THE OCCURRENCE OF SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI* (STEC) AND *SALMONELLA* SPECIES IN CATTLE FEEDLOT RUNOFF

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment
for the Degree of
MASTER OF SCIENCE

Major Program:
Food Safety

October 2013

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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By

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Zoonotic foodborne pathogens such as shiga-toxin producing *Escherichia coli* (STEC) and *Salmonella* on farm environments can potentially contaminate organic manure or agricultural watersheds and subsequently fresh produce during fertilization or irrigation. This study investigated the occurrence of STEC and *Salmonella* serotypes in cattle feedlot runoff samples in two feedlots in North Dakota. Using standard laboratory culture methods this study reported a 39% prevalence of STEC O45, 33 % (O103), 31 % (O157), 27 % (O121), 16 % (O26), 10% (O111), 10% (O113), 10 % (O145) and 39.7 % *Salmonella*. Additionally, occurrence of some STEC serotypes was influenced by feedlot (O111 and O121), sampling location in relation to vegetative filter strips (O157), and sampling time (O45 and O121). Although this study was the first to report occurrence of STEC serotypes including non-O157 serotypes in cattle feedlot runoff, further studies are needed to quantify the pathogen load in runoff prior to disposal.

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to everyone who provided assistance to me throughout this project. My deep gratitude goes to my adviser, Dr. Margaret Khaitsa, for providing skillful guidance, encouragements and her constant support during the course of this project. I am also grateful to the members of my committee, Drs. Charlene Wolf-Hall, Penelope Gibbs, and Shafiqur Rahman for their guidance and support during the course of this project.

I also recognize the financial assistance from North Dakota State University College of Graduate and Interdisciplinary Studies, Equity and Diversity Center, and The Great Plains Institute of Food Safety. I would like to thank the members of Dr. Khaitsa's laboratory for their time spent in providing useful suggestions to improve my project especially the resourceful assistance from the Research Technician, Mrs. Dawn Doetkott. I am also indebted to the secretarial staff of the department of Veterinary & Microbiological Sciences for their general assistance provided during the course of this project.

Finally, I acknowledge the unending support and encouragement from my family members particularly Dr. Ebot S. Tabe (my husband), for his unending support and technical guidance.

DEDICATION

This thesis is dedicated to Dr. Ebot S. Tabe (my husband), you are my inspiration and support and Ms. Arrey M. N. Tabe (my daughter). You brought new sunshine to our lives.

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CHAPTER 1. GENERAL INTRODUCTION

Contamination of water with zoonotic pathogens such as shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella* remains a significant public health concern that has the potential for contamination of vegetable produce with resultant disease outbreaks. Although the gastrointestinal tract of ruminants is the recognized reservoir (Hussein, 2007; Khaitsa et al., 2006; Williams et al., 2008b) for these pathogens, they can persist for a significant period of time in animal manure and contaminated soil, from where they can be transferred to watercourses during periods of heavy rainfall (Maule, 2000). During and following periods of heavy rainfall, pathogens from a range of habitats within the farm environment are readily transferred into watercourses (Williams et al., 2008a). Freshwater ecosystems are often at the interface between agriculture and the wider environment and can play an important role in human exposure to *E. coli* O157, non-O157 serotypes and *Salmonella* through contaminating drinking water, recreational surface waters and shellfish harvesting areas (Callaway et al., 2004; Francis et al., 2012; Smith et al., 2005b; Tauxe, 2002). In addition to causing human illness, contamination of water sources may be important for livestock re-infection (Quilliam et al., 2011). In the past decade, pathogens such as *E. coli* O157 have been isolated from a range of habitats within the farm environment including soil, manure, slurry, drinking troughs, irrigation water, vegetation and farm equipment (Avery et al., 2008; Williams et al., 2008b) with persistence ranging from weeks to years.

The shiga toxin-producing *Escherichia coli* (STEC) group of *E. coli* produce toxins that are anatomically and physiologically similar to those produced by *Shigella dysenteriae* (Gould et al., 2009). STEC, also known as verocytotoxin-producing *E. coli* (VTEC), have the ability to cause hemorrhagic colitis (which is an inflammation of the colon that results in bloody diarrhea),

hemolytic uremic syndrome (acute renal failure that follows bloody diarrhea), hemolytic anemia (fragmenting of red blood cells), and thrombotic thrombocytopenic purpura; a blood disorder characterized by low platelets, low red blood cell count, abnormalities in kidney and neurological functions in humans (Donnenberg and Nataro, 2000; Murinda et al., 2002). In addition, STEC also have also been reported to be the causative agents of diarrhea and disease in other animals including cattle, goats, sheep, cats, pigs and dogs (Zschöck et al., 2000).

Salmonella, which causes salmonellosis, has over 2,600 serotypes that cause a wide range of illness in humans (García-Del Portillo, 2000). Some *Salmonella* serotypes (*S. typhi*, *S. paratyphi*, sheep (*S. abortovis*), and poultry (*S. gallinarum*) are associated more commonly with infection in humans (Bell and Kyriakides, 2002) and are often foodborne or waterborne, though these serotypes are not specific to particular hosts. In the United States (US), *Salmonella* is estimated to cause more than 1.2 million illnesses each year with more than 23,000 hospitalizations and 450 deaths (Scallan et al., 2011a; Scallan et al., 2011b). *Salmonella* infection in humans or animals results in salmonellosis, a disease whose severity varies with the serovar, the strain, the infectious dose, the nature of the contaminated food, and the host status. *Salmonella* infections most often cause gastroenteritis which can range from mild to severe; invasive infections can be severe and potentially life threatening (Scallan et al., 2011b).

Food and environmental safety is a high priority for cattle feedlot managers (Johnson et al., 2003). There has been much speculation on the risk to humans of zoonotic pathogens from feedlot cattle, their carcasses, and the environment. As such agricultural animals are widely recognized as reservoirs of human enteric pathogens (Khaitsa et al., 2006; Nicholson et al., 2005). These pathogens are shed in their feces, which in turn could serve as the primary source for contamination of various food produce. Hence, most cases of human infection by these

pathogens have been linked primarily to the consumption of animal food products (Franz and van Bruggen, 2008). In addition, various pathogens have been recovered from vegetables (Beuchat LR, 2000) and the number of documented disease cases associated with the consumption of raw vegetables increased in the mid-2000s (Sivapalasingam et al., 2004). For instance, several outbreaks of *E. coli* O157:H7 and *Salmonella enterica* serovar *Typhimurium* related infections associated with the consumption of lettuce contaminated with animal waste used as manure were reported (Brandl M. T. Brandl and R. Amundson, 2005). It is perhaps worth noting that people generally acquire salmonellosis through foodborne exposure, although direct contact with infected animals is another possible route (Plym and Wierup, 2006). Therefore, the number and types of pathogens present in livestock waste vary with animal species, feedstuff sources, health status of the animals, and the characteristics of the manure and manure storage facilities (Goyal, 2007).

In bovine manure, *E. coli* O157:H7 is documented to survive for extended periods of time —31 to 48 days (Bolton et al., 2011). *Salmonella* serovar Typhimurium, was capable of survival for a considerable period of time in manure (Barrington et al., 2002) and slurries (Semenov et al., 2011; Semenov et al., 2009). However, once these pathogens get excreted by their animal hosts, their ability to survive is greatly compromised as survival conditions become unfavorable (Franz et al., 2005). In freshly produced manure, pathogen survival is affected by the farm's manure management systems such as whether manure is handled as a solid manure or slurry, as well as if it is applied to the fields as it is produced or after some time in storage, or applied to field by injection or by surface spreading. (Franz et al., 2005; Nicholson et al., 2005). In manure-amended soil, survival times of *E. coli* O157:H7 have been reported to vary considerably, from several weeks (Nicholson et al., 2005) to several months (Bolton et al., 2011).

Long-term survival has also been reported for *Salmonella* serovar Typhimurium (Natvig et al., 2002). Increases in the number of reported salmonellosis outbreaks have intensified efforts to identify environmental sources of *Salmonella.* Previously, *Salmonella* as well as other enteric pathogens have been isolated from both contaminated food and water. Spino (1966) isolated *Salmonella* in the Fargo Red River as far as 73 miles, 4 days' flow, below the Fargo, North Dakota sewage discharge. In the summer and fall of 1968, various *Salmonella* serotypes were isolated from a portion of Lake Mendota, the major recreational lake for Madison, Wisconsin (Claudon et al., 1971). However, the apparent sources of these organisms were a residential storm sewer and a University of Wisconsin Experimental Farms' wash water drain (Claudon et al., 1971).

Livestock producers must deal with the growing challenges associated with manure accumulation, runoff disposal and consequently pollution of ground and surface water with zoonotic pathogens if the consequences of cattle feedlot production systems on the environment have to be reduced (Berry et al., Anonymous). Runoff from cattle feeding operations, manure storage, or manured fields containing pathogens can contaminate surface and ground waters, and further increase the risk for water and/or foodborne illness (Johnson et al., 2003). Although there are many studies that have been conducted to determine the occurrence of foodborne pathogens such as *E. coli* O157 in live cattle and carcasses (Johnson et al., 2003; Sargeant et al., 2003), an increasing number of studies (Renter et al., 2007; Van Donkersgoed et al., 2009) have reported occurrences of these pathogens in the feedlot environment. Not all pathogens possess similar characteristics; some are able to survive for long periods of time in manure while others are susceptible to temperature extremes and manure processing. Therefore, adequate control of

pathogens may require multiple management interventions to achieve significant reduction of pathogens in a manure management system (Woodbury, 2002).

Significance of the research project

Zoonotic foodborne pathogens continue to burden the public health system of the United States (US) (CDC, 2012) and a major source of these pathogens remains animals and their waste products (Ojo et al., 2010). *Campylobacter*, *Salmonella* and STEC are ranked as the most prevalent foodborne pathogens costing the US economy millions of dollars each year (Scallan et al., 2011b). Foodborne pathogens are shed with feces and gastrointestinal secretions or excretions of healthy animals (Oliver, 2009). Information published thus far supports the model in which the presence of pathogens depends on ingestion of contaminated feed, followed by amplification in animal hosts and fecal dissemination in the farm environment. Many foodborne pathogens can have habitats in food-producing animals (for example, skin and gastrointestinal tracts) and in the farm environment (Oliver, 2009). Raw vegetables can also be contaminated when soil is fertilized with improperly composted animal manure (McEwen, 2002).

Odor and water quality problems are the major environmental concerns that are observed during the disposal of manure in livestock production. However, the control of disease causing pathogens in manure is another important issue considered within the concept of manure management. It is worth noting that STEC and *Salmonella* are some of the most important pathogens transmitted during manure disposal. The rapid detection of pathogens in slurry, sometimes used as manure is critical for ensuring environmental and health safety since the pathogen status of the manure will be known prior to disposal. Therefore, it is important to rapidly detect the presence or absence of these pathogens before the disposal of manure to maintain environmental and public health.

Objectives of study

1. To detect the presence of STEC (O26, O45.O103, O111, O113, O121, O145 and O157) and *Salmonella* in run-off samples collected from different locations at two cattle feedlots – in two counties in North Dakota.
2. To determine the prevalence of STEC (O26, O45.O103, O111, O113, O121, O145 and O157) and *Salmonella* isolated from the feedlot run-off samples and whether occurrence of serotypes was influenced by feedlot, time of sampling and sampling location with reference to the position of the vegetative filter strip.

Research questions

1. What is the prevalence of STEC and *Salmonella* serotypes in feedlot runoff from the two feedlots and from the different locations sampled?
2. Is the occurrence of STEC serotypes and *Salmonella* in feedlot runoff influenced by feedlot type, sampling time, or sampling location with reference to the position of the vegetative filter strip?

Organization of the thesis

Chapter 1 provides a general introduction to the study. Chapter 2 comprises a review of the literature that is relevant to the thesis. The review covers aspects of the epidemiology, and disease pathogenesis of the two main pathogens of interest.

Experimental work is described in Chapter 3 and is prepared in the format of a manuscript for submission to the journal of Food Protection; it comprises an abstract, introduction, methods, results, discussion, conclusion, and reference sections. There is an expected overlap in some of the material presented such as in the introduction and reference

sections. Chapter 4 is a general discussion and conclusion that aims to provide context, perspective, and future directions for the body of work described in the experimental chapter.

CHAPTER 2. LITERATURE REVIEW

Ruminants as reservoirs for shiga toxin-producing *E. coli* (STEC) and *Salmonella*

Although STEC are not host specific, they are more prevalent in ruminants than other animals (Hussein and Sakuma, 2005) and most human illness caused by STEC infection has been traced to cattle or cattle products. The microbial population of the ruminant gastrointestinal tract (GIT) is diverse. Although *E. coli* live throughout the GIT of mammals, *E. coli* were not considered "important" ruminal bacteria (de Vaux et al., 2002) because of the competitive nature of the rumen due the presence of other gut flora. High concentrations of volatile fatty acids (VFA) as found in the ruminant GIT are bactericidal (Chaucheyras-Durand et al., 2010) . In addition, the conditions of the lower intestinal tract are favorable for *E. coli* survival, where they can be found at high concentrations ranging from 10^2 to 10^7 cells/g in feces at slaughter (Callaway et al., 2009). The oral cavities of cattle also can contain pathogenic *E. coli* (Smith et al., 2005b), likely due to the process of rumination. However, it should be noted that significant differences can occur between the genotype of *E. coli* O157:H7 isolated from the oral cavity and from feces (Keen and Elder, 2002). Other studies have shown that the terminal portion of the colon was the major site of *E. coli* O157:H7 colonization (Grauke et al., 2002) and the lymphoid tissue located at the recto-anal junction (RAJ) has been demonstrated to be the primary site of colonization of cattle leading to the theory that RAJ colonization may be involved in the phenomenon of "super-shedders" (Cobbold et al., 2007).

Salmonella live in the intestinal tract of various animal species as well, including cattle (Wells et al., 2001), which represents a major reservoir for human foodborne salmonellosis (Wells et al., 2001). Humans become infected with *Salmonella* primarily through fecal contamination of food products or water (Kabagambe et al., 2000; Wells et al., 2001). Another

source of human infection, primarily affecting farm families, employees, and farm visitors is contact with sick animals (Rajić, A et al., 2007). In the US, lactating dairy cows pose a minimal public health risk through milk and dairy products because of commercial pasteurization, although higher risks are associated with farm families and others consuming unpasteurized milk and dairy products (Reviewed in Callaway et al., 2009). Another source of human exposure to *Salmonella* is culled dairy cows through meat contamination during slaughter (Wells et al., 2001) which contributes about 17% of the ground beef available for consumption (Smith et al., 2005b).Fecal *Salmonella* shedding can also augment the risk of within-herd transmission and inadvertent spread to other herds (Wells et al., 2001). In addition to impacting the health and productivity of dairy cattle, these factors lead to an increased risk of zoonotic transmission (Wells et al., 2001) which poses a major public health concern.

Fecal shedding of STEC and *Salmonella* in cattle as a route to human infection

The shedding of pathogenic *E. coli* such as STEC in cattle has been shown to be widespread, but sporadic in occurrence (Meyer-Broseta et al., 2001); highly dependent on season of the year (Gyles, 2007b) and can range from 80% of all feedlot cattle during the summer, to 5-10% shedding during the winter period (Barkocy-Gallagher et al., 2003; Khaitsa et al., 2006). This pattern correlates strongly with an increase in human outbreaks during each summer and or early fall, thus highlighting a linkage between the animal (reservoir) populations and consumers through foodborne outbreaks (CDC, 2006). Temperature or weather has been theorized to be related to or to be the main cause of the seasonality of shedding, (Barkocy-Gallagher et al., 2003; Khaitsa et al., 2006). However, a recent theory proposes that day length and melatonin or seasonal hormones may play a role in this phenomenon. Further research is needed to investigate this intriguing hypothesis (Edrington et al., 2007; Edrington et al., 2006b).

Detection of *E. coli* O157:H7 is also complicated by the fact that fecal shedding can be very sporadic, with an animal testing positive for pathogenic *E. coli* one day, but negative for several days or even weeks (Callaway et al., 2004). Also, diagnosing cattle on the farm or the feedlot as being "infected" by pathogenic bacteria is not an easy task because these pathogens often have little or no effect on the health or production efficiency of the animal (Callaway et al., 2004; Hussein and Sakuma, 2005). However, fecal shedding of foodborne pathogens such as *E. coli* O157:H7 in cattle is directly correlated with levels of carcass contamination (Hussein and Sakuma, 2005; Keen and Elder, 2002), emphasizing that the live animal is a critical link in the production chain (Callaway et al., 2004) and in the transmission of infection to humans.

Transmissions of *E. coli* and *Salmonella* to food produce

Cattle feed has been shown to be a vehicle for the transmission of *Salmonella* as well as *E.coli* (Davis et al., 2003). Introduction of *Salmonella* onto a dairy farm can occur through a variety of routes, including purchased cattle, contaminated feed or water, wild animals such as rodents and birds, human traffic, and insects (Nielsen et al., 2007; Sanchez et al., 2002). Contamination of crops, either by manure used as fertilizer or by irrigation water that has been contaminated by manure run-off, is another key source of transmission (CDC, 2008; Islam et al., 2004; Sivapalasingam et al., 2004). Infected cattle can shed the organism for variable periods and intermittently after either clinically apparent or subclinical infections (Cummings et al., 2010). Such heavy shedders increase the chances of carcass contamination during slaughter.

Persistence of STEC and *Salmonella* in the environment due to cattle runoff

Widespread environmental contamination can result from *Salmonella* and STEC shedding, and these organisms can survive for prolonged periods in suitable conditions outside a host (You et al., 2006). STEC and *Salmonella* have increased probabilities of infecting new

hosts and continuing their life cycle via indirect transmission (Pedersen and Clark, 2007), because of their ability to survive harsh environmental conditions (Winfield and Groisman, 2003). Fertilizing crops with contaminated or non-composted manure slurry may pose a threat if conditions are conducive for bacterial growth (USEPA, 2013). These crops then present a potential source of infection for birds that forage on farmland or to cattle fed with harvested grains or silage that has been grown and stored in conditions that support bacterial growth (Pedersen and Clark, 2007). In addition, contaminated manure slurry, if not managed properly, may pose a threat to human health by seeping into and contaminating ground waters (Pedersen and Clark, 2007). Seepage of manure slurry into recreational waters have also resulted in unintended ingestions and consequently human infection (Ahmed et al., 2009). The survival of enteric microorganisms is influenced by soil and environmental variables, including soil texture and organic matter content, the extent of eutrophication and availability of substrate, pH value, temperature and moisture content (Semenov et al., 2008). The survival of *E. coli* O157:H7 in water (Watterworth L et al., 2006), manure and manure slurry manure-amended soil (Jiang et al., 2002) and sediment (Wang et al., 2011), is well documented with sporadic reports in natural soils (Topp et al., 2003). Temperature was determined to be the most important factor influencing pathogen survival in manure and in manure-or sludge-amended soils, with increasing survival times being a function of decreasing temperature (Semenov et al., 2010; Semenov et al., 2007).

Outbreaks due to *E. coli* O157: H7 infections have always been traced back to consumption of food that has been directly or indirectly contaminated by manure/water containing *E. coli* O157:H7. Animals including deer, horses, dogs, and birds are known to be *E. coli* O157:H7 carriers although, cattle are thought to be the primary carriers of *E. coli* O157:H7 (Chase-Topping M et al., 2008). Using manure as soil amendment or as a fertilizer

source for crops may be the origin of *E. coli* O157:H7 in the environment (Chase-Topping M et al., 2008).from where these pathogens could be mobilized by irrigation water, providing an opportunity for the pathogens to spread into its secondary reservoirs, usually water and soil (Ma et al., 2011). The survival and re-growth of pathogens in these habitats may increase the potential for the pathogen to enter the food chain and thereby constitute a public health risk (Ma et al., 2011). There has been some cases of infection due to direct contact with *E. coli* O157:H7 contaminated soil, and more cases of food poisoning caused by the consumption of vegetables grown in *E. coli* O157:H7 contaminated soils (Mukherjee et al., 2006). Potential pre-harvest sources of contamination include; soil, feces, green or inadequately composted manure, irrigation water, water used to apply fungicides and insecticides, dust, insects, wild and domestic animals, and human handling (Buck et al., 2003).

Non-composted or improperly composted manure can contaminate fruits and vegetables when used as a fertilizer or soil amendment. Runoff water from cattle feedlots and application of contaminated irrigation water to soil also represent possible sources of contamination (Buck et al., 2003). Evidence to support these sources of contamination is largely based on controlled experimental studies in the laboratory and field (Lapidot and Yaron, 2009). For example, *Salmonella enterica* serovar Typhimurium was detected on roots and leaves of lettuce and parsley for up to 63 days and 231 days in soil contaminated with irrigation water and manure compost, respectively (Islam et al., 2004) and (Semenov et al., 2009). Hence, direct contact of aerial tissue with the ground or through rain or irrigated water splashes of soil onto the aerial tissue is likely a significant contributing factor for continued contamination events.

Control of runoff and leaching from stockpiled manure

The primary pollutants in beef manure include nitrogen, pathogens, and phosphorus (Spiehs, 2008). It is known that nitrate (nitrogen) can leach into ground water from stockpiled manure or silage and open lots (Goyal, 2007). It has been reported that nitrate-nitrogen concentrations higher than 10 mg/L in drinking water can harm infants and concentrations higher than 100 mg/L can harm cattle as well (Goyal, 2007). Phosphorus from manure can attach to soil particles and runoff into surface water during rain events (Spiehs, 2008) and as a result excess phosphorus sips into aquatic environments resulting in eutrophication or "algae blooms" and subsequent oxygen depletion (Spiehs, 2008). In addition, disease causing bacteria such as *E. coli*, *Salmonella*, *Cryptosporidium*, and *Giardia* can threaten public health and reduce livestock performance if runoff containing these pollutants reaches human or livestock water supplies (Spiehs, 2008).

To reduce potential contamination of surrounding surface and ground water, under the 1996 hazard analysis and critical control points regulations, the US Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) identified *Salmonella* as a target pathogen for monitoring in slaughter plants (USDA, 2012). This monitoring is likely to result in novel cooperative approaches among producers, veterinarians, and processors to ensure reduction of *Salmonella* shedding in cattle at the time of slaughter. Because cattle are the primary reservoir for human infection with pathogenic *E. coli* and *S. enterica* (Wells et al., 2001), minimizing the presence of these pathogens in the herd will reduce the number of infected cattle sent to slaughter, hence minimizing disease risk to humans (Pedersen and Clark, 2007). As a result, research on farm management practices has attempted to identify factors that affect pathogen prevalence in cattle (Pedersen and Clark, 2007). This has led to the implementation of the

following stringent control practices; i) Control of runoff and leaching from open lots; ii) Proper management of silage storage; iii) Elimination or reduction of cattle access to streams, rivers, lakes, or ponds; iii) Installation of clean-water diversion; and the application of manure at correct rates to fields. Other methods such as aerobic storage, composting, aeration, aeration digestion, chlorination, ozonification, lime stabilization, ultraviolet light (UV) irradiation, and pasteurization of manure, before disposal or use in the fields have been proposed (Goyal, 2007).

Human illnesses due to *E. coli* O157 and non-O157

E. coli O157:H7 was first identified as a foodborne pathogen in 1982 (Meng and Doyle, 2002). Since then, it is recognized as an important foodborne pathogen (Meng and Doyle, 2002). Consumption of undercooked ground beef contaminated by *E. coli* O157:H7 is the leading cause of severe bloody diarrhea and complications such as HC and HUS in the US especially children twelve years and younger (Ennis et al., 2012; Lepšanović et al., 2012; Luchansky et al., 2012). HUS (Lepšanović et al., 2012; Melton-Celsa et al., 2012; Norman et al., 2012), a thrombotic microangiopathy ensues approximately one week after diarrhea onset in approximately 15% of infected children (Chandler et al., 2002; Wong et al., 2000). in general, young children and the elderly are more susceptible in developing HUS and those affected may require dialysis and/ or blood transfusions (Buchanan, 2000). In addition, over the past 15 years, non-O157 serotypes have emerged as important enteric pathogens and numerous outbreaks in countries such as Japan, Argentina, Chile, Germany, Australia, the US and Ireland have been attributed to non-O157 infections (Bettelheim, 2007; Padola et al., 2004; Padola et al., 2002). The serotypes more commonly associated with human infections are: O26:H11/H-, O91:H21/H, O103:H2, O111:H-, O113:H21, O118:H16, O121:H19, O128:H2/H-, O130:H11, O141:H19, O145:H28/H-, O146:H21, O163:H19, O172:H-, and O178:H19.45 (Etcheverría et al., 2010). In Argentina, the

country with the highest incidence worldwide of HUS, isolates obtained from 4824 samples from cattle, foods (hamburger and minced meat), and environment of farms were analyzed to detect STEC. It was determined that HUS was most likely caused by *E. coli* shiga toxin (Stx) absorbed from the gut (Gyles, 2007a; Melton-Celsa et al., 2012).

Salmonella– related illnesses

Salmonella is the etiologic agent for salmonellosis which is the major cause of bacterial enteric illness in both humans and animals (Reviewed in Frendza, 2004). Given that the transmission of *Salmonella* is through contaminated food, water, or contact with infected animals, the primary habitat for *Salmonella* is the intestinal tract of humans and animals (Wells et al., 2001). Most persons infected with *Salmonella* develop diarrhea, fever, and abdominal cramps 12 to 72 hours after infection. The illness usually lasts 4 to 7 days, and most persons recover without treatment. However, in some persons the diarrhea may be so severe that the patient may need hospitalization. In severe cases, the *Salmonella* infection may spread from the intestines to the blood stream, and then to other body sites and can cause death unless the person is treated promptly with antibiotics (CDC, 2006). Generally the young, elderly and people with compromised immune systems are more likely to develop complications from *Salmonella* infection (CDC, 2006).

CHAPTER 3. DETECTION OF SHIGA–TOXIN PRODUCING *ESCHERICHIA COLI* (STEC) AND *SALMONELLA* SPECIES IN CATTLE FEEDLOT RUNOFF

Abstract

Animals and their wastes are a major source of zoonotic foodborne pathogens with *Campylobacter, Salmonella* and shiga-toxin producing *Escherichia coli* (STEC) ranked the most prevalent. These pathogens can contaminate meat and milk products or raw vegetables during processing. The objectives of this study was to detect the presence and determine the prevalence of STEC (O157 and non–O157) serotypes and *Salmonella* species in run-off samples collected from two cattle feedlots in North Dakota. Using Full-Size Portable ISCO 6712 Sampler, runoff samples were collected and transported to the laboratory for processing. Using standard culture methods, STEC and *Salmonella* were isolated from the runoff samples. Polymerase chain reaction (PCR) was used to confirm each STEC serotype. Of 136 samples collected, 106 of 136 (78 %) samples tested positive with at least one of the *E. coli* serotypes: O26 (16%), O45 (39%), O103 (33%), 0111 (10%), O113 (10%), O121 (27%), O145 (10%) and O157 (31%) and 40 % tested positive for *Salmonella*. Feedlot A recorded a significantly higher prevalence of O111 ($P=0.0098$) and O121 ($P=0.0131$) and *Salmonella* ($P<0.05$) compared to Feedlot B. Outflow sampling location had a higher occurrence of O157, and June sampling time had a higher occurrence of O45 and O121. These data provide evidence of the presence of these STEC serotypes and *Salmonella* in feedlot runoff underscoring the need for pretreatment of feedlot runoff before disposal into the environment or use as organic manure.

Introduction

Contamination of water and food with zoonotic pathogens such as *Escherichia coli* and *Salmonella* can have severe public health consequences including, but not limited to large disease outbreaks. Although the gastrointestinal tract of ruminants is a recognized reservoir for these zoonotic pathogens (Khaitsa et al., 2006; Nicholson et al., 2005), *E. coli* and *Salmonella* can persist for a significant period of time in animal manure and contaminated soil, from where they can be transferred to watercourses through runoff during heavy rainfall or thawing of snow (Franz and van Bruggen, 2008; Johnson et al., 2003). Freshwater ecosystems are often at the interface between agriculture activities and environmental concern and their contamination such as of drinking water, recreational surface waters and shellfish harvesting areas can play an important role in human exposure to *E. coli* O157, non-O157 serotypes and *Salmonella* (Quilliam et al., 2011). Both *E. coli* O157 (Ferens and Hovde, 2011) and non-O157 *E. coli* (Renter et al., 2007) have been isolated from a range of habitats within the farm environment including soil, manure, slurry, runoff, drinking troughs, irrigation water, vegetation and farm equipment (Avery et al., 2008; Williams et al., 2008b) with persistence ranging from weeks to years. During and following periods of heavy rainfall, pathogens from all these habitats are readily transferred into watercourses through runoff (Williams et al., 2008a).

In addition to causing human illness, contamination of water sources may be important for livestock re-infection (Quilliam et al., 2011). The shiga-toxin producing *Escherichia coli* (STEC) group produces toxins that are anatomically and physiologically similar to those produced by *Shigella dysenteriae* (Gould et al., 2009). STEC are capable of causing hemorrhagic colitis, hemolytic uremic syndrome, hemolytic anemia, and thrombotic thrombocytopenic purpura (Donnenberg and Nataro, 2000; Murinda et al., 2002). STEC also cause diarrhea and

disease in other animals including cattle, pigs, sheep, goats, cats, and dogs (Zschöck et al., 2000). Non-O157 STEC are known to contribute to this burden of illness but have been under-recognized as a result of diagnostic limitations and inadequate surveillance (Brooks et al., 2005). In recent years, non-O157 STEC strains have been linked to outbreaks and sporadic cases of illness worldwide (Mathusa et al., 2010). In Germany, for example, the STEC strain belonging to serotype O104:H4 was reported as the cause of the 2011 outbreak that affected several people (Frank et al., 2011; Mora et al., 2011). Epidemiologic investigations supported a link between the outbreaks in Germany and France and fenugreek seeds imported from Africa (Mora et al., 2011). However, there was no isolation of the causative strain O104:H4 from any of the samples of fenugreek seeds analyzed (Mora et al., 2011).

The genus *Salmonella* has of more than 2,600 serotypes that cause a wide range of illness in humans (García-Del Portillo, 2000). Most serotypes are not host-specific, however, some are more commonly associated with infection in humans (*S. typhi*, *S. paratyphi*, sheep (*S. abortovis*), and in poultry (*S. gallinarum*) (Bell and Kyriakides, 2002). Sporadic *Salmonella* infections in domesticated animals have been associated with *Salmonella* infections originating from garden birds (Horton et al., 2013). In addition, the risk of gastroenteritis is higher among children living in rural farm areas as opposed to their urban counterparts (Levallois et al., 2013).

Food and environmental safety is a high priority for cattle feedlot producers (Johnson et al., 2003). Numerous studies (Renter et al., 2007; Van Donkersgoed et al., 2009) have reported on the risk to humans of zoonotic pathogens from feedlot cattle, their carcasses, and their environment. These pathogens are shed in their feces and can be transported into surface water via either runoff from feedlot or from cropland application of manure. In either cases manure or runoff may serve as the primary source for contamination of water sources and fresh food

produce. This study examined the burden of *E. coli* and *Salmonella* spp. in two cattle feedlot runoff systems in two separate feedlot facilities located in two different counties in North Dakota. The specific objectives of the study were: 1) To detect the presence of STEC (O26, O45.O103, O111, O113, O121, O145 and O157) and *Salmonella* in run-off samples collected from different locations at two cattle feedlots – in two counties in North Dakota, and 2) To determine the prevalence of STEC (O26, O45.O103, O111, O113, O121, O145 and O157) and *Salmonella* isolated from the feedlot run-off samples and whether occurrence of serotype was influenced by feedlot, time of sampling and sampling location with reference to the position of the vegetative filter strip.

Materials and methods

Sampling sites

Runoff samples were collected from two cattle feedlots in two counties in North Dakota (hereafter named feedlot–A and feedlot–B for purposes of this study). Feedlot–A became operational in 2011 on an area of 115 m by 50 m, with a capacity of 192 beef cattle housed in six (6) pens. Each pen housed approximately thirty two steers. The overall slope of the feedlot area was 5%. A 65 m long and 115 m wide vegetative filter strip (VFS) was constructed immediately after the feedlot pen surface and an alley that ran along the width of the feedlot as illustrated in Figure 1A (Rahman et al., 2013). The VFS was seeded with common cattails grass and graded to a uniform slope of 2% on clay soil. A settling basin constructed at the end of the VFS collected runoff exiting the VFS (Figure 1A).

Feedlot B was started in 2006 and had a capacity of 999 steers housed in five (5) pens with approximately 200 steers per pen. In this feedlot, a two-stage VFS system was constructed (Figure 1B). At the initial stage, runoff from the feedlot ran through an approximately 165 m

long narrow grassed area seeded with smooth brome grass (*Bromus inermis*) and western wheatgrass and reached to a solids separator. At the second stage, runoff from the solids separator was channeled through a pipe and spread onto a vegetative filter strip which was 40m long in the direction of flow. The VFS was established on fine sandy loam soil with an overall slope of 2%. At the end, runoff exiting from the VFS was contained in a retaining pond and used for irrigating croplands. The animals were fed with hay, silage and corn mixed feed depending on their ages.

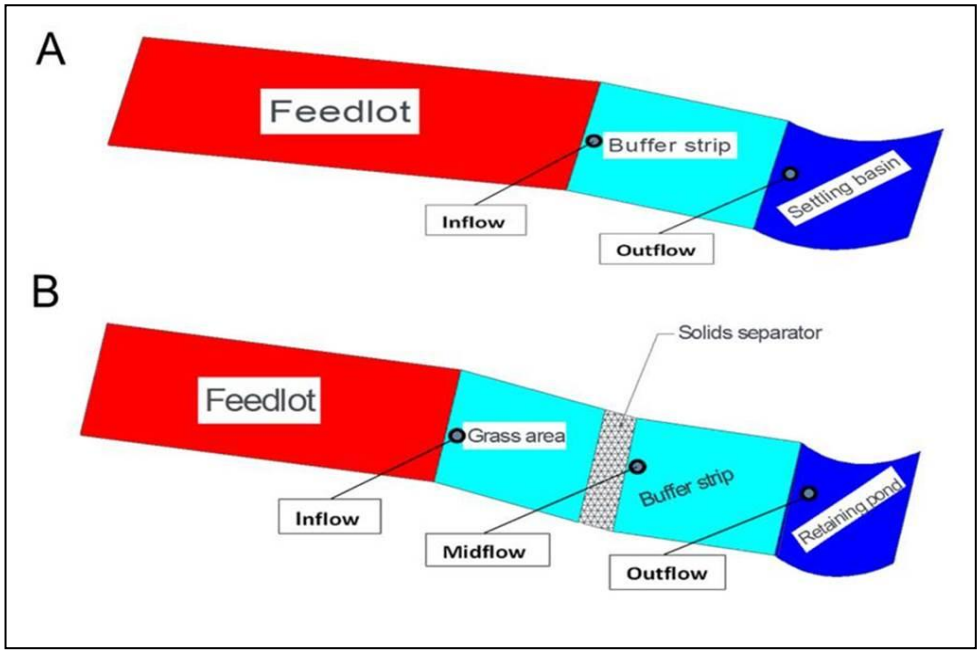

Figure 1. Layout of the feedlot, vegetative filters strip, and water spreading area/settling basin. a) Feedlot A with settling basin, and b) Feedlot B with solid separator and retaining pond. Small circles represent sampling locations. Figures are not to scale. (Rahman et al., 2013).

The two feedlots were located in two counties of ND with two different climatic zones. Feedlot A was located in a county with a continental climate, characterized by cold winters (average temperature -14^{o}C) and warm summers (average temperature 21.4^{o}C). The county receives on average 538.23 mm of rainfall in a year (Godon and Godon, 2002). Feedlot B was

located in a county without a weather station. Data on weather conditions were made by inference from a neighboring county which was 28.324 km (17.6 miles) from the county where feedlot B was located. This neighboring county has a sub humid climate characterized by long, cold winters with about six days per month having temperatures above freezing temperatures. The average annual rainfall was 508 mm, and average temperatures varied from -14.5^{o}C in January to 21.5^{o}C in July (NDDoH, 1995). Runoff samples were collected during the months of April and June 2012, and were received at the lab on April 13, 2012 and on June 11, June 13 and June 20, 2012. The daily precipitation received during the months of April and June 2012 in the county where feedlot A was located is summarized in Table 1 and 2 while the total precipitation for the month of April and June in this county was 28.96 mm (1.14 in) and 56.9 mm (2.24 in), respectively. In the county closest to feedlot B, the daily precipitation received during the months of April and June 2012 is summarized in Table 3 and 4; the total precipitation for the month of April and June in this county was 90.68 mm (3.57 in) and 41.66 mm (1.64 in), respectively. All weather data was obtained from the North Dakota Agricultural Weather Network (http://ndawn.ndsu.nodak.edu/).

Table 1. Weather data for North Dakota county where feedlot A was located during the month of April, 2012.

Date	Max Temp Degrees F	Min Temp Degrees F	Avg Temp Degrees F	Avg Wind Speed (mph)	Rainfall (inch)
1-Apr-12	72	47	59	11.07	0.00
2-Apr-12	60	44	52	10.89	0.00
3-Apr-12	62	31	46	7.80	0.00
4-Apr-12	64	28	46	4.22	0.00
5-Apr-12	65	42	54	10.01	0.00
6-Apr-12	66	41	54	15.81	0.00
7-Apr-12	57	39	48	15.90	0.00
8-Apr-12	59	33	46	12.13	0.00
9-Apr-12	44	24	34	10.60	0.00
10-Apr-12	40	20	30	8.76	0.00
11-Apr-12	50	16	33	3.89	0.00
12-Apr-12	67	36	51	10.74	0.00
13-Apr-12	55	46	50	10.78	0.27
14-Apr-12	70	39	55	5.76	0.00
15-Apr-12	59	35	47	16.16	0.46
16-Apr-12	44	28	36	13.07	0.00
17-Apr-12	61	27	44	9.99	0.01
18-Apr-12	54	39	46	8.53	0.19
19-Apr-12	56	38	47	5.51	0.00
20-Apr-12	58	34	46	5.47	0.00
21-Apr-12	57	38	47	13.05	0.10
22-Apr-12	61	34	48	7.37	0.00
23-Apr-12	74	38	56	7.43	0.00
24-Apr-12	77	49	63	8.31	0.00
25-Apr-12	73	44	59	12.19	0.00
26-Apr-12	60	30	45	10.40	0.00
27-Apr-12	52	43	47	8.91	0.09
28-Apr-12	48	42	45	10.51	0.01
29-Apr-12	55	43	49	12.98	0.00
30-Apr-12	69	47	58	7.71	0.00
Average	60	37	48	9.86	
Total					1.14
Max	77	49	63	16.16	0.46
Min	40	16	30	3.89	0.00
StDev	9	8	8	3.26	

Table 2. Weather data for North Dakota county where feedlot A was located during the month of June, 2012.

Date	Max Temp Degrees F	Min Temp Degrees F	Avg Temp Degrees F	Avg Wind Speed mph	Rainfall inch
1-Jun-12	70	50	60	6.26	0.00
2-Jun-12	80	48	64	4.50	0.00
3-Jun-12	82	59	71	5.26	0.00
4-Jun-12	84	55	69	5.36	0.00
5-Jun-12	88	59	73	5.92	0.00
6-Jun-12	85	67	76	8.13	0.00
7-Jun-12	92	70	81	9.43	0.01
8-Jun-12	88	65	77	9.26	0.00
9-Jun-12	95	66	81	12.12	0.00
10-Jun-12	81	59	70	8.35	0.21
11-Jun-12	64	47	56	14.98	0.00
12-Jun-12	73	41	57	6.56	0.00
13-Jun-12	76	58	67	12.72	0.00
14-Jun-12	82	61	71	10.64	0.87
15-Jun-12	85	59	72	6.23	0.00
16-Jun-12	74	61	68	10.82	0.05
17-Jun-12	83	57	70	9.13	0.00
18-Jun-12	84	65	74	9.86	0.00
19-Jun-12	70	57	64	7.69	0.30
20-Jun-12	74	58	66	7.75	0.78
21-Jun-12	78	54	66	8.28	0.00
22-Jun-12	79	52	66	4.19	0.00
23-Jun-12	84	64	74	7.41	0.02
24-Jun-12	77	59	68	8.29	0.00
25-Jun-12	81	60	70	9.23	0.00
26-Jun-12	84	67	75	13.57	0.00
27-Jun-12	86	60	73	8.25	0.00
28-Jun-12	88	56	72	7.15	0.00
29-Jun-12	88	58	73	3.52	0.00
30-Jun-12	87	58	72	4.66	0.00
Average	81	58	70	8.18	
Total					2.24
Max	95	70	81	14.98	0.87
Min	64	41	56	3.52	0.00
StDev	7	6	6	2.81	

Table 3. Weather data for North Dakota county nearest to where feedlot B was located during the month of April, 2012.

Date	Max Temp Degrees F	Min Temp Degrees F	Avg Temp Degrees F	Avg Wind Speed (mph)	Rainfall (inch)
1-Apr-12	68	48	58	13.61	0.00
2-Apr-12	54	44	49	13.35	0.00
3-Apr-12	62	36	49	9.00	0.00
4-Apr-12	64	30	47	6.66	0.00
5-Apr-12	69	37	53	9.20	0.00
6-Apr-12	68	41	54	13.84	0.00
7-Apr-12	57	32	44	16.87	0.00
8-Apr-12	59	27	43	12.57	0.00
9-Apr-12	47	24	36	11.27	0.00
10-Apr-12	43	18	31	7.35	0.00
11-Apr-12	53	17	35	5.12	0.00
12-Apr-12	55	32	44	10.99	0.21
13-Apr-12	52	42	47	7.83	0.81
14-Apr-12	70	34	52	5.86	0.01
15-Apr-12	53	34	44	19.12	1.45
16-Apr-12	41	30	35	12.75	0.00
17-Apr-12	65	30	47	8.20	0.20
18-Apr-12	53	39	46	9.50	0.02
19-Apr-12	54	42	48	6.22	0.01
20-Apr-12	60	38	49	5.92	0.00
21-Apr-12	56	38	47	15.18	0.14
22-Apr-12	63	31	47	5.74	0.00
23-Apr-12	75	37	56	9.86	0.00
24-Apr-12	83	43	63	7.02	0.00
25-Apr-12	76	46	61	12.72	0.00
26-Apr-12	57	33	45	12.22	0.00
27-Apr-12	49	39	44	13.55	0.46
28-Apr-12	47	37	42	14.09	0.22
29-Apr-12	58	44	51	10.41	0.05
30-Apr-12	71	44	57	8.44	0.00
Average	59	36	47	10.48	
Total					3.57
Max	83	48	63	19.12	1.45
Min	41	17	31	5.12	0.00
StDev	10	8	8	3.61	0.30

Table 4. Weather data for North Dakota county nearest to where feedlot B was located during the month of June, 2012.

Date	Max Temp Degrees F	Min Temp Degrees F	Avg Temp Degrees F	Avg Wind Speed (mph)	Rainfall (inch)
1-Jun-12	66	45	56	7.37	0.19
2-Jun-12	80	45	62	4.62	0.00
3-Jun-12	83	57	70	5.52	0.15
4-Jun-12	85	55	70	4.77	0.00
5-Jun-12	84	57	70	6.94	0.00
6-Jun-12	89	60	75	7.50	0.00
7-Jun-12	92	61	77	9.63	0.00
8-Jun-12	87	60	74	6.43	0.00
9-Jun-12	93	68	81	12.67	0.00
10-Jun-12	72	56	64	10.02	0.21
11-Jun-12	66	45	56	16.09	0.00
12-Jun-12	76	40	58	6.16	0.00
13-Jun-12	82	58	70	9.78	0.00
14-Jun-12	80	60	70	9.70	0.41
15-Jun-12	85	56	70	4.81	0.00
16-Jun-12	75	55	65	10.79	0.00
17-Jun-12	87	53	70	7.07	0.00
18-Jun-12	84	65	75	8.99	0.00
19-Jun-12	66	59	62	10.33	0.42
20-Jun-12	77	56	67	10.11	0.27
21-Jun-12	77	52	65	8.21	0.00
22-Jun-12	80	52	66	5.16	0.00
23-Jun-12	86	65	75	7.06	0.00
24-Jun-12	78	62	70	9.06	0.00
25-Jun-12	83	59	71	7.79	0.00
26-Jun-12	88	68	78	8.65	0.00
27-Jun-12	83	59	71	7.55	0.00
28-Jun-12	92	53	72	3.68	0.00
29-Jun-12	85	58	71	3.13	0.00
30-Jun-12	90	59	75	4.85	0.00
Average	82	57	69	8	
Total					1.64
Max	93	68	81	16	0
Min	66	40	56	3	0
StDev	7	7	6	2.80	

Sample collection

The experimental sites were equipped with automatic samplers (ISCO 6712, Teledyne ISCO Inc., Lincoln, NE, (Figure 2) to collect runoff samples sequentially at one hour interval upon activation of the sampler. In Feedlot A, one sampler was installed to collect runoff at the entry of the VFS (hereafter referred to as inflow), and another sampler was installed at the exit of the VFS to collect runoff leaving the VFS (hereafter referred to as outflow). In feedlot B the first ISCO sampler was installed at the grassed area immediately after the pens (hereafter referred to as inflow); another sampler was installed to collect runoff samples after the solids separator (midflow) and a third sampler collected runoff samples exiting the VFS (outflow).

Figure 2. Full-Size Portable ISCO 6712 sampler used for collecting runoff samples. This device allows the selection of different programming modes to enable different routine applications. Photo courtesy of www.isco.com.

In consideration of the layout of both feedlots, the outflow of feedlot A was equivalent to the midflow of feedlot B. Samplers were powered by heavy duty marine batteries, which were

charged by a solar panel. A detailed description can be found in Rahman et al. (2013). Samples were collected in the months of April and June of 2012 (Table 1 through 4). After collection, samples were transported to the laboratory for processing and data analysis.

Laboratory isolation of *E. coli* O157

The isolation of *E. coli* O157 was performed as previously described (Khaitsa et al., 2006; Tabe et al., 2008). Briefly, for each runoff sample, 90 mL of gram negative (GN) enrichment broth was added to a whirl pak bag (Nasco VWR, USA), into which 10 mL of runoff was added. To prevent the growth of any unwanted bacteria, the GN broth was supplemented with Vancomycin (8.0 mg/L) and Cefixime-Tellurite (0.05 mg/2.5mg/L), and Cefsulodin (10.0 mg/L), all of which *E. coli* O157 is resistant to. Each bag of runoff and broth mixture was incubated at 37°C for 18 to 24 hours. Following enrichment and incubation, immunomagnetic beads functionalized with antibodies against the *E. coli* O antigen were used to retrieve the *E. coli* O157 from the enriched runoff broth according to the manufacturer's protocol (Dynal Biotech ASA). One mL of runoff broth was transferred into pre-labeled microcentrifuge tubes containing 20 µL of the anti–*E. coli* O157 immunomagnetic beads, mixed thoroughly by rotating using a Dynal rotator mixer (Dynal Biotech ASA) for 30 minutes. Then, the tubes were inverted continuously for 3 minutes using a magnetic particle concentrator to capture the beads, and washed 3 times with phosphate-buffered saline plus tween 20 (PBS). The final product was reconstituted in 100 µL of PBS containing tween 20, and vortexed. Twenty µL of aliquot (immunomagnetic beads and captured bacterial cells) was plated and spread onto sorbitol–MacConkey agar (Difco™ Becton Dickinson MD, USA) plates supplemented with Cefixime (0.05 mg/mL) and Potassium Tellurite (2.5 mg/mL; CT–SMAC, Dynal Biotech ASA), and incubated at 37°C for 18-24 hours. Suspected colorless isolates (characteristic of *E. coli*

O157) that did not ferment sorbitol in CT-SMAC agar were sub-cultured on MacConkey and Fluorocult agars (Difco™ Becton Dickinson & Company, MD, USA). Further, latex agglutination test using Remel Kit (Remel, Lenexa, KS) was used to detect the presence of O antigen from *E. coli* O157 isolates that did not ferment sorbitol but fermented lactose within 24 hours and had a negative 4-methylumbelliferyl-β-D-glucuronide (MUG, no fluorescence was produced by the colonies).

Laboratory isolation of non-O157 shiga toxin-producing *E. coli*

For the isolation of non–O157 *E. coli*, runoff samples were prepared using protocols for the isolation of *E. coli* O157:H7 with slight modifications. Briefly, a sterile 15 mL centrifuge tube was loaded with 1 mL of runoff sample and 9 mL of buffered peptone water (BPW) (Difco™ Becton Dickinson & Company, MD) and incubated overnight at 37°C. Immunomagnetic beads functionalized with antibodies against O antigens for different non–O 157 *E. coli* strains (O26, O45, O103, O111, O113, O121, and O145) were used to retrieve their respective strains from the enriched runoff samples and plated on VRB MUG (Violet Red Bile MUG) agar. Suspected non-O157 *E. coli* colonies were subcultured onto Eosin methylene blue (EMB) agar media plates and incubated overnight at 37°C. Colonies with typical *E. coli* characteristics were subjected to biochemical test by stabbing in 7 mL Triple Sugar Iron (TSI) agar slants (Becton Dickinson, Franklin Lakes, NJ), and the results read after 24 hours following an overnight incubation at 37°C. Positive isolates (by production of acid and gas) were further confirmed using the indole test. TSI positive isolates were inoculated into test tubes containing 3 ml of tryptic soy broth and incubated at 37°C for 24 hours. Thereafter, 3–5drops of Kovac's reagent were added to the positive isolate to test for indole production (a red-violet at the surface

layer of the broth). Indole positive samples were stored at −80°C for 3 weeks to 1 month before use.

Laboratory isolation of *Salmonella*

For the isolation of *Salmonella*, runoff samples were cultured in the laboratory using protocols for the detection of *Salmonella* spp as previously described *(Khaitsa et al., 2007)*. Briefly, a sterile 15 mL centrifuge tube was loaded with 1 mL of runoff sample and 9 mL of buffered peptone water (BPW) (Difco™ Becton Dickinson & Company, MD) and incubated overnight at 37°C. This was followed by immunomagnetic beads separation as performed above but with immunobeads (Dynabeads® anti-*Salmonella*, Dynal Biotech, Inc., Lake Success, NY) specific for *Salmonella* species. After the final wash, the beads were transferred to 10 mL of Rappaport Vassiliadis R10 (RV) broth for the resuscitation of potential *Salmonella* bacteria (Becton Dickinson, Sparks, MD) and incubated (with constant gentle shaking) at 42ºC for 24 h. Following incubation, the RV cultures were streaked on modified brilliant green agar (mBGA, Becton Dickinson) and mannitol lysine crystal violet brilliant green agar (MLCB, Oxoid LTD, Basingstoke, UK). Characteristic *Salmonella* colonies were stabbed in 7 mL TSI agar slants (Becton Dickinson,), and the results read after 24 hours of incubation. Tubes with characteristic black pigments due to hydrogen sulfite gas production by *Salmonella* spp were considered positive. Positive *Salmonella* isolates were subcultured in tryptic soy broth supplemented with 5 % glycerol and stored at −80°C for further analyses.

DNA template extraction

Bacterial DNA used for the multiplex polymerase chain reaction (PCR) were prepared from the *E. coli* isolates using the single cell lysing buffer (SCLB) protocol (Marmur, 1961). A single isolated freshly cultured bacteria colony from tryptic soy agar plate was suspended in 40

µL of SCLB in a 0.2 mL microcentrifuge tube. The SCLB master mix consisted of 10 µL of 20 mg/mL proteinase K (Amresco) and 990 µL of 10 mM Tris HCL and 1mM EDTA (TE) buffer solution (Amresco). The entire mixture was placed in a thermocycler (Eppendorf) and run under the following conditions: 80°C for 10 minutes, cooled at 55°C for 10 min, and hold at 4°C. Following lysis, 80 µL of sterile double distilled water was added to the suspension and then centrifuged for 30 sec at 4500 × g. The samples were stored at -20°C until further analyses.

Multiplex polymerase chain reaction (mPCR)

The primer sets specific for the O gene of each serotype and PCR conditions used for the amplification of *E. coli* O157:H7 and non–O157 target genes are shown in Table 5. The *E. coli* isolates were tested in an eight primer multiplex PCR assay for detection and amplification of the *E. coli* serogroups for *E. coli* O26, O45, O103, O111, O113, O121, O145 and O157. Each mPCR reaction was completed on a 25.5 µL reaction mixture containing 2 µL of template DNA, 12.5 µL of multiplex buffer, 0.5 µL of primer (composite) set and 10.5 µL of double distilled water. Each PCR reaction had a positive and negative control. Amplification of target genes was performed following the Qiagen's multiplex kit instructions (Qiagen, Valencia, CA) and included; initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 57.5°C for 60 secs and a final extension for 10 min at 72°C, then cooling to 4°C. The DNA amplicon was electrophoresed in a 1.5% agarose gel for 45 minutes at 170V, stained with Ethidium bromide for 15 minutes, destained with water for 15 minutes, and observed under ultraviolet light using an Ultra Violet AutoChemi System (Tiles Scientific, USA). Gel images were captured electronically and amplicon size were determined with the help of DNA molecular size standards (PCR marker, Promega, Madison WI).

Table 5. Specific primers used for multiplex PCR reactions.

STEC Serotype	Primer sequence
O26	F –CAATGGGCGGAAATTTTAGA
	R − ATAATTTTCTCTGCCGTCGC
O45	F – TGCAGTAACCTGCACGGGCG
	R – AGCAGGCACAACAGCCACTACT
O103	F – TTGGAGCGTTAACTGGACCT
	R – GCTCCCGAGCACGTATAAAG
O111	F – TGTTTCTTCGATGTTGCGAG
	R – GCAAGGGACATAAGAAGCCA
O113	F – TGCCATAATTCAGAGGGTGAC
	R – AACAAAGCTAATTGTGGCCG
O121	F – TCCAACAATTGGTCGTGAAA
	R–AGAAAGTGTGAAATGCCCGT
O145	F – TTCATTGTTTTGCTTGCTCG
	R – GGCAAGCTTTGGAAATGAAA
O157	F – TCGAGGTACCTGAATCTTTCCTTCTGT
	R – ACCAGTCTTGGTGCTCTGACA

Analysis of data

Binary data (present or absent) for each of *E. coli* serotypes and *Salmonella* species were collected and entered into an Excel® (Microsoft 2010) spread sheet. The data were analyzed using Epi Info version™ 7.1.2.0. The prevalence of STEC serotypes (O26, O45, O103, O111, O113, O121, O145 and O157) and *Salmonella* species for the two feedlots and sampling locations (inflow, midflow, and outflow) were computed and summarized in tables and graphically. Chi-square analyses were performed to investigate significant differences at $P < 0.05$ for the occurrence of STEC (O26, O45, O103, O111, O113, O121, O145 and O157) serotypes and *Salmonella* within (at different locations) and between feedlots. Additionally, overall differences in occurrence of STEC between inflow & outflow samples irrespective of feedlot were evaluated. Due to differences in the design of the VFS used for each feedlot as explained previously, for purposes of data analysis, comparison of occurrences of STEC (O26,

O45, O103, O111, O113, O121, O145 and O157) serotypes and *Salmonella* was done between both inflow samples of Feedlot A and B and outflow samples of Feedlot A and midflow samples of Feedlot B (which were equivalent to Feedlot A outflow samples). Additionally, outflow samples from feedlot B were also compared to outflow samples from feedlot A to see if the longer VFS in feedlot B made a significant difference in occurrence of STEC (O26, O45, O103, O111, O113, O121, O145 and O157) serotypes and *Salmonella.*

Results

Objective # 1: Prevalence of STEC (O26, O45.O103, O111, O113, O121, O145 and O157) and Salmonella serotypes in run-off samples collected from different locations at two cattle feedlots – in two counties in North Dakota.

A total of one hundred and thirty six (136) runoff samples were collected from the two feedlots (Feedlot A-91 and Feedlot B-45) in April and June of 2012. Feedlot A had two sampling locations – inflow and outflow while Feedlot B had three sampling locations- inflow, midflow and outflow as indicated earlier in Figure 1A and 1B, respectively. There were no midflow samples collected from feedlot A and feedlot B samples were all obtained at one sampling time. The distribution of samples collected by feedlot, sampling time (all in 2012) and location is shown in Table 6.

Prevalence of STEC serotypes in feedlot runoff

Of 136 samples tested, 22.1% (30) did not test positive for any STEC. Of the negative samples, 73.3% (22 of 30) were from feedlot A and 26.7% (8 of 30) were from feedlot B. When stratified by sampling location, 80% (24 of 30) were from inflow, 6.7% (2 of 30) were from midflow and 13.3% (4 of 30) were from outflow. There was a decrease in the number of negative samples as we moved from inflow to outflow sampling location for both feedlots. Also, 20% (6

of 30) of the negative samples were collected in April while the remaining 80% (24 of 30) were collected in June 2012 (Table 7).

Table 6. Distribution n (%) of feedlot runoff samples by feedlot, sampling time and location (2012).

	Feedlot A (N=91)		Feedlot B (N=45)			N =136
Sampling time	**Inflow n (%)**	**Outflow n (%)**	**Inflow n (%)**	**Midflow n (%)**	**Outflow n (%)**	**Total**
1 – 04/13	18 (19.8%)	-	-	-	-	18
2 – 06/11	18 (19.8%)	-	-	-	-	18
3 – 06/13	10 (11%)	9 (9.9%)	-	-	-	19
4 – 06/20	27 (29.7%)	9 (9.9%)	27 (60%)	12 (26.7%)	6 (13.3%)	81
Subtotal	73 (80.2%)	18 (19.8%)	27 (60%)	12 (26.7%)	6 (13.3%)	136
Total	91 (66.9%)		45 (33.1%)			136

Table 7. Distribution n (%) of negative samples by feedlot, sampling location and time.

	Feedlot A(N=22)		Feedlot B (8)			N =30
Sampling time	**Inflow n (%)**	**Outflow n (%)**	**Inflow n (%)**	**Midflow n (%)**	**Outflow n (%)**	**Total**
1 – 04/13	6 (27.3%)	-	-	-	-	6
2 – 06/11	2 (9.1%)	-	-	-	-	2
3 – 06/13	1 (4.5%)	0 (0%)	-	-	-	1
4 – 06/20	9 (40.9%)	4 (18.2%)	6 (75%)	2 (25%)	0 (0%)	21
Subtotal	18 (81.8%)	4 (18.2%)	6 (75%)	2 (25%)	0 (0%)	30
Total	22 (73.3%)		8 (26.7%)			30

In addition, 78 % (106 of 136) samples tested positive for at least one of the *E. coli* serotypes: O26 (22 of 136 – 16%), O45 (53 of 136 – 39%), O103 (45 of 136 – 33%), 0111 (13 of 136 – 10%), O113 (14 of 136 – 10%), O121 (37 of 136 – 27%), O145 (13 of 136 – 10%) and O157 (42 of 136 – 31%) (Figure 3). Feedlot A had a prevalence of 75.8% (69 of 91)

for STEC while Feedlot B had a prevalence of 82.2% (37 of 45) STEC. Although these two feedlots had two different management practices and feed ration, but the occurrence of STEC are very high in both feedlots. Overall, between one to seven STEC serotypes were isolated from each positive runoff sample from both feedlots. 77.9% (106 of 136) tested positive for at least one STEC serotype (Figure 5, and 6). A total of 47.1% (64 of 136) samples were positive for more than one STEC serotype. However, no sample was positive for all serotypes tested in this study as illustrated by the color coded table (Figures 4 and 5).

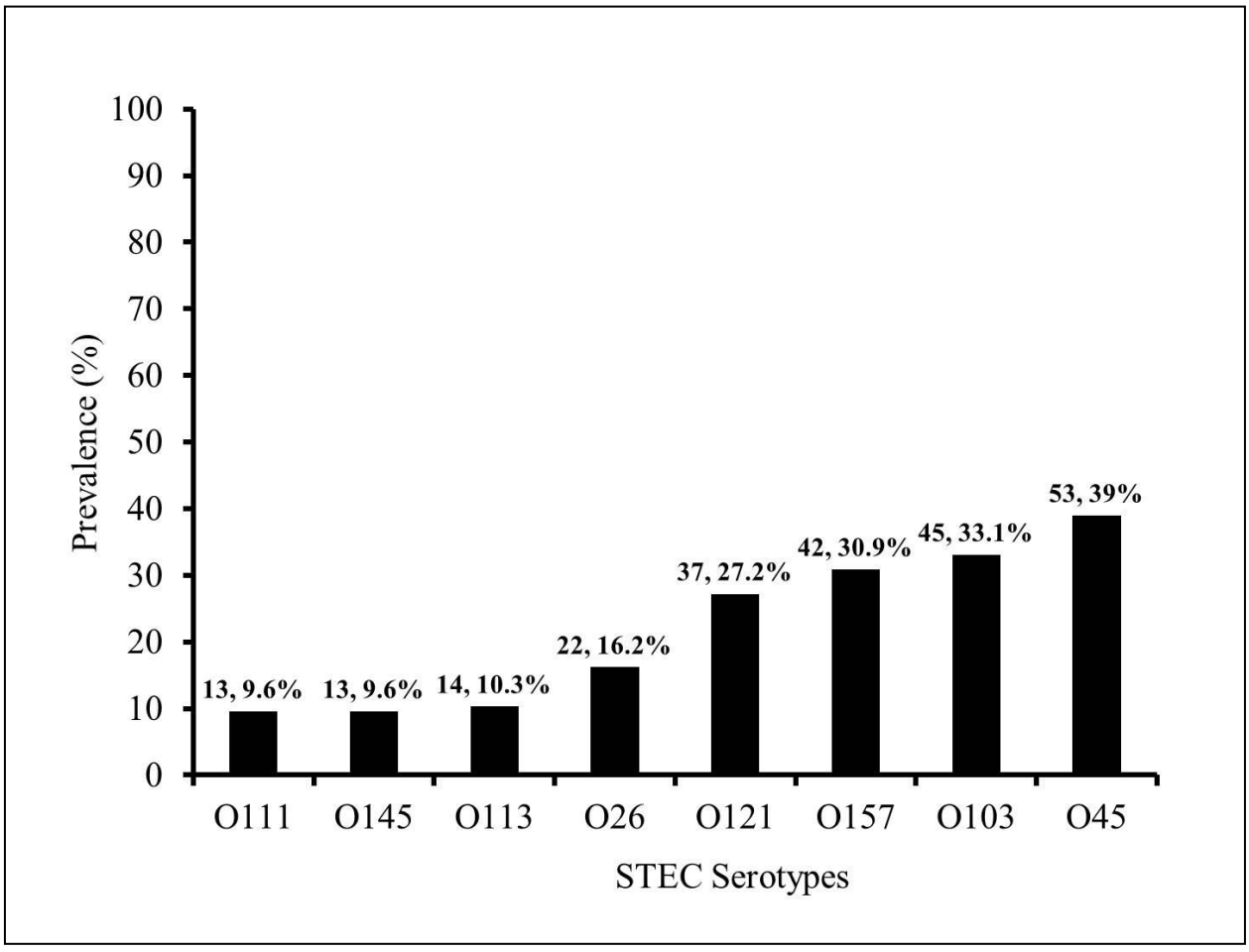

Figure 3. Distribution (n, %) of STEC serotypes detected in feedlot runoff samples tested in this study (N-136).

Of the 64 samples that tested positive for more than one STEC serotype, 67.2% (43 of 64) were from feedlot A and 32.8% (21 of 64) were from feedlot B. Figure 6 shows the frequency of samples that tested positive for at least one STEC serotype. In addition, 57.8% were positive for at least 3 STEC serotypes with 75.7 % from feedlot A and 24.3% from feedlot B (Figure 7). When stratified by location, 64.9% were from inflow, 2.7% from midflow and 32.4% from outflow samples (Figure 8), which is expected since inflow runoff samples are coming directly from the feedlot surface. Again when stratified by time, 5.4% (2 of 37) and 94.6% (35 of 37) of the samples were collected in April and June, respectively (Figure 9). Greater number of STEC serotypes in runoff samples during June is likely due to temperature and greater runoff volume. The lone sample that tested positive for seven STEC serotypes was collected in the inflow location of feedlot A at sampling time 2 (Figure 4). Worthy of note is the fact that the number of samples positive for at least 3 STEC serotypes increased with time (Figure 9).

SN	ID	O26	O45	O103	O111	O113	O121	O145	O157
1	A-IN -1-1	0	0	0	0	0	0	0	0
2	A-IN -1-2	0	0	0	0	0	0	0	0
3	A-IN -1-3	0	0	0	0	0	0	0	0
4	A-IN -1-4	0	0	0	0	0	0	0	0
5	A-IN -1-5	0	0	1	0	0	0	1	1
6	A-IN -1-6	0	0	0	1	0	0	0	1
7	A-IN -1-7	0	0	0	0	0	0	0	0
8	A-IN -1-8	0	0	1	0	0	0	0	0
9	A-IN -1-9	1	0	0	0	0	0	0	0
10	A-IN -1-10	0	0	1	0	0	0	0	0
11	A-IN -1-11	0	0	1	1	0	0	0	1
12	A-IN -1-12	0	0	1	0	0	0	0	0
13	A-IN -1-13	0	0	0	0	0	0	0	0
14	A-IN -1-14	0	0	1	0	0	0	0	0
15	A-IN -1-15	0	0	1	0	0	0	0	0
16	A-IN -1-16	0	0	0	0	0	0	0	1
17	A-IN -1-17	0	0	1	0	0	0	0	0
18	A-IN -1-18	0	0	1	1	0	0	0	0
19	A-IN -2-1	0	1	0	0	0	0	0	0
20	A-IN -2-2	0	0	0	0	0	0	0	0
21	A-IN -2-3	0	1	0	0	0	0	0	0
22	A-IN -2-4	0	1	0	0	0	0	0	0
23	A-IN -2-5	0	0	0	0	0	0	0	0
24	A-IN -2-6	0	1	0	0	0	0	0	0
25	A-IN -2-7	0	1	0	0	1	1	1	0
26	A-IN -2-8	0	1	0	0	0	0	0	0
27	A-IN -2-9	0	1	0	1	1	1	0	0
28	A-IN -2-10	1	1	0	1	1	1	0	0
29	A-IN -2-11	1	1	0	0	0	0	0	0
30	A-IN -2-12	0	1	0	0	1	0	0	0
31	A-IN -2-13	0	0	0	0	1	0	1	0
32	A-IN -2-14	1	1	0	1	1	1	0	0
33	A-IN -2-15	1	1	1	0	0	0	0	0
34	A-IN -2-16	1	1	1	1	1	1	0	1
35	A-IN -2-17	0	1	1	0	1	0	0	1
36	A-IN -2-18	1	1	1	1	1	1	0	0
37	A-IN -3-1	1	0	0	0	0	1	0	0
38	A-IN -3-2	0	1	0	0	0	1	0	1
39	A-IN -3-3	1	1	0	1	0	1	1	1
40	A-IN -3-4	0	0	0	0	0	0	1	0
41	A-IN -3-5	0	0	0	0	0	1	0	1
42	A-IN -3-6	0	1	0	1	1	1	0	0
43	A-IN -3-7	0	0	0	0	0	1	0	0
44	A-IN -3-8	0	0	0	0	0	1	0	0
45	A-IN -3-9	0	0	0	0	0	1	0	0
46	A-IN -3-10	0	0	0	0	0	0	0	0

SN	ID	O26	O45	O103	O111	O113	O121	O145	O157
46	A-IN -3-10	0	0	0	0	0	0	0	0
47	A-OUT-3-1	0	0	1	0	0	1	1	0
48	A-OUT-3-2	0	0	1	0	0	1	1	1
49	A-OUT-3-3	0	0	1	0	0	1	0	1
50	A-OUT-3-4	1	1	0	1	1	1	0	1
51	A-OUT-3-5	1	1	0	1	0	1	0	1
52	A-OUT-3-6	0	1	1	0	1	1	0	0
53	A-OUT-3-7	1	0	0	0	0	1	0	1
54	A-OUT-3-8	1	0	1	1	0	1	0	0
55	A-OUT-3-9	0	1	1	0	0	1	0	1
56	A-IN-4-1	0	0	0	0	0	0	0	0
57	A-IN-4-2	0	1	1	0	0	0	0	0
58	A-IN-4-3	0	1	1	0	0	1	0	0
59	A-IN-4-4	0	0	0	0	0	0	0	0
60	A-IN-4-5	0	1	1	0	0	0	0	0
61	A-IN-4-6	0	1	1	0	0	1	0	0
62	A-IN-4-7	0	1	1	0	0	1	0	0
63	A-IN-4-8	1	1	1	0	0	0	1	1
64	A-IN-4-9	0	0	1	0	0	0	0	0
65	A-IN-4-10	0	0	1	0	0	1	0	0
66	A-IN-4-11	0	0	1	0	0	0	0	1
67	A-IN-4-12	0	0	0	0	0	0	0	0
68	A-IN-4-13	0	0	0	0	0	0	0	1
69	A-IN-4-14	0	0	1	0	0	1	1	0
70	A-IN-4-15	0	0	0	0	0	0	0	1
71	A-IN-4-16	0	0	0	0	0	0	0	0
72	A-IN-4-17	0	0	0	0	0	0	0	0
73	A-IN-4-18	0	0	0	0	0	0	0	0
74	A-IN-4-19	0	0	0	0	0	0	0	0
75	A-IN-4-20	0	0	1	0	0	1	0	0
76	A-IN-4-21	0	0	0	0	0	0	0	0
77	A-IN-4-22	0	0	1	0	0	0	0	1
78	A-IN-4-23	0	1	1	0	0	1	0	1
79	A-IN-4-24	0	0	0	0	0	0	0	1
80	A-IN-4-25	0	0	0	0	0	0	0	1
81	A-IN-4-26	0	1	0	0	0	1	0	0
82	A-IN-4-27	0	0	0	0	0	0	0	0
83	A-OUT-4-1	0	0	0	0	0	0	0	1
84	A-OUT-4-2	0	0	0	0	0	0	0	0
85	A-OUT-4-3	0	0	0	0	0	0	0	1
86	A-OUT-4-4	1	0	0	0	0	0	1	1
87	A-OUT-4-5	0	0	0	0	0	0	0	1
88	A-OUT-4-6	0	0	0	0	0	0	0	0
89	A-OUT-4-7	0	0	0	0	0	0	0	0
90	A-OUT-4-8	1	0	0	0	0	0	0	0
91	A-OUT-4-9	0	0	0	0	0	0	0	0

Figure 4. Color coded presentation of STEC frequency distribution per runoff samples in feedlot A. The red and green boxes represent positive and negative results, respectively, for each runoff sample tested. SN= serial number for each sample for convenience; ID= experimental identification for each sample; A=Feedlot; IN = Inflow sample; OUT = Outflow sample; First number (1, 2,3 or 4) = Sampling time; Sampling time 1 = April 13, 2012; Sampling time 2 = June 11, 2012; Sampling time 3 = June 13, 2012; Sampling time 4 = June 20, 2012; Second number (1, 2,3…n) = Sample serial number.

SN	ID	O26	O45	O103	O111	O113	O121	O145	O157
1	B-IN-4-1	0	1	1	0	0	1	0	0
2	B-IN-4-2	0	0	1	0	0	0	0	1
3	B-IN-4-3	0	0	0	0	0	0	0	0
4	B-IN-4-4	0	1	1	0	0	0	0	0
5	B-IN-4-5	0	1	1	0	0	0	0	0
6	B-IN-4-6	1	1	0	0	0	0	0	1
7	B-IN-4-7	0	1	0	0	0	0	0	0
8	B-IN-4-8	0	0	1	0	0	0	0	1
9	B-IN-4-9	0	1	0	0	1	1	0	0
10	B-IN-4-10	0	1	0	0	0	0	1	0
11	B-IN-4-11	0	0	0	0	0	0	0	1
12	B-IN-4-12	0	0	0	0	0	0	0	0
13	B-IN-4-13	0	0	1	0	0	0	0	0
14	B-IN-4-14	0	0	1	0	0	0	0	0
15	B-IN-4-15	0	0	0	0	0	0	0	0
16	B-IN-4-16	1	0	0	0	0	0	0	0
17	B-IN-4-17	0	0	0	0	0	0	0	0
18	B-IN-4-18	0	1	0	0	0	0	0	0
19	B-IN-4-19	0	1	0	0	0	0	0	0
20	B-IN-4-20	0	0	0	0	0	0	0	0
21	B-IN-4-21	0	0	0	0	0	0	0	0
22	B-IN-4-22	0	0	0	0	0	1	0	0
23	B-IN-4-23	0	0	0	0	0	0	1	0
24	B-IN-4-24	0	1	1	0	0	0	0	0
25	B-IN-4-25	0	1	1	0	1	0	0	1
26	B-IN-4-26	0	0	0	0	0	0	0	1
27	B-IN-4-27	0	1	1	0	0	0	0	1
28	B-M-4-1	0	0	0	0	0	0	0	0
29	B-M-4-2	0	0	0	0	0	1	0	1
30	B-M-4-3	1	1	0	0	0	0	0	0
31	B-M-4-4	1	0	0	0	0	0	0	0
32	B-M-4-5	0	0	0	0	0	0	0	0
33	B-M-4-6	0	1	0	0	0	0	0	0
34	B-M-4-7	0	1	0	0	0	0	0	0
35	B-M-4-8	0	1	0	0	0	0	0	0
36	B-M-4-9	0	0	0	0	0	0	1	1
37	B-M-4-10	0	0	0	0	0	1	0	1
38	B-M-4-11	0	1	1	0	0	0	0	1
39	B-M-4-12	0	0	0	0	0	0	0	1
40	B-OUT-4-1	0	1	0	0	0	0	0	1
41	B-OUT-4-2	0	1	0	0	0	0	0	1
42	B-OUT-4-3	0	1	0	0	0	0	0	0
43	B-OUT-4-4	0	1	1	0	0	1	0	0
44	B-OUT-4-5	1	1	0	0	0	0	0	1
45	B-OUT-4-6	1	1	1	0	0	0	0	0

Figure 5. Color coded presentation of STEC frequency distribution per runoff sample in feedlot B. The red and green boxes represent positive and negative results, respectively, for each runoff sample tested. SN= serial number for each sample for convenience; ID= experimental identification for each sample; B=Feedlot; IN = Inflow sample; OUT = Outflow sample; M=Midflow sample; First number (4) = Sampling time 4 = June 20, 2012; Second number (1, 2, 3…n) = Sample serial number.

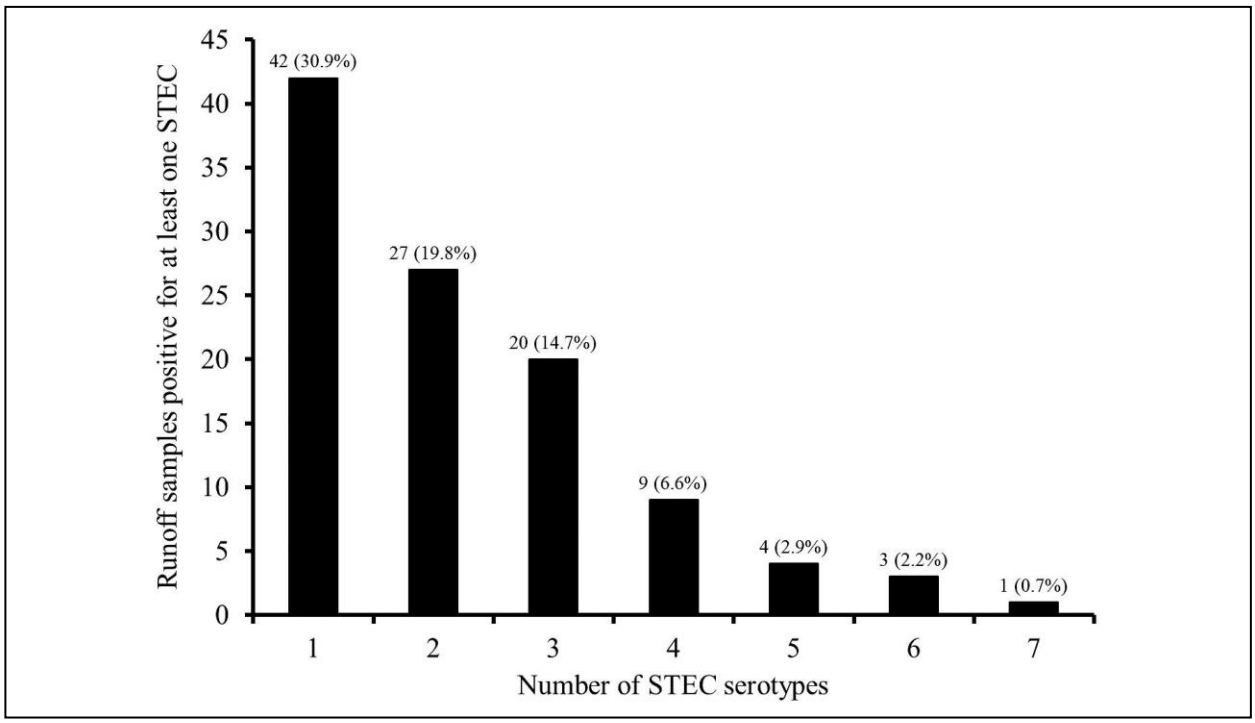

Figure 6. Distribution (n,%) of various (STEC) serotypes within the total number of runoff samples from both feedlot A and B that tested positive for at least one STEC serotype.

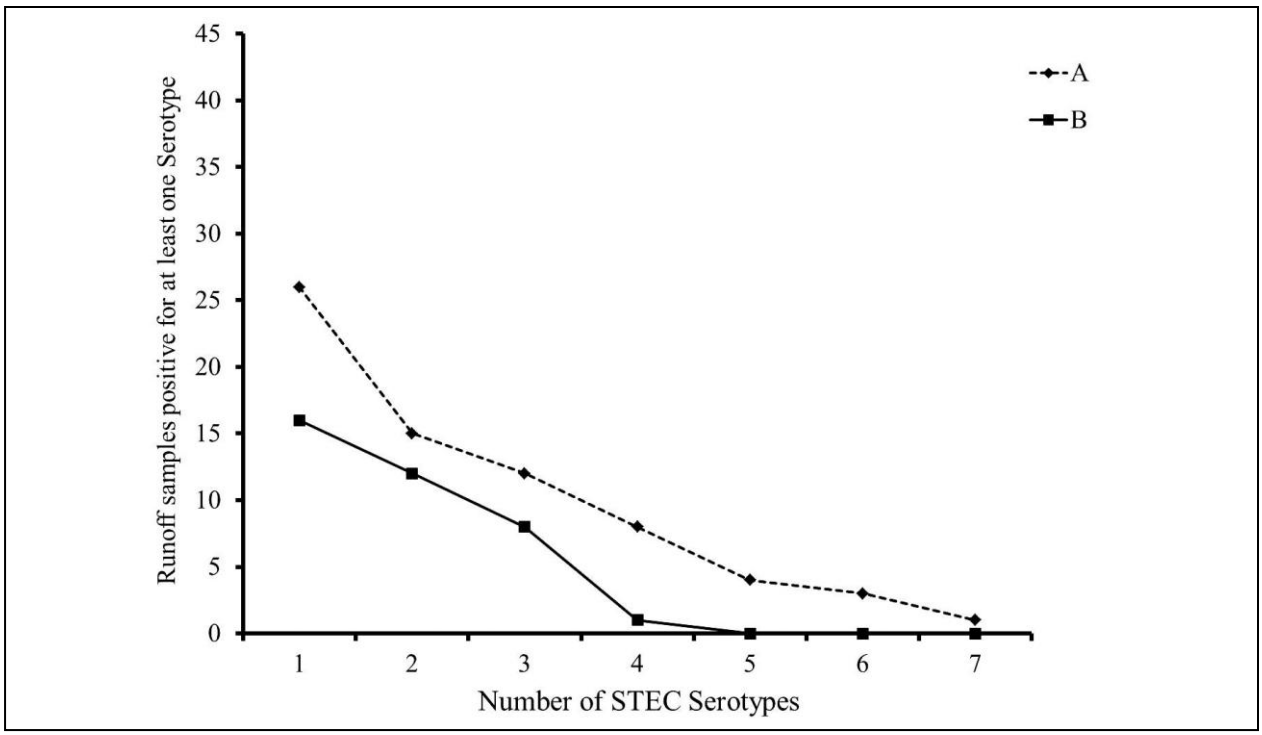

Figure 7. Distribution by feedlot of various (STEC) serotypes within the total number of runoff samples that tested positive for at least one STEC serotype (106 of 136).

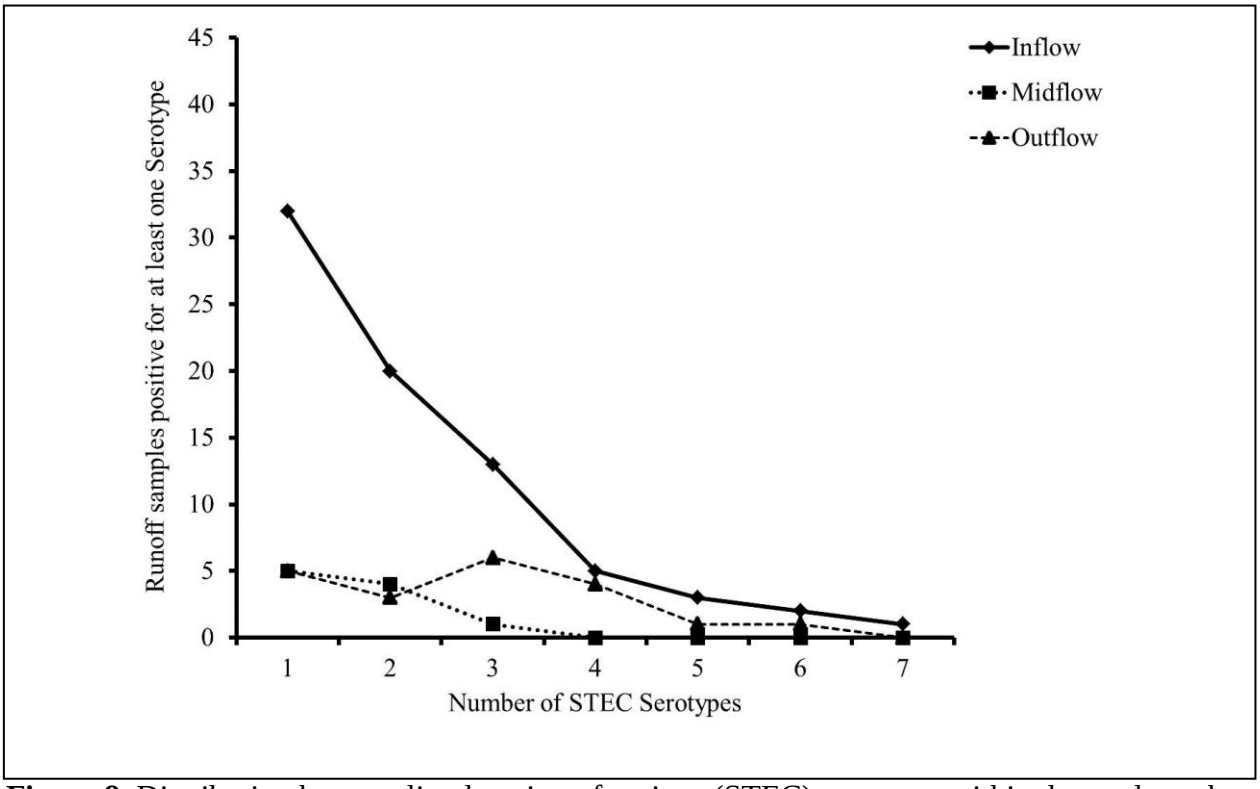

Figure 8. Distribution by sampling location of various (STEC) serotypes within the total number of runoff samples that tested positive for at least one STEC serotype (106 of 136).

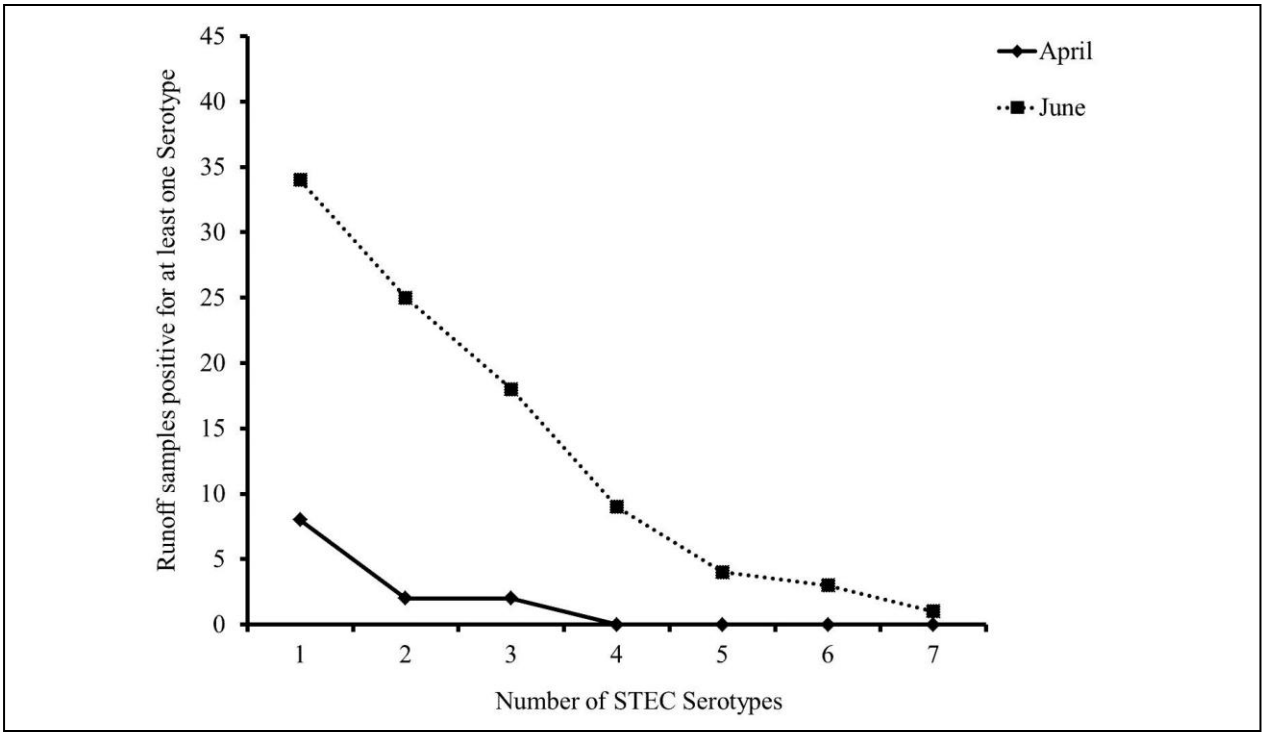

Figure 9. Distribution by sampling time of various (STEC) serotypes within the total number of runoff samples that tested positive for at least one STEC serotype (106 of 136).

Prevalence of *Salmonella* in feedlot runoff

Salmonella was isolated from 40% (54 of 136) runoff samples with Feedlot A recording a prevalence of 51.6 % (47 of 91) while Feedlot B reported a significantly lower prevalence of 15.5 % (7 of 45) (*P*=0.00004) relative Feedlot A (Table 8). Since samples were collected from Feedlot B only once, there was no available data to perform analysis to determine the effect of time on *Salmonella* prevalence in this feedlot (B).

Table 8. Prevalence and distribution of *Salmonella spp* in feedlot run-off from feedlot A and B in North Dakota.

Location	Sampling Site	Site Distribution (%)	Total
Feedlot A	Inflow	38 (52.1%)	47 (51.6%)
	Outflow	9 (50%)	
Feedlot B	Inflow	3 (11.1%)	7 (15.56%)
	Outflow	2 (33.3%)	
	Midflow	2 (16.7%)	

Objective # 2: Whether STEC (O26, O45.O103, O111, O113, O121, O145 and O157) and Salmonella serotypes isolated from the feedlot run-off samples were influenced by feedlot, time of sampling and sampling location with reference to the position of the vegetative filter strip.

When stratified by feedlots, 47.3% (43 of 91) and 46.7% (21 of 45) of the samples were positive for more than one STEC serotype in Feedlot A and B respectively. In addition, serotypes, O111 (*P*= 0.0098) and O121 (*P*= 0.0131), reported a significantly higher prevalence in Feedlot–A relative to Feedlot–B (Table 9). There was also a significant difference in the prevalence of STEC O157 (*P*=0.0064) (Figure 10) in the outflow relative to the inflow location (Table 9). When adjusted for feedlots, the outflow location in Feedlot A had a significantly higher occurrence of STEC serotype O157 (*P*=0.0073) (Figure 11) but this was not observed for the rest of the serotypes (Table 10). In addition, a statistically significant proportion of samples in Feedlot A were positive for *Salmonella* spp in April (77.78 %, 14 of 18) than in June (45.21 %, 33 of 73) (*P*= 0.0133).

Table 9. Results of chi-square analyses to determine the association between STEC serotype occurrence and feedlot, and STEC serotypes occurrence and sampling location.

STEC serotypes	n (%) Positive		χ^2 - value	Odds ratio	95% CI	P- value
	Feedlot A (N=91)	**Feedlot B (N=39)**				
O26	16 (17.58%)	4 (10.26%)	1.13	0.5357	0.17 – 1.72	0.4269_a
O45	30 (32.97%)	17 (43.59%)	1.33	1.5712	0.73 – 3.39	0.2480
O103	32 (35.16%)	11 (28.21%)	0.60	0.7243	0.32 – 1.64	0.4396
O111	13 (14.29%)	0 (0.00%)	6.19	0.0000	Undefined	0.0098^{*}_a
O113	12 (13.19%)	2 (5.13%)	1.84	0.3559	0.08 – 1.67	0.2271_a
O121	31 (34.07%)	5 (12.82%)	6.15	0.2846	0.10 -0.80	0.0131^{*}
O145	10 (10.99%)	3 (7.69%)	0.33	0.6750	0.18 -2.60	0.7536_a
O157	27 (29.67%)	12 (30.77%)	0.02	1.0535	0.47 – 2.38	0.9003
	Inflow (N=100)	**Outflow (N=30)**				
O26	12 (12%)	8 (26.67%)	3.8133	2.6667	0.97 – 7.32	0.0508^{*}
O45	38 (38%)	9 (30%)	0.6398	0.6992	0.29 – 1.68	0.4238
O103	36 (36%)	7 (23.33%)	1.6726	0.5411	0.21 – 1.38	0.1959
O111	10 (10%)	3 (10%)	0.0000	1.0000	0.26 – 3.90	1.0000_a
O113	12 (12%)	2 (6.67%)	0.6831	0.5238	0.11 – 2.48	0.5199_a
O121	25 (25%)	11 (36.67%	1.5687	1.7368	0.73 – 4.14	0.2104
O145	9 (9%)	4 (13.33%)	0.4815	1.5556	0.44 – 5.46	0.4958
O157	24 (24%)	15 (50%)	7.4286	3.1667	1.35 – 7.41	0.0064^{*}

$_a$ - Fisher's Exact *P*- value reported.
* - significant *P*- value

Table 11 presents the distribution of STEC as a function of sampling time. Although shiga-toxin *Escherichia coli* serotypes were present in runoff in April, the occurrence of certain STEC serotypes — O45 and O121— increased significantly as from April to June (Figure 12). When we compared the outflow samples of feedlot A with the outflow samples of feedlot B, there was a statistically significant higher occurrence of STEC O45 in feedlot B (*P*=0.0015) (Table 12).

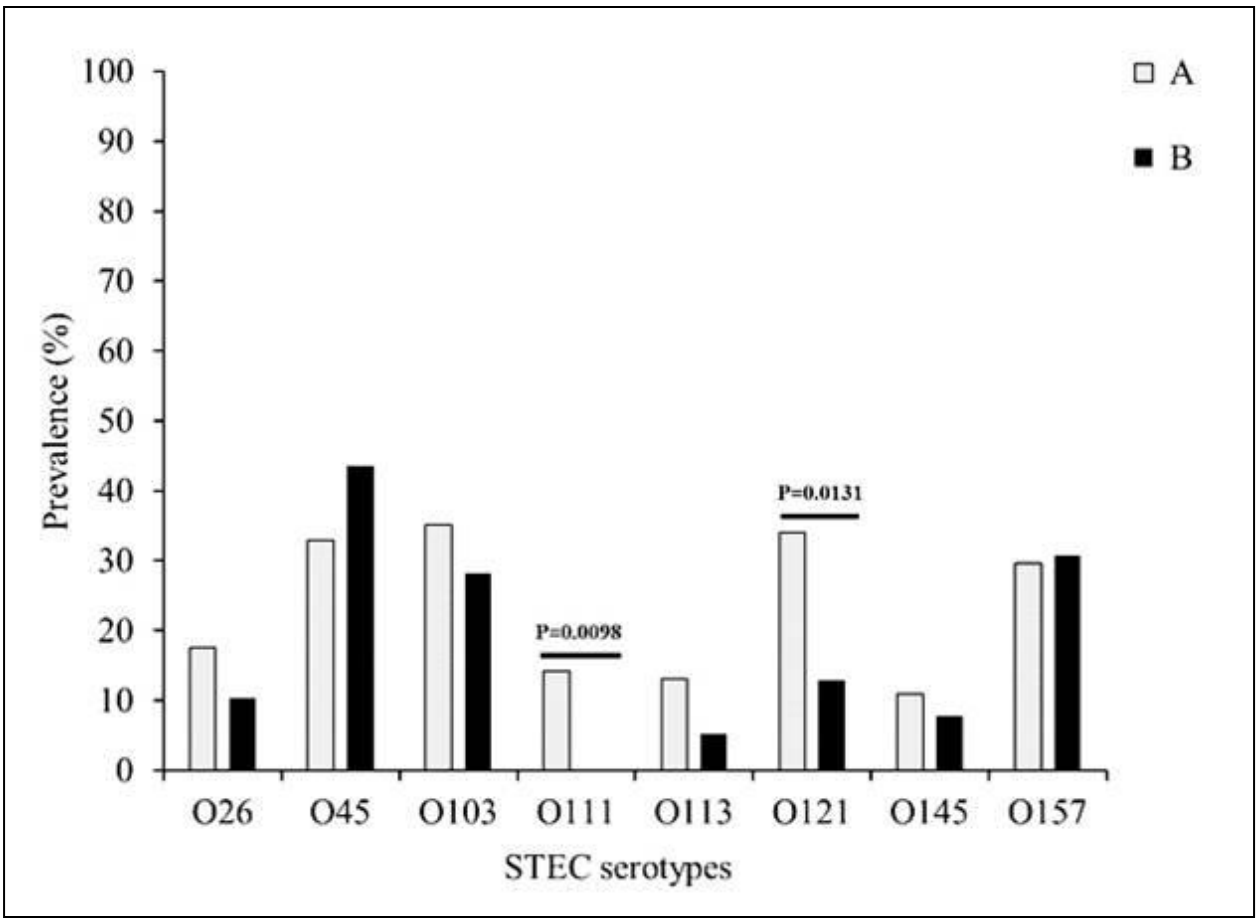

Figure 10. Results of chi-square analyses to determine the association between feedlots (A and B) and STEC serotypes occurrence. The *P*- value of statistical significant difference between in and out flow is also presented.

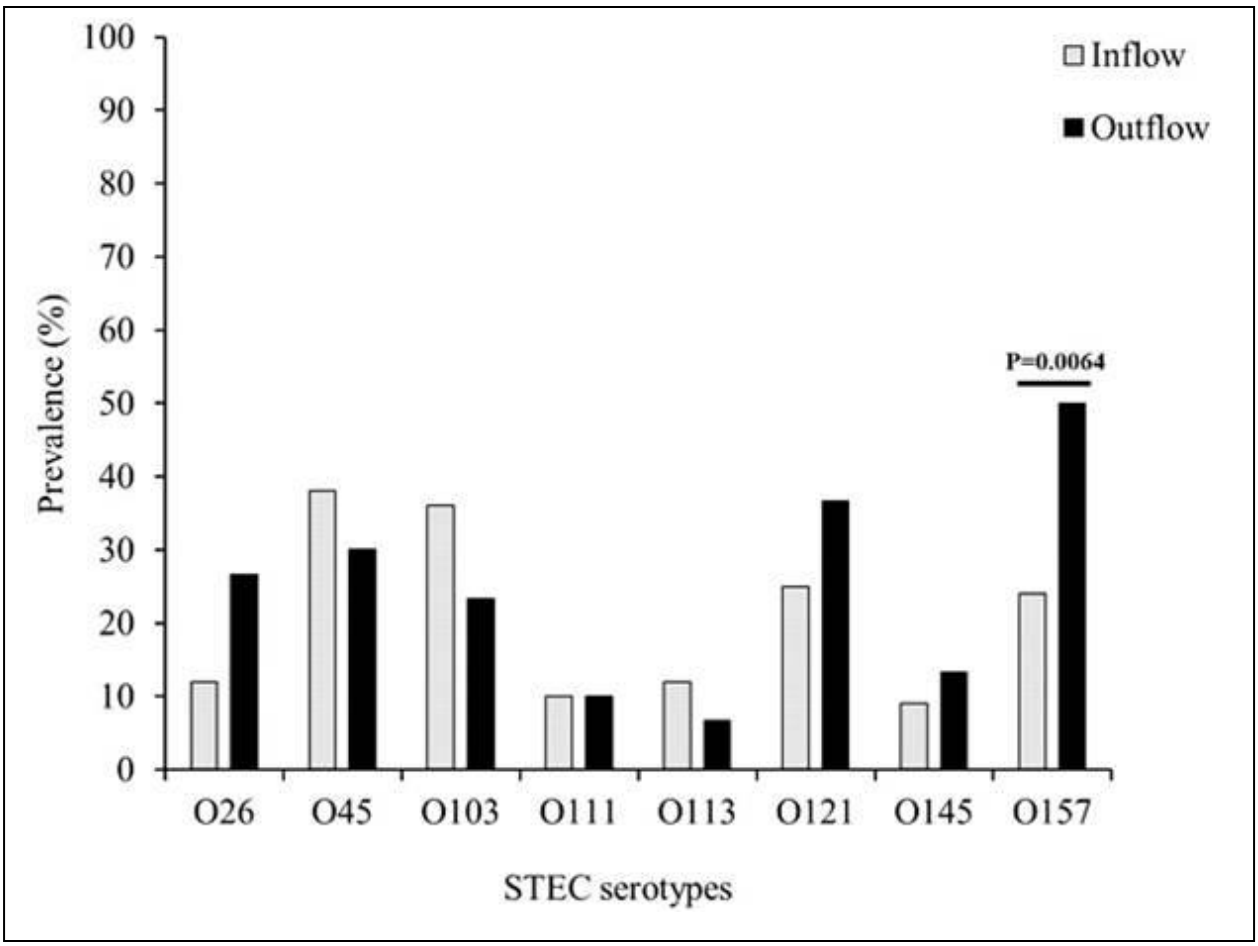

Figure 11. Results of chi-square analyses to determine the association between sampling locations and STEC serotypes occurrence. The *P*- value of statistical significant difference between feedlots is also presented.

Table 10. Results of chi-square analyses to determine the association between sampling location and STEC serotypes occurrence while adjusting for feedlots.

STEC serotypes	n (%) Positive		χ^2 - value	Odds Ratio	95% CI	P-value
	Inflow	Outflow				
Feedlot A (N=91)						
O26	10 (13.70%)	6 (33.33%)	3.8415	3.150	0.96 – 10.31	0.0508
O45	26 (35.62%)	4 (22.22%)	1.1723	0.5165	0.15 – 1.73	0.4028_a
O103	26 (35.62%)	6 (33.33%)	0.0330	0.9038	0.30 – 2.69	0.8558
O111	10 (13.70%)	3 (16.67)	1.260	0.1039	0.31 – 5.15	0.7160_a
O113	10 (13.70%)	2 (11.11%)	0.0844	0.7875	0.16 – 3.96	1.0000_a
O121	22 (30.14%)	9 (50%)	2.5364	2.3182	0.81 – 6.63	0.1112
O145	7 (9.59%)	3 (16.67%)	0.7395	1.8857	0.44 – 8.15	0.4081_a
O157	17 (23.29%)	10 (55.56%)	7.2050	4.1176	1.40 – 12.08	0.0073^*
Feedlot B (N=39)						
O26	2 (7.41%)	2 (33.33%)	0.7738	2.500	0.31 – 20.27	0.5733_a
O45	12 (44.44%)	5 (41.67%)	0.0261	0.8929	0.23 – 3.53	0.8717
O103	10 (37.04%)	1 (8.33%)	3.3801	0.1545	0.02 – 1.38	0.1219_a
O111	0 (0.00)	0 (0.00)			0.26 – 3.90	
O113	2 (7.41%)	0 (0.00)	0.9369	0.0000	Undefined	1.0000_a
O121	3 (11.11%)	2 (16.67%)	0.2294	1.6000	0.23 – 11.08	0.6342_a
O145	2 (7.41%)	1 (8.33%)	0.0100	1.1364	0.09 – 13.89	1.0000_a
O157	7 (25.93%)	5 (41.67%)	0.9663	2.0408	0.49 – 8.57	0.4553_a

$_a$ - Fisher's Exact *P*- value reported.

* - significant *P*- value

Table 11. Results of the chi-square analysis to determine the association between sampling time and STEC serotypes occurrence for feedlot A

STEC serotypes	n (%) Positive		χ^2 - value	Odds Ratio	95 % CI of OR	P-value
	April (N=18)	June (N=73)				
O26	1 (5.56%)	15 (20.55%)	2.2397	4.40	0.54 – 35.7	0.1795_a
O45	0 (0%)	30 (41.10%)	11.0353	undefined	Undefined	0.0004_a^*
O103	9 (50%)	23 (31.51%)	2.1660	0.460	0.16 – 1.31	0.1411
O111	3 (16.67%)	10 (13.70%)	0.1039	0.7937	0.19 – 3.24	0.7160_a
O113	0 (0)	12 (16.44%)	3.4084	undefined	Undefined	0.1142_a
O121	0 (0)	31 (42.47%)	11.5932	undefined	Undefined	0.0002_a^*
O145	1 (5.56%)	9 (12.33%)	0.6772	2.3906	0.28 – 20.20	0.680_a
O157	4 (22.22%)	23 (31.51%)	0.5965	1.6100	0.48 – 5.43	0.5697

$_a$ - Fisher's Exact *P*- value reported.
* - significant *P*- value

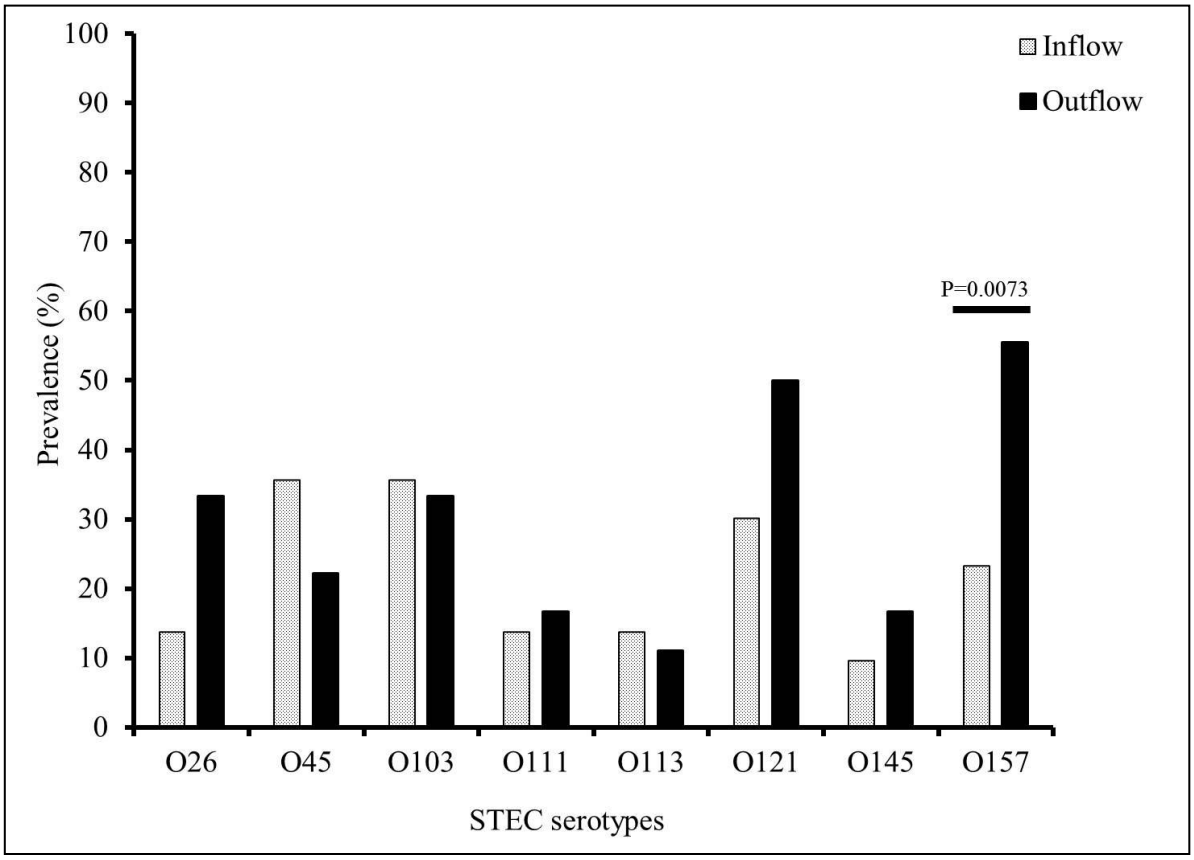

Figure 12. Results of chi-square analyses to determine the association between sampling location and STEC serotypes while adjusting for feedlot A.

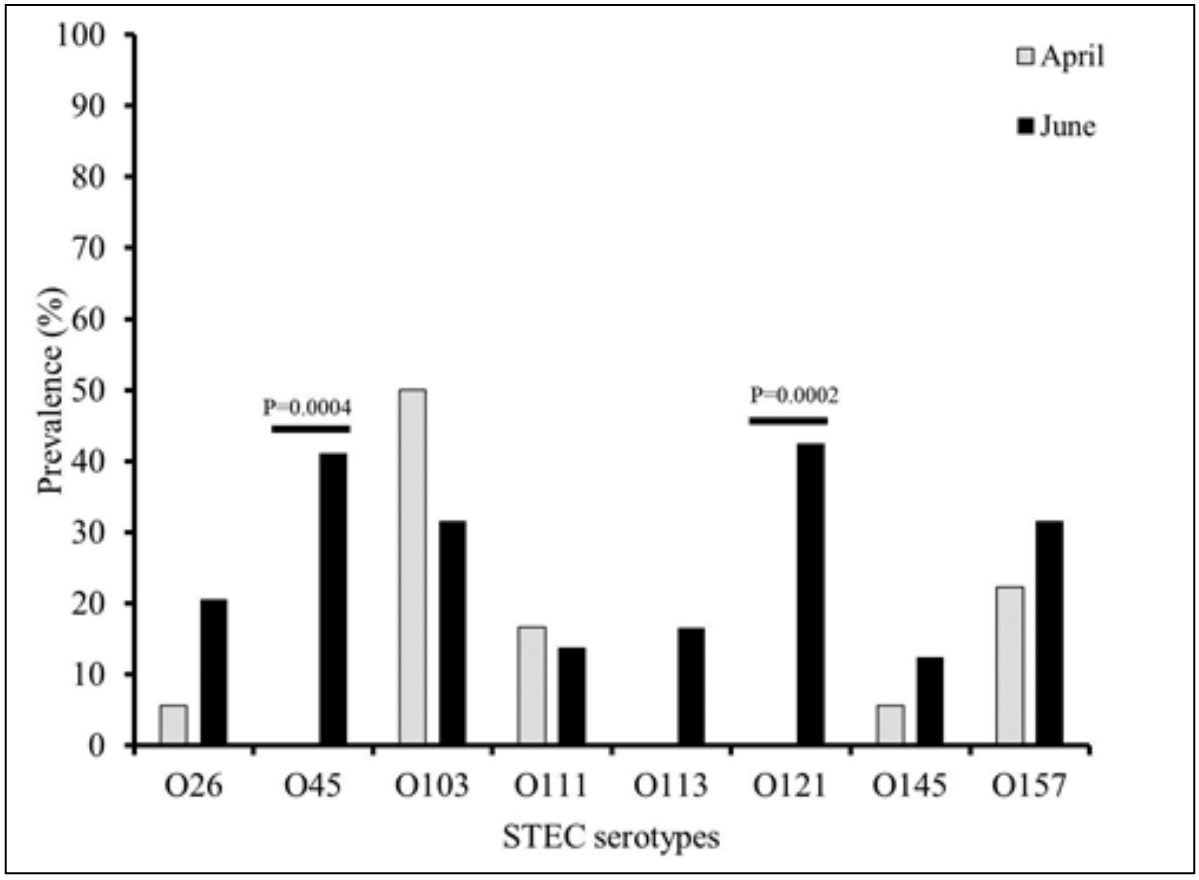

Figure 13. Results of the chi-square analysis to determine the association between sampling time and STEC serotypes occurrence.

Table 12. Chi-square analysis to determine association between STEC serotypes occurrence and outflow sampling location.

STEC serotypes	n (%) Positive		χ^2 - value	Odds Ratio	95% CI of OR	P-value
	Feedlot A (N=18)	Feedlot B (N=6)				
O26	6 (33.33%)	2 (33.33%)	0	1.0	0.14 -7.10	1.0000_a
O45	4 (22.22%)	6 (100%)	11.2	undefined	Undefined	0.0015_a^*
O103	6 (33.33%)	2 (33.33%)	0	1.0	0.14 – 7.10	1.0000_a
O111	3 (16.67%)	0 (0%)	1.1429	undefined	Undefined	0.5464_a
O113	2 (11.11%)	0 (0%)	0.7273	0	Undefined	1.0000_a
O121	9 (50%)	1 (16.67%)	2.0571	0.2	0.02 – 2.07	0.3408_a
O145	3 (16.67%)	0 (0%)	1.1429	0	Undefined	0.5464_a
O157	10 (55.56%)	3 (50%)	0.0559	0.8	0.13 – 5.01	1.0000_a

$_a$ - Fisher's Exact *P*- value reported

* - significant *P*- value

Also worthy of note is the fact that some STEC serotypes (O45, O103, O111, O113 and O121) for feedlot A and (O111, O113 and O145) for feedlot B reported no occurrence in the outflow sampling locations of both feedlots at sampling time four. There was a higher occurrence of STEC O45 in feedlot B (P = 0.0002) (Table 13).

Table 13. Chi-square analysis to determine association between STEC serotypes occurrence and sampling time four for both feedlots A and B.

STEC serotypes	n (%) Positive		χ^2 - value	Odds Ratio	95% CI of OR	P-value
	Feedlot A (N=9)	Feedlot B (N=6)				
O26	2 (22.22%)	2 (33.33%)	0.2273	1.75	0.17 – 17.69	1.0_a
O45	0 (0%)	6 (100%)	15.0000	undefined	undefined	0.0002_a *
O103	0 (0%)	2 (33.33%)	3.4615	undefined	0.14 -7.10	0.1429_a
O111	0 (0%)	0 (0%)				
O113	0 (0%)	0 (0%)				
O121	0 (0%)	1 (16.67%)	1.6071	undefined	undefined	0.4_a
O145	1 (11.11%)	0 (0%)	0.7143	1.00	undefined	0_a
O157	4 (44.44%)	3 (50%)	0.0446	1.25	0.16 – 9.92	1.0_a

$_a$ - Fisher's exact *P*- value reported.
* - significant *P*- value

Discussion

The goal of this study was to detect the presence of shiga toxin-producing *Escherichia coli* (STEC) serotypes and *Salmonella* in cattle feedlot runoff samples collected from two feedlots located in two counties in North Dakota. For the first time this study was able to characterize the occurrence of various STEC serotypes and *Salmonella* in runoff from feedlot facilities in North Dakota. Our study detected the presence of multiple STEC serotypes and *Salmonella* in cattle feedlot runoff suggesting that these zoonotic pathogens may be present in both cattle feces and the farm environment thus predisposing ND agricultural watershed to livestock fecal pathogen contamination during and following heavy rainfall. Other studies (Quilliam et al., 2011; Renter et al., 2007) have also reported presence of non-O157 STEC in

farm environments in other areas. (Cooley et al., 2013) reported the non-O157 STEC in plant, water, soil and fecal samples from a leafy greens production region in Carlifornia. Renter et al (2007) reported presence of non-O157 STEC (O2:H7, O15, O51, O111, O113, O139, O145, and O165) in feces from feedlot cattle in Alberta. Also, a study by Ahmed et al (2009) reported the prevalence of enterohaemorrhagic *E. coli* (EHEC O157 LPS, EHEC VT1, and EHEC VT2 genes) in surface waters (ponds and creeks) in Brisbane, Australia. Additionally, Quilliam et al., 2011conducted a comprehensive review of literature on studies that had reported presence of human pathogens at the agricultural-environment interface including detection of *Escherichia coli* O157 in freshwater ecosystems. These authors concluded that enteric pathogens such as *E. coli* and *Salmonella* can be shed along with livestock waste on the farm environment from where they can be transported during or following heavy rainfall to surrounding pasturelands, produce fields and watersheds.

Detection of STEC serotypes in runoff from feedlot facilities in ND was a significant finding in the wake of the ruling by US Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS, 2012) to regulate the presence of non–O157 STEC belonging to serotypes O26, O45, O103, O111, O121, and O145 (referred to as the *Big Six*) in non–intact beef products (77 FR 31975). This study reported presence of all the *Big six* serotypes plus O113 and O157; moreover, other studies have reported a correlation between presence of these pathogens in cattle feces and recovery from carcasses (DebRoy et al., 2011) and in raw-milk cheeses (Madic et al., 2011) which underscores the public health importance of this finding.

This study utilized recently developed polymerase chain reaction (PCR) tests for non–O157 STEC (DebRoy et al, 2011) to determine the presence of O26, O45, O103, O111, O113, O121, O145 in feedlot runoff samples. It is possible that recent improvements in sensitivity and

specificity of diagnostic tests for non-O157 STEC enabled this study to detect presence of these pathogens in feedlot run off. It is highly probable that with continued advances in diagnostic testing for non-O157 STEC, we will see more reports of increased detection of these pathogens in feedlot environments and other probable reservoirs such as soil, wild life and water samples (Cooley et al, 2013). More studies such as the one published by (Rodney Moxley et al., 2013) that continue to evaluate diagnostic sensitivity & specificity of several methods for non-O157 STEC are anticipated.

This study showed an overall *E. coli* prevalence range of 10 to 39% in feedlot runoff obtained from the two feedlots in North Dakota. This is in concordance with the worldwide ranges of *E. coli* O157 prevalence of 0.2 to 48.8% and non-O157 prevalence of 0.4 to 74% reported by (Hussein and Sakuma, 2005). In fact, a study conducted by Brooks et al (Brooks et al., 2005), reported the following prevalence of these STEC serotypes in people: O26 (22%), O111 (16%), O103 (12%), O121 (8%), O45 (7%), and O145 (5%) during the summer; young persons 3–37 years of age had increased risk of hemolytic uremic syndrome (HUS) and bloody diarrhea. The distribution of the serotypes in Brook's *et al*'s study underscores the public health importance of non-O157 STEC detected by the present study, suggesting an active circulation of the STEC serotypes with greater possibility of transmission to consumers. Data generated by this study on the runoff pathogen burden is essential for estimation of the risk level posed by feedlot runoff to the food safety industry and to public health.

The overall prevalence of STEC in feedlot B (82.2%) was higher than for feedlot A, which might be due to the age of feedlots. Feedlot-A is relatively newer than feedlot-B. Also, variation of runoff resulting from rainfall in two feedlots might play some role. Normally, increased soil moisture favors bacterial growth and multiplication and could have accounted for

this difference. Although feedlot B was older than feedlot A, there was a higher prevalence of *Salmonella* and STEC O111 and O121 in Feedlot A relative to Feedlot B. The reasons for these differences are unknown. However, the disparity in sample volume from the two feedlots could be the reason for this difference. Again, the disparity in rainfall amounts at the two locations of the feedlots could also have contributed to this difference. The majority of samples were collected in the month of June and feedlot-A recorded higher rainfall amounts (2.24 in) relative to feedlot- B (1.14 in) for the month of June (ND Agricultural Weather Station, 2012). In this study, feedlot runoff was collected during rainfall; it is possible that more runoff was able to wash away more manure (STEC reservoir) where rainfall amounts were heavier thus leading to a higher prevalence than where rainfall was less.

It was interesting to note that for feedlot A, although STEC serotypes and *Salmonella* were present in runoff in April samples, the occurrence of certain STEC serotypes — O45, O113 and O121— increased significantly among the samples obtained in June. This observation could be explained by the difference in sample volume during the two months or due to more precipitation during the month of June (2.24 in) that led to more runoff and consequently more STEC recovery. Also, it is possible that warmer temperatures associated with the summer months may favor bacterial growth and multiplication hence more STEC recovery in June could be a reflection of seasonal shedding of these pathogens in cattle feces and therefore manure as has been reported to occur with *E. coli* O157:H7 (Edrington et al., 2006a).Unfortunately, the design of this study and the small sample size did not enable us to determine seasonality of STEC in feedlot run off.

The purpose of the vegetative filter strip (VFS) was to reduce pollutants and pathogens from feedlots as the runoff migrated from inflow through the VFS to the outflow. Based on this

study, outflow locations recorded a higher prevalence of O26, O121, O145 and O157 compared to the inflow sampling locations though only O157 was statistically significant. This is probably reflecting the ability of runoff to transfer pathogens over long distances. Also, the fact STEC O157 had a significantly higher occurrence at the outflow sampling location could be an indication that STEC O157 could be more waterborne than the rest of the STEC serotypes tested in this study or buffer area might also be the source for STEC serotypes and can be transported by runoff. Although it is possible that the VFS contributed to a reduction in pathogen levels, its direct effect on STEC serotypes occurrence among the sampling locations could not be evaluated by this study. Additional studies are needed to determine the effect of the VFS on STEC serotypes variability as well as pathogen load reduction.

In cattle manure, *E. coli* O157:H7 (Semenov et al., 2008; Semenov et al., 2009) and non-O157 (Bolton et al., 2011) are documented to survive for extended periods of time. Additionally, *Salmonella* serovar Typhimurium, was also reported to survive for a considerable periods of time in manure (USEPA, 2013) and slurries (Semenov et al., 2011; Semenov et al., 2009). This might explain why we were able to recover STEC and *Salmonella* in feedlot run off both in the inflow and outflow samples of both feedlot A and B. In our study, we reported a prevalence rate of 40 % (54 of 136) in both feedlots. However, we were unable to determine the relatedness of STEC and *Salmonella* serotypes isolated from inflow and outflow samples. Nevertheless, presence of STEC and *Salmonella* in both inflow and outflow samples and at both feedlots underscores the need for controlling runoff from feedlots, barnyards and other livestock facilities in order to prevent excess pathogens in manure from reaching rivers, streams and lakes (MDoA, 2013). Reducing the pathogen level in feedlot runoff before disposal or use as manure

is vital towards reduction of contamination of fresh produce especially in organic farming practices that use animal manure.

Although the present study targeted these particular STEC serotypes in runoff samples, the presence of all the serotypes suggests the possibility of environmental persistence of these bacteria. The diversity in serotypes among the non−O157 STEC recovered from the runoff samples in this study was notable and could represent potential variability in the risk to human health (Brooks et al., 2005). There was also disparity in the prevalence of STEC serotypes among the various locations (inflow and outflow) that were sampled. The reason for this disparity is not known and the role of VFS could not be assessed; however, this observation could be an indication of environmental diversity of STEC serotypes present. In addition, from Feedlot−A, more STEC isolates were detected from the inflow relative to the outflow samples. A possible reason is that inflow location was in close proximity to the pens, which might have contributed greater number of isolates as had been reported by Brooks et al (2005) where samples collected from cattle pens had higher number of isolates than sites further from cattle pens.

The detection of *Salmonella* from the runoff samples was not surprising considering their ubiquitous nature (Thomas et al., 2013). However, there is a paucity of data on *Salmonella* prevalence in feedlot runoff, particularly in North Dakota. The data presented in this study showed that *Salmonella* was present in feedlot runoff samples with a higher prevalence reported in feedlot−A than feedlot−B. However, there is need to repeat this study on a larger scale to validate these results as the total number of samples tested especially from feedlot B was limited. The study was limited to only two facilities in North Dakota which were not selected randomly but rather based on willingness to participate in the study. This as a limitation to the study and

therefore caution should be taken before extrapolation of the results over a larger geographical area.

Conclusion

It has been known that cattle manure and feedlot runoffs are a major source of zoonotic foodborne pathogens. Livestock animals and the farm environment can act as reservoirs for many foodborne pathogens including STEC and *Salmonella*. However, our study was the first to detect the presence of STEC serotypes and *Salmonella* in runoff from cattle feedlots in North Dakota. Survival and presence of these organisms in feedlot runoff may signify water systems contamination which has important public health implications for spread of these pathogens to crops either by direct application of manure, by irrigation with contaminated water or directly to man by contact with animals or contaminated soil. Therefore, the control of disease causing pathogens in manure is an important consideration within the concept of manure management. Further pretreatment of feedlot runoff may be needed before disposal into the environment or use as organic manure. Also, the role of VFS in reducing STEC or *Salmonella* pathogen loads or diversity may require re-evaluation.

Limitations of the study design

1. Convenience sampling rather than a randomized sampling was used in selecting the participating feedlots hence the findings of this study can only be limited to feedlots studied.
2. Sample collection was only for a short period and depended on the amount of rainfall which was not the same for both locations of the feedlot facilities hence inferences about seasonal variations and pathogen persistence times cannot be made from these data.
3. The study made use of vegetative filter strips that were different by design for each feed lot. This also limited the ability to compare the findings from both feedlots.

4. Microbiological tests performed did not quantify pathogen load hence the effectiveness of the VFS in reducing pathogen load could not be determined by this design.
5. The laboratory methods used are time consuming. This is therefore not a rapid method for the detection of *E. coli* O157 and non-O157 STEC.
6. The immunomagnetic beads used to concentrate STEC serotypes were not specific. The multiplex PCR was able to identify STEC serotypes (O45, O113 and O121) than were not concentrated by immunomagnetic beads.
7. The sensitivity and specificity of the Qiagen multiplex polymerase chain reaction kit are not known, making calculations of false positives and negatives difficult.

Ideas for future studies

1. Characterize the distribution of STEC and *Salmonella* serotypes on a large scale in other feedlot facilities within the State of North Dakota or other States in the US.
2. Determine the relatedness of STEC serotypes in the Inflow and outflow collection locations by multilocus sequence typing or pulsed field gel electrophoresis. Additionally, soil samples from VFS could be tested for presence and variety of STEC in addition to inflow and outflow locations & relatedness of these pathogens compared to those obtained from other locations.
3. Confirm *Salmonella* by serotyping the *Salmonella* isolates; determine the relatedness of *Salmonella* serotypes in the Inflow and outflow collection locations by techniques such as pulsed field gel electrophoresis.
4. Quantify the pathogen load before and after the VFS to verify its effectiveness in pathogen load reduction.
5. Perform the same study with randomly selected feedlot facilities for a longer length of time to identify seasonal variations if any exist.

CHAPTER 4. GENERAL DISCUSSION AND CONCLUSION

Feedlot runoff is one of the routes through which enteric pathogens including those of public health significance may be indirectly transferred to produce fields from domesticated animal waste where they are deposited or stored on adjacent agricultural land (Erickson and Doyle, 2012) or watersheds . Numerous studies (Forslund et al., 2011; Jay et al., 2007) have reported that enteric pathogens can contaminate land, surface waters, and ground waters adjacent to produce fields in both horizontal and vertical movement. When this happens, the risk of pathogen contamination of fresh produce in particular will be dependent on a number of factors, including, but not limited to pathogens in soil particles, the interval between manure application and rainfall, the directionality of water flow due to topography of the slope, and the density of vegetation between the waste source and the produce fields (Ferguson et al., 2007; Lewis et al., 2010). It has been reported that there are a large number of zoonotic pathogens that inhabit and grow in the gastrointestinal tract of livestock and are shed in their feces asymptomatically, often in very large numbers (Erickson and Doyle, 2012; Smith et al., 2005a).

The viability of these organisms in livestock makes domesticated animals like cattle suitable reservoirs for *E. coli* O157:H7 (Khaitsa et al., 2006; Smith et al., 2005a) and *Salmonella* (Thomas et al., 2013; Zweifel et al., 2004b) as has been previously reported. In North Dakota, studies have reported seasonality in *E. coli* O157:H7 and *Salmonella* prevalence and peak prevalence have been recorded in summer as opposed to the winter months (Khaitsa et al., 2006; Khaitsa et al., 2007). Despite this documented trend, no study had reported the occurrence of non-O157 STEC in any livestock or feedlot facility in North Dakota before. This study is the first of its kind to present evidence of the existence of non-O157 STEC serotypes in the feedlot environment. In the wake of USDA-FSIS (2012) ruling to regulate the presence of non–O157

STEC belonging to the six serotypes in non–intact beef products, targeting these serotypes (the big six) in addition to O157 STEC and *Salmonella* as reported in this study provides relevant data that will inform future studies involving non-O157 STEC in North Dakota.

The prevalence of STEC (O157 and non-O157) serotypes and *Salmonella* as was reported in this study was in line with studies that have previously reported the prevalence of these pathogens in humans (Espié et al., 2008), in cattle and in feedlot environments (Masana et al., 2011; Zweifel et al., 2004a; Zweifel et al., 2005) . In addition, the persistence of zoonotic pathogens in manure used for agricultural purposes is an indication that pretreatment before disposal of animal feces as waste or before being used as manure is essential. This also underscores the importance of conducting future studies aimed at detecting these pathogens in livestock housed in feedlot facilities, fresh food produce and retail beef products. An understanding of the occurrence and distribution of the non-O157 serotypes and their diversity in the environment will also impact public health policies in food safety.

This study does have some limitations and therefore caution should be exercised during extrapolation of the findings to other feedlot facilities. The facilities used for this study were sampled by convenience rather than randomly. In addition, samples collection was done for a short period and depended on the amount of rainfall. We recommend that additional studies be conducted on randomly selected feedlot facilities, with samples collected for a longer period of time. This will provide data that can be extrapolated to other facilities and also determine trends in the occurrences of these STEC serotypes and *Salmonella.*

Furthermore, the vegetative filter strips used for the two facilities were of different designs thus limiting the comparability of the findings from both facilities. Another study could be designed that uses the same vegetative filter strips to increase comparability of findings from

the different facilities. Also quantifying the pathogen load by microbial plate counts for the different sampling locations would also provide information on the effectiveness of the VFS in reducing pathogen loads. Another limitation of this study was lack of sufficient funding which prevented us from serotyping the *Salmonella* spp isolated in the runoff samples.

Further studies will be needed to determine the serotypes of the *Salmonella* isolates. Also, establishing a phylogenetic relationship between STEC (O26, O45, O103, O111, O113, O121, O145 and O157) and *Salmonella* spp isolated at the different sampling locations using tools like pulse gel field electrophoresis (PGFE) or multilocus sequence typing (MLST) will help inform the genetic diversity of the non- O157 STEC in feedlot environment.

This study has characterized the occurrence of STEC (O26, O45, O103, O111, O113, O121, O145 and O157) and *Salmonella* in feedlot runoff in two feedlot facilities in North Dakota. The findings from this study indicate that cattle feedlot runoff could be a vehicle for the transmission of STEC serotypes and *Salmonella*. These results warrant proper treatment of runoff before it is used as manure or disposed especially in agricultural watershed which are for cropland irrigation. It is therefore important that policies be made to regulate management of runoff from beef production facilities with consideration of the public health impact of presence of these pathogens.

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