year. Calves were classified into six breed groups: breed group 1 (80 to 100% Angus and 0 to 20% Brahman), breed group 2 (60 to 79% Angus and 21 to 40% Brahman), breed group 3 ("Brangus" = 62.5% Angus and 37.5% Brahman), breed group 4 (40 to 59% Angus and 41 to 60% Brahman), breed group 5 (20 to 39% Angus and 61 to 80% Brahman), breed group 6 (0 to 19% Angus and 81 to 100% Brahman) (Figure 6-1B). Calves were followed from birth through their first year of life with samples collected four times approximately every
three months. Calves were kept with their dams until weaning, which took place in August during the third sampling period. To keep track of individual animal identity the fecal samples were collected from the recto-anal junction (RAJ) of calves using sterile cotton swabs (Fisherbrand®) and placed in sterile 15mL conical tube (Falcon®). The fecal samples were collected in four different seasons of the growing period viz, March (n=259), June (n=263), August (n=261) and December (n=193). Due to culling of calves for reasons unrelated to this study, only 193 calves were available for the December sample. The sampling scheme resulted in 188 animals that had all four sampling time points. All samples were transported on ice and processed the same day using the protocol described below.

**Identification of Cefotaxime Resistant Animals**

We used a combination of culture-based and nucleic acid-based methods for the detection and enumeration of cefotaxime resistant bacteria from the fecal samples. Fecal swabs were suspended with 2 mL of Tryptic Soy Broth (TSB) broth and spread plated onto MacConkey agar containing cefotaxime (Becton Dickinson Company, MD, USA) after 10-fold serial dilutions (up to 10^-2). We incubated plates at 37ºC and enumerated bacterial colonies after 24 hours. We purified up to six colonies on MacConkey agar containing cefotaxime and froze them in 15% glycerol for future use.

**Statistical Analyses**

We used simple logistic regression to determine: 1) the differences in herd prevalence of cefotaxime resistance among sampling periods, and 2) the association between prevalence of cefotaxime resistance and calf breed group (groups 1-6) or sex of calf (bull or heifer). For animals with four consecutive samples collected (N=188), the
results of the microbiological testing between two sampling dates were used to determine the proportion of calves that were not previously shedding and did not shed (−/−), were not previously shedding and became shedders (−/+), those that remained shedders (+/+), and those calves that were previously shedding and are no longer shedding (+/−) cefotaxime resistant bacteria. Significant increases and decreases in prevalence between any two consecutive sampling periods were determined by using McNemar’s test for matched pairs. All statistical analysis was conducted using STATA software package (STATA® MP 11.2, StataCorp, College Station, Texas, USA) with an significance threshold of α < 0.05.

**PCR Characterization of the Cefotaxime Resistant Bacteria**

We screened cefotaxime resistant isolates for the presence of *bla*CTX-M gene by using the primers, KCP 685 (5’-TTTGCAGTGTGCAGTACCAGTAA-3’) and KCP686 (5’-CGATATCGTTGGTGCTGACCATA- 3’) (~544 bp) [292] (Table 6-1). Frozen 2689 bacterial isolates were revived on cefotaxime containing MacConkey agar plates and subjected to colony PCR for the detection of *bla*CTX-M gene. The PCR products from 60 *bla*CTX-M positive isolates were purified using the QIAquick PCR Purification Kit (Qiagen®). The purified PCR products were sequenced using Sanger sequencing protocols. The raw unedited sequences were analyzed using Mega5® and Bio Edit® softwares. Briefly, the sequences were analyzed by Jukes & Cantor (JC) model with uniform distribution and maximum likelihood tree was constructed from the sequences using Bootstrap method with 1000 bootstrap replications.
Identification of Bacterial Isolates by 16S rRNA Gene Sequencing

Resistant bacterial colonies on MacConkey agar were purified and genomic DNA was extracted from 60 blaCTX-M positive isolates using QIAamp DNA Mini Kit (Qiagen®). Then 16S rDNA gene was amplified using primers, KCP 812 (5' - CAG GCC TAA CAC ATG CAA GTC - 3') and KCP813 (5' - GGG CGG WGT GTA CAA GGC - 3') (~1300 bp) [293] (Table 6-1). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen®) and sent to the Sanger sequencing center (CGRC) at the University of Florida for sequencing. The resultant sequences were aligned using Bio Edit® and analyzed using Jukes & Cantor (JC) model with Gamma distribution in Mega5®. The maximum likelihood tree was constructed from the sequences using a Bootstrap method with 1000 bootstrap replications.

Multi-drug Resistance

To determine the public health importance, we tested cefotaxime resistant isolates from beef calves for the presence of multi-drug resistance against 10 antibiotics from nine different classes. The blaCTX-M positive bacterial colonies on MacConkey agar were purified and inoculated overnight in Luria Bertanii (LB) broth containing cefotaxime (4µg/mL). After the incubation approx. 5 x 10⁵ cells were plated on LB agar containing antibiotics. The following antibiotics were used Cefotaxime (4 µg/mL), Erythromycin (20 µg/mL), Nalidixic acid (30 µg/mL), Tetracycline (15 µg/mL), Ampicillin (50 µg/mL), Kanamycin (50 µg/mL), Potassium Tellurite (2.5 µg/mL), Rifampicin (100 µg/mL), Chloramphenicol (35 µg/mL) and Polymixin B (50 µg/mL). The plates were incubated at 37°C and the isolates were classified as negative (-), mild resistant (+) and
highly resistant (++) based on the diameter of the growth on the antibiotic containing media after overnight incubation.

**Metagenomic Analysis**

To determine the abundance of microflora and its effect on the cefotaxime resistance (CefR), we did metagenomic analyses of the fecal samples from the calves using the latest technology of 454 pyrosequencing (Macrogen Inc., South Korea). The sequenced-based metagenomics involves; extracting DNA from a given sample, fragmenting it, randomly sequencing the extracted DNA, and then comparing the sequence output to a database to identify resistance genes. The two most common techniques used for sequence-based metagenomic sequencing are Roche 454 Pyrosequencing and Illumina GAIIx Sequencing [294]. We used 48 samples which were collected at four different sampling points from 12 animals including both cefotaxime positive (CefR) and cefotaxime negative samples (CefS) to identify and compare the bacterial flora in beef calves. We included fecal samples from male and female calves representing the six breed groups.

**Metagenomic DNA Extraction and Pyrosequencing**

The DNA was extracted from the fecal samples with PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer’s protocol. Library was prepared using PCR products according to the GS FLX plus library prep guide. Libraries were quantified using Picogreen assay (Victor 3). The emPCR, corresponding to clonal amplification of the purified library, was carried out using the GS-FLX plus emPCR Kit (454 Life Sciences). Briefly, libraries were immobilized onto DNA capture beads. The library-beads obtained were added to a mixture of
amplification mix and oil and vigorously shaken on a Tissue Lyser II (Qiagen) to create "micro-reactors" containing both amplification mix and a single bead. Emulsion was dispensed into a 96-well plate and the PCR amplification program was run according to the manufacturer's recommendations. Twenty monogram aliquot of each sample DNA was used for a 50 ul PCR reaction. The 16S universal primers 27F (5’-GAGTTTGATCMTGGCTCAG-3’), 518R (5’-WTTACCGCGGCTGCTGG-3’) were used for amplifying of 16S rRNA genes. FastStart High Fidelity PCR System (Roche) was used for PCR under the following conditions: 94°C for 3 minutes followed by 35 cycles of 94°C for 15 seconds; 55°C for 45 seconds and 72°C for 1 minute; and a final elongation step at 72°C for 8 minutes. After the PCR reaction, the products were purified using AMPure beads (Beckman coulter) and sequenced using the following method by Macrogen Ltd. (Seoul, Korea). Following PCR amplification, the emulsion was chemically broken and the beads carrying the amplified DNA library were recovered and washed by filtration. Positive beads were purified using the biotinylated primer A (complementary to adaptor A), which binds to streptavidin-coated magnetic beads. The DNA library beads were then separated from the magnetic beads by melting the double-stranded amplification products, leaving a population of bead-bound single-stranded template DNA fragments. The sequencing primer was then annealed to the amplified single-stranded DNA. Lastly, beads carrying amplified single-stranded DNA were counted with a Particle Counter (Beckman Coulter). Sequencing was performed on a Genome Sequencer FLX plus (454 Life Sciences), and each sample was loaded in 123
1 region of a 70 mm-75 mm PicoTiter plate (454 Life Sciences) fitted with a 8-lane gasket.

Selection of 16S rRNAs and Taxonomic Assignment

Using the basic local alignment search tool (BLAST), all the sequence reads were compared to Silva rRNA database. Sequence reads which had sequence similarity with less than 0.01 E-value were admitted as partial 16S rRNA sequences. Non-16S rRNA sequence reads comprised less than 1% of all reads. Taxonomic assignment of the sequenced read was carried out using NCBI Taxonomy Databases. The five most similar sequences for each sequence read were found by their bit scores and E-value from BLAST program. Needleman-Wunsch global alignment algorithm was used to find the optimum alignment of two sequences along their entire length. A pairwise global alignment was performed on selected candidate hits to identify the best aligned hit. The taxonomy of the sequence with the highest similarity was assigned to the sequence read. By the similarity, we assigned the taxonomy down to these taxonomical hierarchies; species with more than 97% similarity, genus 94%, family 90%, order 85%, class 80%, and phylum 75%.

Operational Taxonomic Unit (OTU) Analysis for Community Richness

CD-HIT-OTU software was used for clustering. Mothur software was used for analyzing microbial communities and Shannon-Weaver diversity index and Simpson index were used for species diversity. Statistical analysis of the metagenomic data for the microflora diversity and richness within the calves for different ages was analyzed using SAS (Version 9.4) and an R program{R Development Core Team, 2010 #228} was used to generate heat maps for the various OTUs present in our fecal samples.
Mean proportions and 95% CIs were used to describe the changes in proportions of the 16S rDNA reads assigned to different OTUs present in the fecal samples. Mean proportions of the bacterial taxa (OTUs) within the study groups were analyzed using a generalized linear mixed model in SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). The heat maps were generated by the free online G-plot package in R-program.

Evaluation of Environmental Factors on CefR

The role of environmental temperature, rain rate and surface precipitation in the dynamics of cefotaxime resistance was evaluated by collecting the metrological data from the government data base. The data for Gainesville temperature in year 2013 was collected from National Climatic Data Center, US Department of Commerce http://www7.ncdc.noaa.gov/CDO/cdoselect.cmd?datasetabbv=GSOD&countryabbv=&georegionabbv. The rain rate (precipitation) data were collected from the Tropical Rainfall Measuring Mission (TRMM) satellite of NASA, USA. The Accumulated TMI Surface Precipitation data were collected from TRMM Online Visualization and Analysis System (TOVAS) (NASA, USA) for the year 2013 http://gdata2.sci.gsfc.nasa.gov/daac-bin/G3/gui.cgi?instance_id=TRMM_Monthly

Results

Prevalence of Cefotaxime Resistance is High from March to August

The prevalence of cefotaxime resistance (CefR) is high in beef calves even though they were not exposed to any of the cephalosporin antibiotics. The cefotaxime resistance was 59.8%, 53.2%, 73.9% and 6.2% in March, June, August and December sampling respectively (Figure 6-2A). The dynamics of cefotaxime resistance (CefR) in calves that were sampled four times was studied using McNemar’s test to determine if
the marginal prevalence of CefR significantly increased or decreased between sampling points of the study (Figure 6-2B). The proportion of cattle that became colonized, stopped shedding, or remained colonized or not colonized between four sampling points was stratified by age, breed group and sex to determine if calf characteristics influenced the likelihood of CefR shedding over time. By the first sampling period in March, 59.8% of the animals had become colonized, followed by a net decrease of 11% driven primarily by a large proportion of previously colonized animals becoming uncolonized no longer shedding CefR (20%), though 16.5% of calves became colonized between March and June sampling time (Table 6-2). Furthermore, the number of animals which remained uncolonized after a previous single colonization in March increases from 23.4% in June to 63.8% in December. Similarly, those animals colonized with CefR in March, 21.8% of them remained colonized in June, and followed by a marked increase to 70% in August and then decreasing back to 20 % in December, respectively. This high rate of shedding followed by a period of recovery leads to a relatively lower prevalence at the age of one year in December sampling, where there is no net gain in animals becoming newly colonized. These dynamics are shown graphically in Figure 6-2B where a large epidemic wave ultimately leads to a large proportion of the animals losing colonization and remaining cefotaxime susceptible, with a much smaller proportion of the animals gaining and losing colonization resulting in a stabilization of herd prevalence at the final sampling in December.

**Effect of Animal Physiological Factors on CefR Prevalence**

We compared the prevalence of cefotaxime resistance (CefR) with animal physiological factors like breed and sex of the animal. There was no significant
association between prevalence and breed and that all breeds shed the highest CefR in August sampling except breed group 6 which shed highest CefR in June (Figure 6-3A). None of the animals from breed group 1 were shedding CefR in December. Overall the prevalence of CefR in breed group 5 and 6 was higher than other breeds. There was no significant difference in the CefR prevalence in males or females (Figure 6-3B), however, both groups shed higher CefR in August and the prevalence of CefR was higher among males in December sampling.

**Microbial Factors Influencing the Cefotaxime Resistance**

Previous studies have shown that *bla*CTX-M beta-lactamases confer resistance to critically important cephalosporin drugs [61] and these genes could potentially be transferred among between animals and humans [295]. The frozen isolates were thawed and screened for the presence of *bla*CTX-M which is known as the most common resistance gene against third generation cephalosporins. We sequenced the *bla*CTX-M gene from 60 positive isolates (Figure 6-4A) by Sanger sequencing and observed three variants of *bla*CTX-M gene viz., *bla*CTX-M-1, *bla*CTX-M-15 and *bla*CTX-M-32 in 63%, 25% and 12% of the isolates, respectively (Figure 6-4A). We observed that the isolates from March sampling contained predominantly the *bla*CTX-M-1 (84%) and *bla*CTX-M-32 (16%) type of the resistance gene while the August isolates had predominantly the *bla*CTX-M-15 (100%) type of the gene. We didn’t have any of the isolates from December sampling positive for the *bla*CTX-M gene. Next, we sequenced the 16S rRNA gene from these 60 isolates to identify them at the species level (Figure 6-4B). We found that most of the isolates were *Escherichia coli* (81%) followed by *Pseudomonas sp.* (10%), *Achromobacter sp.* (7%) and *Ochrobactrum sp.* (2%) (Figure 127)
6-4B). These are environmental and soil bacteria known to have antibiotic resistance. The predominance of E. coli containing the \textit{bla}CTX-M genes indicates that there is a higher chance of this resistance gene being spread to other bacterial genera with the passage of time because \textit{Enterobacteriaceae} members have the highest potential to spread resistance.

\textbf{Cefotaxime Resistant Isolates also Show High Level of Multi-drug Resistance}

The problem of antibiotic resistance is complicated by the selection of bacteria resistant to several other classes of antibiotics and making it more harmful to the public health [296]. We used 10 antibiotics and classified an isolate multi-drug resistant if it was resistant to three or more antibiotics. We observed that all of our 60 isolates were resistant to five or more antibiotics (multi-drug resistant) (Figure 6-5A). In this study we found that 18 \%, 45 \%, 35 \% and 2\% of our isolates were resistant to five, six, seven and eight antibiotics tested, respectively (Figure 6-5B). We couldn't find resistance against two synthetic antimicrobials viz., Potassium Tellurite and Nalidixic acid.

\textbf{Effect of Microflora on CefR}

Microbial community structure has been found to affect the antibiotic resistance in soil [139] and in dairy cattle [288]. Metagenomics allows the cost-effective characterization of complex microbial communities including bacterial, viral, or fungal flora present in biological or environmental samples [294]. To understand the change in the microflora in different time points and its effect on the Cefotaxime resistance in beef calves, we analyzed the fecal microbiota of calves over the four different time points by using the next gen sequencing technology. The proportionate abundance of different bacterial OTUs in the samples was analyzed in the 48 samples using the GLIMMIX.
procedure of SAS (SAS Institute Inc., Cary, NC). We found higher abundance of Fusobacteria (P= 0.041), Elusimicrobia (P= 0.0481) and Chlamydia (P= 0.0025) in the CefR samples while as, CefS samples had higher abundance of Spirochetes (P= 0.022) (Table 6-4). We also found that the microflora of the animal changes with sampling time and calf age with Desulfovibrionales (P= 0.0132), Entomoplasmatales (P= 0.0072), Pasteurellales (P= 0.00155) and Sphinobacteriales (P= 0.028) being the predominant bacteria in the March; whiles as, June sampling had predominantly Proteobacteria (P= 0.0003) and Sphinobacteriales (P= 0.028). The August sampling had higher proportion of Lachnospiraceae (P= 0.0064), but the December sampling had predominantly Spirocahetes (P= 0.039) and Actinobacteria (P= 0.079) (Table 6-4). We also generated heat map from the fecal samples using the G- plot package of R-program to show the difference of microflora in samples collected from cefotaxime resistant and susceptible samples (Figure 6-6) and difference among samples collected at four different time points (Figure 6-7). There is a difference in microflora between susceptible and resistant animals but the overall abundance of OTUs is low which makes it hard to visualize the differences on the heat map.

Effect of Climate and Diet Change on Cefotaxime Resistance CefR

We collected data from the governmental agencies (NASA and National climatic data center) and found that the temperature and precipitation were high in summer of 2013 and maybe associated with the CefR microflora of beef calves at the farm. Previous studies have shown the effect of environment and diet on the microflora of animals [297-299] and in our study we speculate that the observed high prevalence of cefotaxime resistance in June and August compared to the December sampling might
be associated with the environmental factors like high temperature and surface precipitation (Figure 6-8, Figure 6-9). The beef calves were housed with their dams until weaning in August. We presume that the reduced cefotaxime in December may be due to the climate change or change in the microflora after weaning as the diet of animal changes after weaning.

**Discussion**

Antibiotic resistance causes more than 23000 deaths and $55bn in US (overall societal costs) and resistance against several antibiotics including cephalosporins has been reported from hospitals in more than 100 countries around the globe [39]. Even though the food animals are blamed for the antibiotic resistance in humans [256,257], the commensal bacteria can exchange ESBLs genes and other resistance determinants even in the absence of antibiotic selection [245] so the origin and transmission of antibiotic resistance genes remains debatable [106]. In this study we used beef calves who had no history of prophylactic antibiotic use and followed them for one year to understand the various factors that affect the cefotaxime resistance in beef cattle. The high prevalence of cefotaxime resistance in early stages of life indicates that origin of antibiotic resistance is independent of antibiotic usage. High prevalence of resistance in warmer climate compared to cold winter indicates there is a role of environment and similar to previous studies could be independent of antibiotic usage in the antibiotic resistance. Previous studies have shown resistant *E. coli* isolates from cattle feces independent of antimicrobial usage on the farm [258]. Furthermore, antibiotic resistance including resistance to β-lactams has been reported in several studies even before such
drugs were used in animals [243,300] and beef calves are often raised on range land with minimal use of antibiotics [244].

We did not find a significant difference among breed groups in the prevalence of cefotaxime resistance. To understand the relationship between animal genetic factors and cefotaxime resistance, we used calves from a multi-breed herd where Angus and Brahman cattle are raised and each animal has a variable proportion of the genetic make-up from the two breeds (Figure 6-1B). All the breeds shed higher CefR in August and breeds 5 and 6 shed higher CefR than other breeds, although the prevalence levels were not significantly different. We conclude that shedding of resistant bacteria is mainly influenced by the microflora which may in turn be influenced by the environment and feed. There was no significant difference between males and females with respect to cefotaxime resistance, indicating that animal factors are not the sole contributors to antibiotic resistance.

The calves in this study were weaned in August and left on the pasture with the diet solely consisting of grasses on the pasture and no milk. Thus, we presume that it might have affected the cefotaxime resistance because the diet has been shown to affect the gut microbiota in animals [301]. The high prevalence of CefR in warmer climates may be associated with environmental factors like high surface precipitation and higher microflora in the environment. In addition, rains lead to leaching of metals that can cause multi-drug resistance [296]. The low prevalence of CefR in the dry and cold weather of December in the Florida sampling supports this argument, although further studies need to be conducted to draw a definitive conclusion.
This study reports the presence of cefotaxime resistant genes in bacteria which are commensal and part of animal microflora. The sequencing of the isolates showed majority of the isolates were *E. coli* (82%) (Figure 6-4B) which is consistent with the previous studies where the increased prevalence of ESBL genes has been reported from *E. coli* isolates [62]. Our study has a major public health importance because we isolated ESBL genes similar to the ones present in isolates from human hospitals [45,302]. The major b-lactamase gene responsible for resistance against third generation cephalosporins is *bla*CTX [60,292] and we amplified the ESBL gene (*bla*CTX-M) (Figure 6-4A) from the isolates obtained from beef calves using the previously reported primers [292]. The genes isolated from calves belonged to three main types, M-1, M-15 and M-32. We have observed dominance of *bla*CTX M-1 type in March and M-15 type in August, which indicates that microflora is evolving and gives resistance to calves without antibiotic selection. The predominance of change in gene subtype is relevant to human medicine as several studies have reported higher incidence of M-15 variant in human hospitals [105] and *E. coli* is the main etiological agent [303].

The therapeutic options are severely limited due to the emergence of multidrug resistant bacteria that cause treatment failures and public health crises [256] and multidrug resistance among ESBL producing bacteria like *E. coli* is of a great concern [242]. In a recent study, 412 multi-drug resistant ESBL producing bacteria from agricultural, urban and pristine soils were isolated, of which 80% were resistant to 16-23 antibiotics, comprising almost all classes of antibiotic [242]. In another study, multi-drug resistance
(> or =3 to 6 antimicrobials) was seen in 40% of *E. coli* isolates from healthy lactating cattle with isolates exhibiting resistance to ampicillin (48%), ceftiofur (11%), chloramphenicol (20%), florfenicol (78%), spectinomycin (18%), and tetracycline (93%) antibiotics [98]. Similarly, an increase in the rate of ESBL-producing *E. coli* from 0.2% in 2001 to 9.7% in 2010 has been reported in Japan [304]. In our study, we found multi-drug resistance (up to against 8 antibiotics tested) among our calf isolates (Figure 6-5A, 6-5B). Isolates were highly resistant to cefotaxime (100% of the isolates tested), ampicillin (100%) and polymixin B (37%) while (35%) of the isolates were resistant to seven antibiotics tested. The higher prevalence of multi-drug resistant bacteria among young beef calves is alarming for public health because these resistant isolates and genes may reach to human hospitals via horizontal transmission of resistance genes [42,276], contamination of beef [305] or accidental iatrogenic introduction and it would be impossible to treat infections [306,307].

Metagenomic analysis techniques have been used to understand the role of microflora in antibiotic resistance in environment like soil [139], glaciers [272] and fecal samples [287]. The importance of utilization of next-gen sequencing technology to study antibiotic resistance is evident by the fact that most of the bacteria are unculturable and antibiotic resistance is present in natural environments like soil [106], water [308], manure [288] and health care facilities like hospitals [303,306]. In this study, we demonstrated the role of microflora in the dynamics of cefotaxime resistance and we our results indicate differences in microflora in cefotaxime resistant samples compared to susceptible samples (Table 6-3). We used samples from all four sampling points to
determine the change in microflora and its effect on resistance (Table 6-4). The CefR group has higher abundance of Fusobacteria, which are associated with mucus membrane and are responsible for certain periodontal diseases and necrotic lesions [309] whereas, Elusimicrobia are usually referred to as termite bacteria and are associated with hind-gut of the insects [310]. The observation of cefotaxime resistance in such environmental bacteria isolated from calves without antibiotic selection points out to the speculation that antibiotic resistance develops in nature and some of the resistant bacteria can have serious public health implications if they gain access to human hospitals. We also observed that different microflora was predominant at different sampling time points (Table 6-4) indicating that microflora is dynamic and develops as the animals grow or changes with the sampling times. Bacteria are almost present in every ecosystem and every animal of the world but most of the bacterial taxa are yet to be fully identified [106,290], so even if we had significantly different microflora in the different samples, their overall prevalence is so low that it was not clearly distinguishable on the heat map.

In our study we could not find a significant effect of animal factors on cefotaxime resistance but our results indicate that environment including diet affects the microflora thereby affects the resistant bacteria. Effect of climate on microflora like heterotrophic microorganisms has been shown in previous studies in marine food webs [311] where increased temperature has been implicated to change microbial community structure, cell size and biogeography of microbial species. Also climate changes like increase in temperature and/or UV radiation has been shown to alter the bacterioplankton
assemblage and microbial processes in a lagoon ecosystem [297]. The calves in our study were weaned in August and the diet consisted of mainly pasture grass after that with no milk and no supplementation. The diet, health and environmental disturbances trigger alterations in intestinal microbiota composition [298] which we presume would be the reason for reduced prevalence in December sampling. The December climate in north central Florida is dry and cold and in a study involving effect of climate factors such as wind and rain on the microbial counts, heterotrophic bacteria were found to have an inverse relationship with average dry interval humidity ($r^2$=0.61, $p=0.04$) and a similar trend was observed for Pseudomonas spp. [312], thus we presume that the lower prevalence of CefR in the December sampling could be an overall effect of changing diet and climate.

Cefotaxime resistance has a major impact on public health because ESBL producers isolated from animal products meat belong to the same phylogenetic groups as human pathogens [57] and resistance genes have been isolated from the environment [139]. These situations may lead to either the resistant bacteria gaining entrance into human hospitals or human pathogens acquiring resistance from environment and then causing untreatable infections which is aggravated by incorrect prescriptions up to 30–50% of the time in human hospitals [313]. To prevent the emergence and spread of resistance in humans, we need to understand the prevalence and dynamics of cefotaxime resistance in beef cattle and evaluate the effect of the environment on the dynamics of antibiotic resistance especially against cephalosporins.
Table 6-1. Primers used to amplify *bla*CTX-M and 16S rRNA genes

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer name</th>
<th>Sequences</th>
<th>Size (bp)</th>
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<td>16Sr RNA-R</td>
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<tr>
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<td>500 bp</td>
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<tr>
<td><em>bla</em>CTXM pan-R</td>
<td>KCP686</td>
<td>CGATATCGTTGGTGTTGCCATA</td>
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Table 6-2. Prevalence of CefR

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<th>August</th>
<th>December</th>
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<td>41</td>
<td>56.1</td>
</tr>
<tr>
<td>group 6</td>
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p= Phylum, c= Class, o= Order, f= Family, g= Genus
### Table 6-4. Difference in microflora of Cefotaxime resistant versus susceptible samples

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*p= Phylum, c= Class, o= Order, f= Family, g= Genus*
Figure 6-1. Farm sampling (A) Number of samples collected. (B) Breed group composition of the beef calves
Figure 6-2. CefR in four sampling time points (A) Prevalence of CefR and (B) Dynamics of the CefR shed by calves in first year of life
Figure 6-3. Effect of animal factors on CefR (A) Prevalence of CefR in six different breed groups (B) Prevalence of CefR in male and female calves
Figure 6-4. Genetic characterization of CefR (A) Phylogenetic tree of blaCTX-M gene (B) Phylogenetic tree of 16S rRNA gene of CefR isolates
Figure 6-5. Multi-drug resistance of CefR isolates (A) Level of drug resistance against different antibiotics (B) Percentage of isolates resistant to multiple antibiotics
Figure 6-6. Microflora difference between cefotaxime resistant and susceptible samples
Figure 6-7. Microflora difference at four different sampling times
Figure 6-8. Effect of environment on CefR (A) Temperature and (B) rain rate in Gainesville FL in year 2013
Figure 6-9. Effect of Accumulated surface precipitation on CefR (A) March (B) June (C) August (D) December in Gainesville FL in year 2013
There is a wide knowledge gap about the role of animal factors in the dynamics of major human pathogens like Shiga toxin producing *E. coli* (STEC) and antibiotic resistance. In the first study, the role of animal factors in STEC dynamics was investigated. This finding suggests that animal factors especially animal age can play an important role in the STEC dynamics in cattle. It was shown that heifers shed lower STEC in the first year and shed higher STEC in the second year and overall in the herd, the STEC shedding peaked every two years as animal’s aged. These findings provide clear implications for the development of on-farm mitigation strategies. By segregating animals with the highest risk of infection and highest rates of shedding, it could be possible to lower the concentrations of STEC at the pre-harvest level and prevent transmission from cattle to humans.

Thus, the reduction of pathogens such as STEC at the pre-harvest level represents an opportunity to control the exposure to contaminated animal products in order to reduce zoonotic transmission from animals to humans.

In our next study, a cohort of beef calves from an early age and followed them for one year to study the effect of animal factors like age, breed, sex and microflora on STEC dynamics was employed. Our findings provide conclusive evidence that animal age has an effect on STEC dynamics. It was shown that animal breed and sex were not significantly associated with STEC shedding. The results from metagenomic analyses indicate that microflora has an effect on STEC shedding and diverse microflora reduces
STEC shedding. The microflora of a beef calf develops with age becoming more diverse resulting in reduced level of STEC shedding.

One of the major concerns for public health is antibiotic resistance, thus in this study, the prevalence of cefotaxime (a 3rd generation cephalosporin) resistance on different farms. Cattle shed resistant bacteria even in the absence of antibiotic exposure and environmental and soil bacteria, which are regarded as commensals, have influence on the cefotaxime resistance in cattle.

A cohort study of beef calves from the birth was conducted for one year in order to study the role of animal and environmental factors in the dynamics of cefotaxime resistance. Cefotaxime resistance was higher in summer months and it decreased in the December. After conducting the metagenomic analysis of the fecal samples, it has been shown that microflora affects the cefotaxime resistance and that calf microbiota changes during the sampling times.
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of chromID ESBL, a chromogenic medium for detection of Enterobacteriaceae 
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coli} strains obtained from Kenyan patients during an 18-year period. BMC 
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BIOGRAPHICAL SKETCH

Raies Mir started his education in Kashmir valley of India. Raies was always interested in science and loved animals. Raies obtained his DVM from the SKUAST-K University in Kashmir. He became interested in microbiology and pursued his master's degree (M. V. Sc.) in veterinary bacteriology at Indian Veterinary Research Institute. Raies served as Research Assistant at University of Wisconsin-Madison for one year before joining the University of Florida for his PhD program in the Department of Animal Sciences. Raies has been awarded and recognized by several academic institutions and some of the awards include Junior Research Fellowship, National Eligibility Test for Lectureship, UF Graduate Alumni Fellowship award, William C. and Bertha M. Cornett Fellowship. Raies served as Vice-President of ASGSA in the Department of Animal Sciences and served a graduate student Mentor for the students with learning disability at UF for two years.